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The Response of *Staphylococcus aureus* to Culture in a Low-Fluid-Shear Environment

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The Response of *Staphylococcus aureus* to Culture in a Low-Fluid-Shear Environment

by

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Dedication

To my grandparents, Clayton and Bonnie Jones, whose unwavering support and pride continually drove me.

To my mother, Carolyn, step-father, John, and brother, Jordan, for your endless encouragement and belief in me.

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The Response of *Staphylococcus aureus* to Culture in a Low-Fluid-Shear Environment

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The opportunistic pathogen, *Staphylococcus aureus*, encounters a wide range of fluid shear levels within a human host. As an intestinal colonizer, *S. aureus* experiences the low-fluid-shear levels consistent with the site; however, as the intestinal presence of the organism is infrequently associated with disease, very few investigations have examined how this environmental parameter impacts the lifecycle of the bacteria. Therefore, the responses of *S. aureus* to culture in this environment were documented by means of the physiologically-relevant, low-fluid-shear conditions generated by the rotating-wall vessel bioreactor. Exposure to the low-shear environment initiated the formation of a novel attachment-independent biofilm in which the *S. aureus* cells were completely encased in an extracellular matrix that conferred increased antibiotic resistance. Further analysis of the cells within the biofilm revealed a decrease in

carotenoid pigmentation and growth, an increase in susceptibility to oxidative stress, and increased killing by whole blood as compared to controls. In addition, low-shear-cultured *S. aureus* displayed increased macrophage clearance and a decreased ability to invade epithelial cells. Whole genome microarray analysis revealed that low-shear-cultured responsive genes were associated with fermentative respiration, consistent with the metabolic profile of *S. aureus* within a biofilm. Additional molecular investigations revealed the decreased expression of the RNA chaperone gene, *hfq*, which parallels the low-shear response of certain Gram-negative microorganisms. This is the first report describing an Hfq association with fluid shear in a Gram-positive organism, indicating that the ability to sense and respond to mechanical stimuli is evolutionarily-conserved among structurally diverse prokaryotes. Collectively, these findings demonstrate the ability of low fluid shear to serve as an environmental cue directing *S. aureus* toward a colonization phenotype in which it decreases known virulence components, reduces its growth, and remains protected within a biofilm matrix.

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List of Abbreviations

Agr	Accessory Gene Regulator
AIP	Auto inducing Peptide
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CHIPS	Chemotaxis Inhibitory Proteins of Staphylococci
ClfA	Clumping Factor A
ClfB	Clumping Factor B
Cna	Collagen-Binding Protein
CspA	Cold Shock Protein A
DMEM	Dulbecco's Modified Eagle Medium
EbpS	Elastin-Binding Protein
EPS	Extracellular Polymeric Substance
ESEM	Environmental Scanning Electron Microscope
ETA	Exfoliative Toxin A
ETB	Exfoliative Toxin B
FBS	Fetal Bovine Serum
FnbpA	Fibronectin-Binding Protein A
FnbpB	Fibronectin-Binding Protein B
GCOS	GeneChip Operating Software
H_2O_2	Hydrogen Peroxide

IgG	Immunoglobulin G
LB	Lennox Broth
LD_{50}	Lethal Dose, 50%
LSMMG	Low-Shear Modeled Microgravity
M9	M9 Minimal Salts Media
MEM	Minimal Essential Media
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity of Infection
mRNA	Messenger RNA
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide plus Hydrogen
NASA	National Aeronautics and Space Administration
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PTSAgs	Pyrogenic Toxin Superantigens
qRT-PCR	Quantitative Real-Time PCR
ROS	Reactive Oxygen Species
RWV	Rotating-Wall Vessel
SAK	Staphylokinase
SCC	Staphylococcal Cassette Chromosome

SCIN	Staphylococcus Complement Inhibitor
SCV	Small Colony Variant
SE	Staphylococcal Enterotoxin
SE	Staphylococcal Enterotoxin-like
SigB	Sigma Factor B
sRNA	Small RNA
SSSS	Staphylococcal Scalded-Skin Syndrome
STS	Shuttle Transport System
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSST	Toxic Shock Syndrome Toxin
US	United States
USSR	Union of Soviet Socialist Republics
UTR	Untranslated Region

INTRODUCTION

Chapter 1: Bacterial Responses to Spaceflight and a Spaceflight Analog

1.1 INTRODUCTION

A microorganism's capacity to sense and adapt to changing conditions both in the environment and host ensures its persistence. For example, during colonization or infection of a host, a bacterium can be subjected to alterations in nutrient availability, pH, oxidation, osmolarity, temperature, and mechanical forces such as fluid shear. Changes in these various environmental conditions have been demonstrated to initiate microbial responses (14, 62, 187) and may serve as cues directing the behavior of the microorganism towards either colonization or the initiation of infection (27, 152). Understanding the response of individual pathogens when introduced to a microenvironment of the host and subsequent comparison of these responses among various microorganisms will provide insight into their pathogenicity and will potentially reveal an evolutionarily conserved response mechanism(s).

1.2 Bacterial Responses to Spaceflight

The ability of a cell to sense and respond to mechanical stimuli is referred to as mechanotransduction. These stimuli have been attributed to alterations in mechanical forces on the cell membrane and can be produced by changes in atmospheric (233), hydrostatic (242), and osmotic pressure (41), increases in fluid shear (167), and decreases in fluid shear induced during culture in spaceflight (153, 225) and spaceflight analogues (77, 148, 153, 225). Early studies of these stimuli focused on the characterization of bacterial responses to osmotic pressure changes and revealed the presence of mechanosensitive channels (146). These channels, located in the bacterial membrane, have been classified into three categories, MscL, MscS, and MscK, based on their structure (143). The mechanosensitive channels sense physical perturbations in cell membrane tension and respond by controlling ion flow (gating), thus maintaining the physical integrity of the cell (143). The presence of these mechanosensitive channels enables the survival of microorganisms during changing levels of osmolarity.

Objects in orbit around the Earth experience a constant state of free fall that is commonly described as microgravity (79). Investigations of the microbial response to this reduced gravity environment coincided with the capability of researchers from the United States (US) and Union of Soviet Socialist Republics (USSR) to send humans and payloads into orbit (136, 137, 240, 241). In 1960, prior to man's first flight, scientists from the USSR launched *Escherichia coli*, *Aerobacter aerogenes*, and *Staphylococcus* into orbit aboard an unmanned satellite (240, 241). Their results, published two years

later, concluded that the microgravity environment of space did not affect the viability of these bacteria (240, 241). In 1961, the USSR again launched *E. coli* along with a human cosmonaut aboard Vostok 2. Following spaceflight, a variant colony type was identified and was determined to be the result of spaceflight factors (100). USSR researchers continued to focus on *E. coli* throughout the course of multiple Vostok flights, finding a significant increase in the levels of phage induction correlating with the duration of time spent in microgravity (238, 239). In 1967, the National Aeronautics and Space Administration (NASA) of the United States launched the unmanned Biosatellite 2, which exposed various biological specimens, including *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), to the microgravity environment of space for 45 hours (136, 137). Interestingly, for both microorganisms an increase in population density was noted for the in-flight samples (136, 137). *Bacillus subtilis* was cultured onboard Apollo 16 and 17 and resulted in the finding that microgravity did not affect the developmental process of spore formation (24). However, when assessed after culture aboard the Apollo-Soyuz Test Project, the colony forming ability of *B. subtilis* spores was found to be reduced among spaceflight samples (51). With evidence mounting that bacteria were able to sense and respond to the microgravity environment of spaceflight, the concern of both the US and USSR space programs shifted to how these variations could impact crew health. Over the course of numerous spaceflights, researchers from various countries analyzed changes in antibiotic resistance in *E. coli* and *Staphylococcus aureus* (114, 205-207). Specifically, the minimal inhibitory concentration (MIC) of oxacillin, chloramphenicol, and erythromycin for *S. aureus* and colistin and kanamycin

for *E. coli* were evaluated among in-flight cultures as compared to controls (205, 206). These investigations documented increased bacterial resistance to all antibiotics tested for both *S. aureus* and *E. coli* (205, 206). Additionally, the researchers observed a thickening of the cell wall that accompanied the increase in resistance of *S. aureus* once returned from flight (205, 206). Various other microbial properties were recorded during this time, including increased conjugation in *E. coli* (34) and increased growth kinetics in *B. subtilis* (142), in response to microgravity.

Data from various Space Shuttle flights also indicated a microgravity-associated microbial response, including decreased lag growth phases, increased exponential growth phases, and increases in cell population, for *E. coli* and *B. subtilis* (93). Additionally, McLean and colleagues demonstrated biofilm formation during spaceflight using *P. aeruginosa* (139).

The most thoroughly analyzed microbial response to spaceflight conditions are those of the bacterium *S.* Typhimurium. In 2006, on board Space Shuttle Atlantis (flight STS-115), *S.* Typhimurium was launched into low-Earth-orbit (225). The bacteria were activated to grow in-flight and either fixed and returned for additional analysis or returned without fixation to be used in a murine model of salmonellosis (225). Mice infected with bacteria cultured in-flight displayed a decreased time-to-death, increased percent mortality, and a decrease in the lethal dose required to kill 50% of the mice (LD_{50}) (225). Analysis of the fixed returned samples revealed the differential expression of 167 genes and 73 proteins and led to the identification of a possible role for the regulatory protein, Hfq, in the bacterial response to culture in the spaceflight environment

(225). Hfq is an RNA chaperone protein, which functions by facilitating the binding of messenger RNA (mRNA) with small non-coding RNA (sRNA) (23). Hfq is considered a post-transcription regulator in numerous microorganisms (23) and an important virulence regulator in *S.* Typhimurium (197). Wilson and colleagues identified Hfq as a regulator of the spaceflight response based on the following evidence: (a) its expression was decreased, (b) the expression of 64 genes belonging to its regulon were differentially altered, (c) sRNAs that are known to interact with Hfq showed differential regulation, and (d) numerous genes whose response pathways are influenced by Hfq were differentially regulated in response to the spaceflight culture environment (225). This was the first report elucidating both the molecular response connected with a regulatory mechanism and alterations in bacterial virulence as a consequence of growth in the spaceflight microgravity environment.

A follow-up investigation on Space Shuttle flight STS-123 confirmed the previous virulence findings (226). Additionally, the investigations on flight STS-123 included the culture of *S.* Typhimurium in M9, a minimal salts media, Lennox Broth (LB), and a combination termed LB-M9 that consisted of LB media supplemented with five inorganic salts present at a higher concentration in M9 as compared to LB as determined by chemical analysis (226). Interestingly, mice infected with *S.* Typhimurium cultured during spaceflight in M9 and LB-M9 media revealed no difference in time-to death or LD_{50} compared to mice inoculated with identically cultured *S.* Typhimurium cultured on Earth (226). Furthermore, the fold increase in the LD_{50} of *S.* Typhimurium cultured in M9 medium relative to culture in LB medium for ground

cultures was 5.7 compared to 56.8 for spaceflight cultures signifying that, while the culture medium did cause a change in LD_{50}, the microgravity environment significantly enhanced the difference (226). In addition, this work also documented the differential expression of 38 genes and 81 proteins, including sets belonging to the Hfq regulon, in *S.* Typhimurium cultured in M9 media in microgravity (226). Taken together, it was determined that media ion concentration influences the spaceflight-related virulence response of *S.* Typhimurium.

Recently, the transcriptional and proteomic response of *P. aeruginosa* cultured aboard STS-115 was reported. Culture in the microgravity environment of space stimulated a bacterial response resulting in the differential regulation of 167 genes and 28 proteins when compared to identically cultured ground controls (38). The most noteworthy change in bacterial regulation was the down-regulation of Hfq and the coinciding differential regulation of 38 transcripts belonging to the Hfq regulon (38). This is the first account of a common molecular regulatory mechanism, shared among different bacterial species, in response to the spaceflight environment.

All of the above descriptions of bacterial responses to microgravity involved the culture of the microbe in liquid media. Other investigations have been performed in which bacteria have been grown on agar plates during spaceflight. For example, when *B. subtilis* and *E. coli* were cultured in low-Earth-orbit on agar plates, no difference in growth was detected as compared to ground controls (93). Moreover, the exposure of *Cupriavidus metallidurans* to microgravity while on agar plates failed to demonstrate differences in cell number, size and morphology, with only an increase in 16 proteins

(118). Taken together, these studies suggest that alterations in fluid-shear levels, imparted by microgravity, are responsible for the responses of microorganisms to this environment.

1.3 Modeling Microgravity: The Rotating-Wall Vessel Bioreactor

Numerous constraints accompany investigations of bacterial responses to microgravity, such as payload restrictions on size, weight, and power, the time and labor requirements of the crew, the significant cost, the limited flight opportunities, and thus the constraints on reproducibility. Due to the difficulty of spaceflight experiments involving cells, NASA scientists developed a cell culture system to serve as a spaceflight analog (231). The rotating-wall vessel (RWV) bioreactor (Fig. 1.1A) was constructed to model aspects of the microgravity environment, specifically pertaining to fluid dynamics.

A cell in liquid media in microgravity experiences two unique aspects important in modeling this environment: 1) remaining in a constant state of suspension and 2) experiencing a quiescent surrounding, devoid of shearing, turbulent forces (97). It is these aspects of spaceflight that the RWV bioreactor effectively models.

The components of the RWV bioreactor system include the vessel, rotation base unit with oxygen pump, and power supply. The vessel is a thin, cylindrical disc to which the cell culture media is introduced by syringe via ports on the vessel's face. Once attached to the base unit, the power supply is turned on initiating rotation of the vessel and the supply of oxygen. Gas exchange within the vessel occurs through a gas permeable membrane on the inner backside of the vessel. The entire system can be

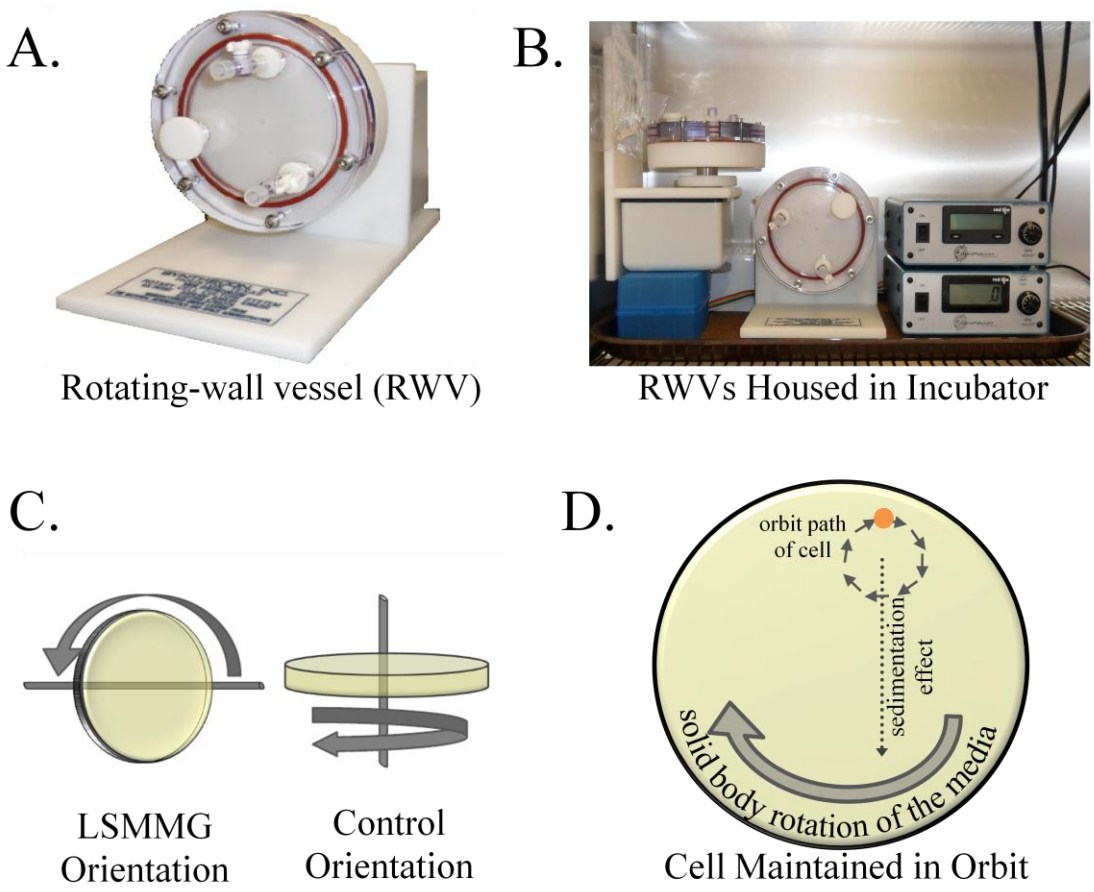


FIGURE 1.1. Operation of the Rotating-Wall Vessel Bioreactor (Synthecon, Houston, TX)**.** (A) Image of the NASA-designed RWV apparatus in which the low-shear modeled microgravity (LSMMG) environment is achieved. (B) RWV culture system in the incubator with their respective base units and power supply systems. (C) The altered positioning of the RWV that results in the two culture orientations, depicting the axis of rotation. The LSMMG environment is achieved by rotation of the RWV on an axis parallel to the ground, whereas the axis of rotation in the control orientation is perpendicular to the ground. (D) Depiction of the orbital path of a cell when cultured in the LSMMG orientation. The continued combination of the sedimentation effect, whereby gravity and lack of motility causes a cell to settle to the bottom of the vessel, and the clock-wise solid body rotation of the media results in the continuous suspension of the cell in an orbit.

housed in an incubator to allow for optimal cell growth at a fixed temperature (Fig. 1.1B). Upon fully filling the vessel with culture media and ensuring the removal of all air space and bubbles, the vessel is attached to its base unit and rotated on an axis parallel to the ground. As the vessel rotates, its rotational velocity is transferred radially inward until the relative fluid motion ceases at which point, solid body rotation of the fluid is achieved (98). A cell within this environment experiences the sedimentation effect imparted by gravity. As it begins to fall toward the bottom of the vessel, "settle out", it is carried back upward by the solid body rotation of the media and thus remains suspended in the fluid in an orbital path (Fig. 1.1C), thereby modeling the first aspect of the microgravity environment described above.

The second environmental aspect experienced by a cell in microgravity that needs to be modeled is that of quiescent, low-fluid-shear flow of the liquid. The continuous acceleration of the microorganism through the media results in the cell attaining terminal velocity (231) and thus incurring a shear force on its surface imparted by the passing fluid. The terminal velocity of a sphere, i.e. a bacterial cell, can be determined by equation 1, where Vs is the terminal velocity, Δd is the difference in the density of the fluid and density of the particle, r is the particle's radius, μ is the viscosity of the fluid, and g is the acceleration due to gravity (231). Since the Reynolds number is extremely

$$Vs = \frac{2\,\Delta d\, g\, r^2}{9\,\mu} \qquad (1)$$

low for a bacterial cell (laminar flow) (175), the Navier-Stokes equation can be applied to determine the fluid shear force on the cell membrane, *F*, as shown in equation 2, where μ is fluid viscosity, *r* is the particle's radius, and *Vs* is the particle's terminal velocity (78).

$$F = 6\pi\mu rVs \qquad (2)$$

Using these assumptions, the shear experienced by a bacterial cell within the RWV has been mathematically modeled and is less than 1 dyne/cm^2 (148). Therefore, during culture in the RWV bioreactor, a microorganism experiences a quiescent, low-shear, low-turbulent environment analogous to the second aspect of spaceflight. As it is important to note the low-shear effects of the fluid on the cells, the term Low-Shear Modeled Microgravity (LSMMG) has been adopted for use in accurately describing the environment produced by the RWV bioreactor (227).

In order to assess the response of microorganisms to the LSMMG environment, a suitable control culture is required. A standard static or shake flask culture of the bacterium in question would not suffice as multiple variables, such as aeration, would be altered. Therefore, early analysis with the RWV vessels demonstrated that an optimal control could be achieved by simply altering the orientation of the vessel to rotate on an axis perpendicular to the ground (Fig. 1.1D) (55). Due to the altered orientation of the RWV, the bacterial cell is no longer suspended in the fluid and the low-shear condition has been disrupted (55). Moreover, the cells in the control vessel are subjected to an identical culture volume in identical hardware, equal speeds of rotation, the same levels of aeration, and are operated side by side in the incubator ensuring an equivalent culture

temperature. The use of a reoriented vessel serving as the control has previously been utilized and validated by multiple investigators (27, 54, 128, 152, 227).

1.4 Bacterial Responses to Low-Shear Modeled Microgravity

The NASA-designed RWV bioreactors were initially intended as a spaceflight analog for eukaryotic cells (77, 231) but have since been used to examine bacteria (4, 37, 52, 152), fungi (91), and archaea (46) in response to this environment. In the mid-1990's, Fang and colleagues were the first to put a bacterium inside the RWV and were primarily focused on the effects of LSMMG on secondary metabolite production (52, 54, 55). Over the course of their studies, they noted that the low-fluid-shear environment of the RWV did not alter gramicidin production from *Bacillus brevis* (54), decreased beta-lactam production by *Streptomyces clavuligerus* (55), inhibited *Streptomyces hygroscopicus'* production of rapamycin (53), and prevented microcin B17 production from *E. coli* (52). A summary of bacterial responses to the LSMMG environment since the work of Fang and colleagues can be found in Table 1.

The low-fluid-shear environment obtained within the RWV is not merely relevant to microgravity but also has been proposed to serve as a model for low-shear sites within the human body (4, 84, 151, 177). It has been suggested that if intestinal epithelial cells lacked micovilli, these cells would experience a fluid shear force in the range from 1 to 5 dynes/cm^2; however, due to the presence of the microvilli, the shear rates between the brush border microvilli of intestinal epithelial cells is less than 1 dyne/cm^2 (76). Additionally, other areas within a human, such as *in utero* and within mucin, have been

characterized as low-shear environments (11, 36, 202). Therefore, the low-shear environment created by the RWV serves as physiologically relevant model for sites a bacterium may encounter during the course of infection and/or colonization in a human host.

Pioneering work expanded this area of research by connecting the LSMMG response of an enteric pathogen, *S.* Typhimurium, to a human host and the spaceflight environment (152, 227). The low-shear conditions within the RWV were found to have profound effects on the behavior of *S.* Typhimurium, including an increase in its virulence potential (152). Mice challenged with LSMMG-cultured *S.* Typhimurium suffered an increased percent mortality, increased time to death, and required a lower LD_{50} as compared to control cultures (152). Further characterization of *S.* Typhimurium in response to LSMMG demonstrated increased growth, increased resistance to acid, thermal, and osmotic stress, increased survival in macrophages and the differential expression of 163 genes (152, 227, 228).

The success of the flight analog studies using the RWV resulted in the aforementioned two spaceflight experiments involving *S.* Typhimurium. One outcome of these investigations was the documented increased virulence of the bacterium in response to spaceflight, paralleling the bacterium's response to LSMMG as produced by the RWV (152, 225) and validating its use as a spaceflight analog. Additionally, bacteria returned from spaceflight revealed the role of Hfq as a regulator of this response (225). Using the RWV bioreactors, the role of Hfq was further confirmed as *hfq* mutants cultured under LSMMG no longer displayed increased acid resistance or increased intracellular

replication in macrophages (225). Furthermore, the RWV was used to supplement the understanding of the role of media composition in affecting the spaceflight response of *S.* Typhimurium. Initial investigations of *S.* Typhimurium in response to both spaceflight and the RWV bioreactor were conducted using LB, a nutrient rich media (152, 225). As previously mentioned, when cultured in spaceflight in a minimal salts media, M9, or LB-M9, which combined LB with the five inorganic salts in higher concentration in M9, the virulence of *S.* Typhimurium was not increased (226). As *S.* Typhimurium cultured in the RWV with LB media revealed an alteration in the organism's acid tolerance response, Wilson, et al. used the RWV to determine the impact of adding combinations of the inorganic salts used in the LB-M9 media in order to evaluate the influence of each salt. It was concluded that the presence of phosphate was sufficient to disrupt the increased acid tolerance phenotype (226). This result further confirms the role of ion concentration in governing the response of *S.* Typhimurium to both spaceflight and LSMMG.

As with spaceflight, *P. aeruginosa* has been characterized in response to culture in the RWV bioreactor. The exposure of *P. aeruginosa* to LSMMG resulted in the formation of a biofilm in the fluid phase of the vessel (36). Additional analysis of the phenotype of low-fluid-shear cultured *P. aeruginosa* revealed increased elastase, rhamnolipid, and alginate production, as well as increased resistance to oxidative and thermal stress (36, 37). Investigation into the molecular response of the bacterium to this environmental parameter identified the differential expression of 134 genes (37). Interestingly, *hfq* and transcripts belonging to the Hfq regulon were differentially regulated (37).

Numerous strains of *E. coli* have been cultured under LSMMG and the response seems to vary with the strain and media type. Investigations with *E. coli* MG1655 yielded decreased growth and the down-regulation of 14 genes, yet resistance to thermal, osmotic, acid and antibiotic stress was unaltered when cultured in Luria Broth under low-fluid-shear conditions (211). However, when cultured in a minimal MOPS media, there was no difference in growth and only the differential expression of 35 genes for this same strain (211). Preliminary analysis with the *E. coli* AMS6, cultured in minimal media, demonstrated an increased resistance to acid and osmotic stress in response to the low-shear conditions (127). A follow-up study included the addition of a glass microcarrier bead, to which the bacteria could attach, into the LSMMG orientated vessel. Under these conditions, attachment of *E. coli* to the beads led to a significantly higher abundance of biofilm production, which conferred increased resistance to osmotic, ethanol, and antibiotic stress (128). Additionally, analysis by different researchers confirmed the finding that *E. coli* AMS6, when cultured in minimal media, is more resistant to osmotic and acid stress as well as thermal and oxidative stress (4). Furthermore, these investigators reported that while adherent-invasive *E. coli* O83:H1 was not altered in terms of growth, acid or osmotic resistance, it did demonstrate increased resistance to thermal and oxidative stress in minimal media after culture under LSMMG (4). Interestingly, low-shear-cultured *E. coli* O83:H1 displayed increased adherence to epithelial cells although invasion rates were unchanged as compared to controls (4).

Gram-positive microorganisms have also been studied to determine their response to low-fluid-shear culture in the RWV, although to a lesser extent. For example,

Streptococcus pneumoniae displays an altered genetic expression profile (3) and the ability of *Bacillus thuringensis* to undergo conjugation and plasmid transfer is uninhibited (15) in response to LSMMG conditions. The role of media composition has been well documented in the low-shear response of Gram-negatives. As staphylococci have complex nutrient requirements, Vukanti, et al. cultured *S. aureus* in a diluted rich media under LSMMG and reported an increase in growth and membrane integrity (221). In contrast, Rosado, et al. noted no difference in growth or antibiotic resistance in three differing strains of *S. aureus* cultured in low-shear (184). However, a decrease in both staphyloxanthin and hemolytic activity was seen in all three strains along with varying degrees of differential gene expression upon culture in the low-shear RWV (184).

To ensure prolonged survival, bacteria are able to sense and respond to various environmental parameters, of which the low-shear modeled microgravity condition created by the RWV bioreactor is no exception. Figure 1.2 details a proposed mechanism for the method by which a bacterial cell may sense and respond to changes in fluid shear coupled to signal transduction events (153). While there are sizeable differences in the adaptations this environment imparts on bacterial cells, common trends emerge. Among Gram-negatives, there are multiple of examples of increased resistance to environmental stressors post low-shear culture (4, 37, 127, 152, 227). Furthermore, changes in biofilm formation have been noted in both *P. aeruginosa* (36) and *E. coli* (128) in response to LSMMG. The media composition has been shown to affect the LSMMG response of both *S. Typhimurium* and *E. coli* albeit in an opposing fashion (4, 211, 226). The increased virulence and acid stress upon *S.* Typhimurium cultured in LB in both true

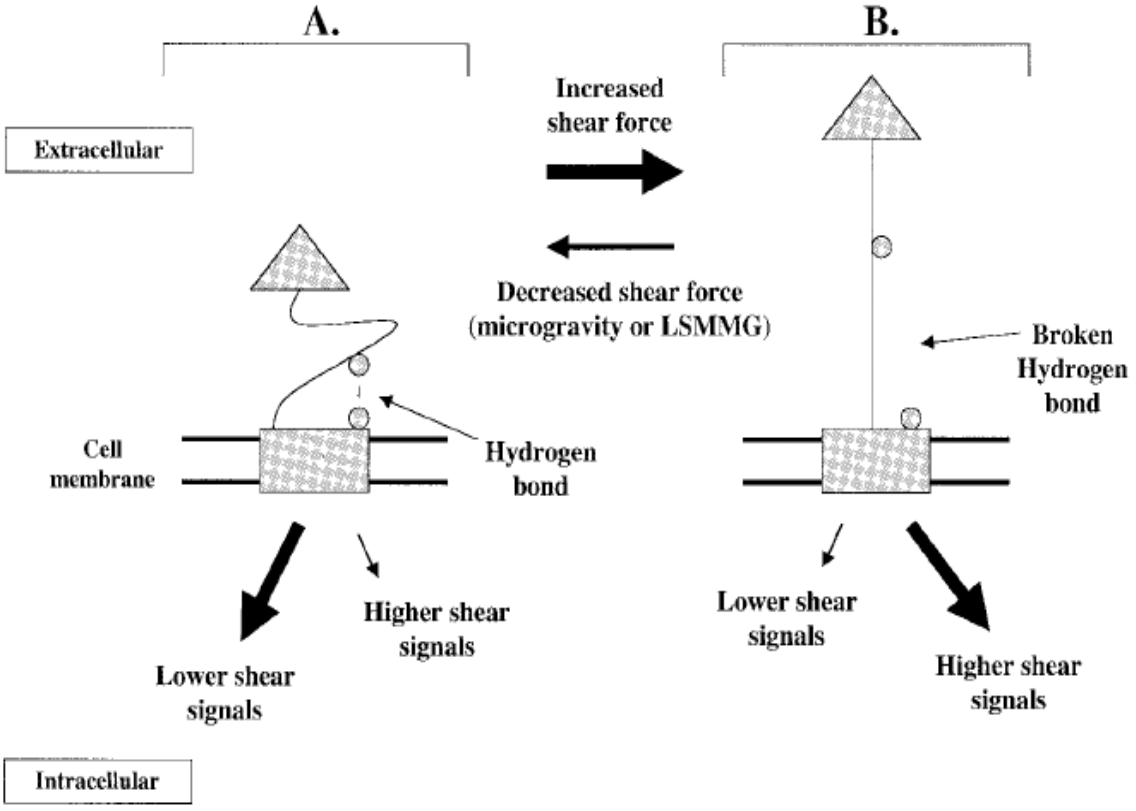


FIGURE 1.2. Proposed Model Depicting a Possible Means by Which Bacteria Sense Changes in Fluid-Shear Force Coupled with Signal Transduction Events (A) Low-shear modeled microgravity and (B) high-shear forces are translated intracellularly, via changes in noncovalent bonds, resulting in a response at the molecular level. (Reproduced with permission from The American Society for Microbiology). (Nickerson, 2004)

microgravity and LSMMG wanes to insignificant when the bacterium is cultured in a minimal media in these conditions (226). However, in a minimal media, *E. coli* displays an increased resistance to acid, osmotic, thermal and oxidative stress (4, 127). Another indicator that microorganisms cultured in the RWV are sensing and responding to this low-fluid-shear environment is demonstrated by the differential gene expression displayed by multiple bacterial species (3, 37, 184, 211, 220, 228). While the altered genetic expression profiles of these bacteria do not suggest any common phenotypic response, there is evidence that some of these microbes are using a conserved molecular response to low-fluid-shear, as Hfq and the Hfq regulon has been implicated in the response of both *S.* Typhimurium and *P. aeruginosa* (37, 225).

Table 1. Bacterial Responses to LSMMG (all results observed using a complex culture media unless otherwise noted)

Bacterial Species	Response				Reference
	Increased	**Decreased**	**Differential**	**Unaltered**	
S. Typhimurium χ^{3339}	Virulence in a mouse model Resistance to acid stress Macrophage survival		Expression of 38 proteins		Nickerson, 2000[152]
		LPS production	Expression of 163 genes		Wilson, 2002[228]
	Resistance to thermal and osmotic stress *Growth	Resistance to oxidative stress			Wilson, 2002[227]
		Hfq Expression			Wilson, 2007[225]
				*Resistance to acid stress	Wilson, 2008[226]
S. Typhimurium 14028	Virulence in a mouse model and cellular invasion		Expression of 22 genes and 125 proteins		Chopra, 2006[33]
E. coli AMS6	*Resistance to acid and osmotic stress			*Growth	Lynch, 2004[127]
	Biofilm formation Resistance to osmotic, ethanol and antibiotic stress				Lynch, 2006[128]
E. coli E2348/69 *E. coli* 86-24	Intimin production			Growth, Intimin production Growth	Carvalho, 2005[26]

E. coli MG1655		Growth Expression of 14 genes	*Differential regulation of 35genes	*Growth Resistance to osmotic, acid, thermal, and antibiotic stress	Tucker, 2007[211]
E. coli K12			Expression of 430 genes		Vukanti, 2008[220]
E. coli 083:H1	*Resistance to thermal and oxidative stress *Adhesion to epithelial cells			*Growth, resistance to acid and osmotic stress *Invasion of epithelial cells	Allen, 2008[4]
P. aeruginosa PA103				Growth and exotoxin A production	Guadarrama, 2005[75]
P. aeruginosa PA01	Biofilm formation in the fluid phase of LSMMG culture. Elastase production Rhamnolipid production		Expression of 15 genes by qPCR	Growth Quorum sensing molecules Pyocyanin production	Crabbe, 2008[36]
P. aeruginosa PA01	Hfq expression Alginate production Resistance to oxidative and thermal stress		Expression of 134 genes by microarray	Growth Resistance to acid stress	Crabbe, 2010[37]
S. pneumoniae TIGR4			Expression of 46 genes	Growth	Allen, 2006[3]
B. thuringensis GBJ001, GBJ002				Conjugation	Beuls, 2009[15]
S. aureus RF1, RF6, RF11		Staphyloxanthin production Hemolytic activity	Expression of 25, 12, and 3 genes respectively	Growth Antibiotic resistance	Rosado, 2010[184]
S. aureus 25923	**Growth and membrane				Vukanti, 2012[221]

	integrity		
Y. Pestis KIMD27	Hela cell rounding	Macrophage proliferation assay	Lawal, 2010[115]

*Results obtained by culture of the bacteria in a minimal media.
**Results obtained by culture of the bacteria in a diluted complex media.

Chapter 2: *Staphylococcus aureus*

2.1 INTRODUCTION

On April 9, 1880, at the Ninth Surgical Congress in Berlin, a Scottish surgeon, Alexander Ogston, presented his findings concerning the involvement of micrococci in abscesses and blood poisoning (159). Ogston reported that pus from abscesses contained micrococci that divided in every direction, resulting in masses that "looked like bunches of grapes" (159). Through an elegant series of animal investigations involving injection of pus from the abscesses into white mice and guinea pigs, Ogston noted that when the pus contained micrococci, an abscess always developed at the site of injection and most often resulted in the animal displaying symptoms of blood poisoning (159). Subsequently, after infection, Ogston was able to isolate micrococci from the blood and tissues which he reported being in "cloudlike masses" (159). Additionally, Ogston documented that pure cultures of micrococci, cultured in the eggs of hens, were able to produce an abscess upon injection into an animal (159). The conclusions from Ogston's presentation, which were published later that year, recognized that 1) micrococci are the most frequent cause of acute abscess formation, 2) micrococci can cause blood poisoning, 3) it is extremely difficult to completely remove micrococci from wounds, and 4) although it seems innocuous in superficial wounds, micrococci play an important role in acute abscesses (159, 161). In an 1883 addendum to his 1882 publication, "Micrococcus

Poisoning", Ogston called his micrococcus *Staphylococcus*, meaning 'bunch of grapes' as has had been suggested to him by Professor of Greek studies, W.D. Geddes (149, 160). In 1886, Anton J. Rosenbach, who was able to culture and isolate *Staphylococcus* on solid media, further classified two strains based on pigmentation and named them *Staphylococcus pyogenes aureus* and *Staphylococcus pyogenes albus*, from the Latin "aurum", for gold, and "albus", for white (185). Today the two strains are known as *Staphylococcus aureus* and *Staphylococcus epidermidis*. Although it has been well over 100 years since Alexander Ogston's description of the involvement of *S. aureus* in disease, a question posed by him remains unanswered: how can a microorganism linked with such acute pathology be otherwise innocuous while associated with a host?

2.2 CHARACTERISTICS OF *S. AUREUS*

S. aureus is a Gram-positive, nonmotile coccus. This bacterium divides in three perpendicular planes with the cells remaining attached, which results in the formation of clusters. *S. aureus* is a facultative anaerobe, utilizing oxygen for aerobic respiration but also fermenting lactic acid under oxygen-limiting conditions (126). It can be distinguished from other staphylococci by a positive catalase or coagulase test, the fermentation of mannitol, and hemolytic activity (126). Furthermore, colonies of *S. aureus* are most often golden in color (126).

S. aureus is able to survive and proliferate in a wide range of environmental conditions. For instance, growth of the organism has been reported to occur at temperatures ranging from 6 to 48 degrees Celsius (117) and at salt concentrations as

high as 15% (72). Additionally, this bacterium can survive in a wide pH range from 4 to 10 (203). As humans and animals are natural reservoirs of *S. aureus*, viable cells can be isolated from virtually any environmental surface that has been in contact with mammals (126, 224). *S. aureus* is also located in air, water, and soil samples (182).

There is a great deal of phenotypic and genetic diversity among *S. aureus* strains. Phenotypic variation is vast, including, but not limited to, differences in pigmentation (74), hemolytic ability, and the capability to form a biofilm (39). Not surprisingly, the genotypes of isolates from bovine and other animals differ dramatically from those isolated from humans, likely as a result of host specificity (92). A comparison of human isolates indicates 10 dominant lineages of *S. aureus*, with each lineage consisting of a unique set of hundreds of core variable genes (121). These core variable genes are scattered throughout the staphylococcal chromosome, insinuating common ancestry with early evolutionary divergence (121). Among human isolates, there are substantial discrepancies in virulence regulators and the carriage of mobile genetic elements (13).

The genome of *S. aureus* is roughly 2.8 megabases, of which approximately 85% codes for proteins (111). The GC content of the organism is low, with an average of 33% among strains (111). Although there are variations among strains, most strains do possess integrated bacteriophages, transposons, insertion sequence elements, and multiple pathogenicity islands (111). Additionally, different strains carry distinct plasmids (111). These assorted mobile genetic elements most often include genes which encode virulence factors and antibiotic resistance cassettes.

Staphylococcal cassette chromosome (SCC) elements are genomic islands that are dispersed throughout the chromosome of the bacterium. The most representative of these SCC elements is the cassette encoding broad-spectrum beta-lactam resistance, SCC*mec* (88). The presence of one of the many types of SCC*mec* in the chromosome of *S. aureus* characterizes that strain as methicillin-resistant *S. aureus* (MRSA) by encoding an altered penicillin-binding protein with low affinity for beta-lactams. This allows for normal cell wall formation in the presence of these antibiotics (66). Although the SCC*mec* has been acquired by countless *S. aureus* strains, it is not the only means by which this microbe can achieve antibiotic resistance. *S. aureus* also produces beta-lactamases, and modifying enzymes that result in antibiotic inactivation as well as efflux pumps which can extrude antibiotics from the cell (125).

2.3 *S. AUREUS* EPIDEMIOLOGY

S. aureus is an opportunistic pathogen with vast medical significance. The capacity of the organism to adapt and proliferate in extensively varied conditions is responsible for its diversity in disease-related pathologies. Bacterial dissemination establishes the disease, which ranges from moderate suppurative infections of the skin and soft tissues, as well as food poisoning, to far more acute diseases, such as toxic shock syndrome, osteomylitis, pneumonia, endocarditis, meningitis, and sepsis (126). In 2005, as a result of *S. aureus* infection, there were approximately 14 million outpatient visits (80), an estimated 478,000 hospitalizations (99) and 19,000 deaths (101) in the United States alone. Although *S. aureus* is a dangerous pathogen, it maintains a commensal

association with approximately 30 – 50% of the healthy population, in whom 10 – 20% are permanently colonized (126). The primary site of colonization is the nasal pharynx (126). *S. aureus* also is commonly isolated from stool samples and rectal sites (1, 40). Moreover, studies have documented the repeated isolation of the same strain from the nose and stool samples/rectal sites of the same individual over long periods of time (1, 40, 181), suggesting that the bacterium either repeatedly passes from the nose through the digestive tract or maintains long term presence in the gastrointestinal tract. The axillae, skin, anus, and vagina also serve as colonization reservoirs (1, 126).

2.3.1 Gastrointestinal Disease Associated with *S. aureus*

S. aureus colonizes the intestinal tract of 20% of the general, healthy population (1) with only a few isolated reports associating *S. aureus* with gastrointestinal enteritis and colitis among immunocompromised individuals (204). *S. aureus* is repeatedly linked as a causative agent of food poisoning (83, 117); however, symptoms arise from the ingestion of preformed staphylococcal enterotoxins in contaminated food rather than by the presence of the organism itself (83, 117). Onset of staphylococcal food poisoning symptoms, including abdominal cramps, nausea, vomiting, and occasionally diarrhea, is rapid occurring 30 minutes to 2 hours after consumption (83, 117). The disease itself is self-limiting, and symptoms fade within approximately 24 hours of onset (83, 117). Numerous studies documenting *S. aureus* food poisoning cases have been successful in associating the enterotoxin-producing strain from infected individuals with the food handler who carried the stain and, thus, provided the source of contamination (83, 112).

Interestingly, long-term analysis of individuals who have suffered food poisoning as a result of *S. aureus* has revealed that these individuals are healthy carriers of the strain associated with the disease for years after the initial symptoms (83, 112).

2.4 IMMUNE EVASION BY *S. AUREUS*

Persistence in human and animal hosts is critical to the survival of *S. aureus*. The microorganism therefore has a vast arsenal of immune evasion mechanisms. In order to avoid detrimental immune modulators, *S. aureus* produces physical barriers such as a polysaccharide capsule and bound surface proteins that bind immunoglobulins in a manner that host immune cells will not recognize, as well as many enzymes and other secreted factors that inhibit specific immune function (155). These various means of circumventing host immunity, as detailed in Figure 2.1, are necessary for colonization and are implicated in increased virulence (155).

The complement system is essential for clearing pathogens in both innate and acquired host immunity (183). As the complement system is so central in immunity, *S. aureus* has multiple mechanisms to inhibit the complement cascade. Staphylococcus complement inhibitor (SCIN) is a small protein excreted by *S. aureus* that binds and stabilizes two complement components, resulting in an ineffective convertase required for complement activation (183). Furthermore, staphylokinase (SAK) is an enzyme produced by *S. aureus* that binds host plasminogen and converts it to the active protease, plasmin (90). This bacterial bound plasmin efficiently cleaves complement components (90). In addition to SCIN and SAK, fibrinogen-binding protein can also bind

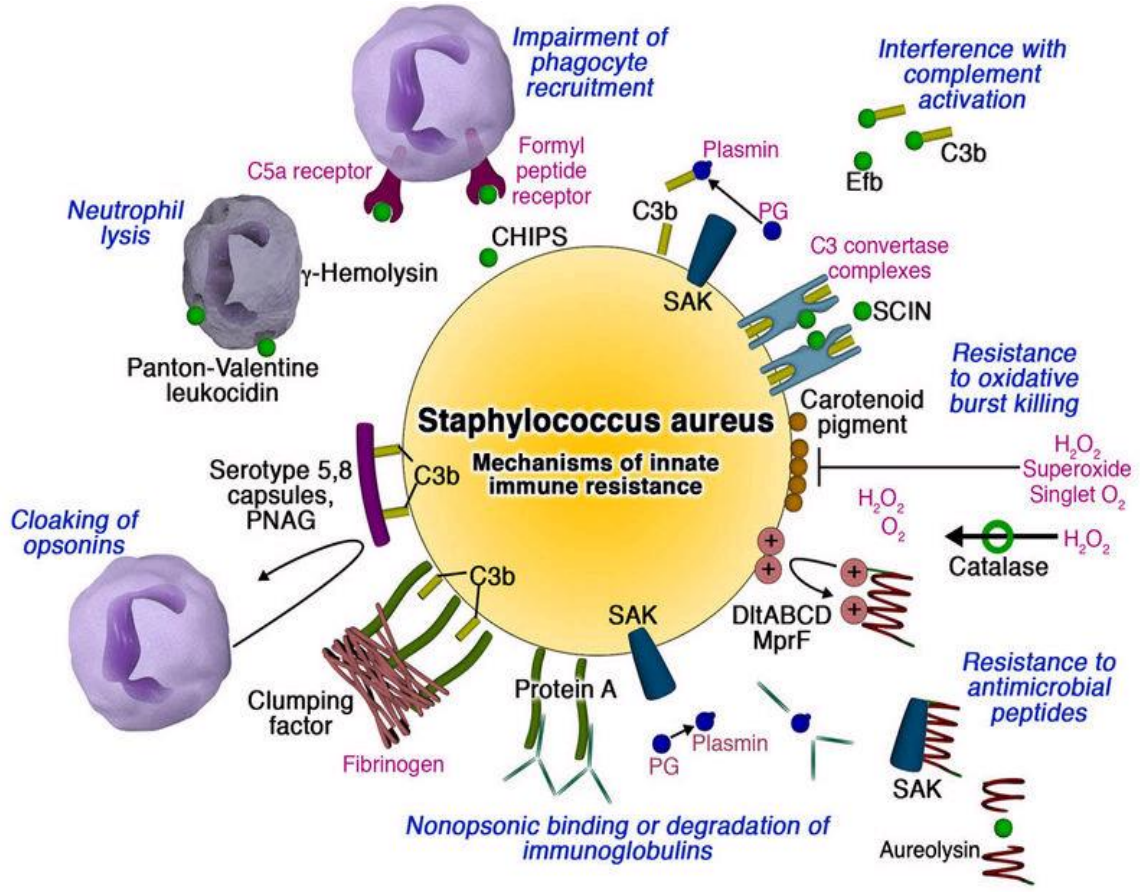


FIGURE 2.1. The Numerous Mechanisms of Immune Resistance by *Staphylococcus aureus*. *S. aureus* has an arsenal of host immune avoidance mechanisms including complement inactivation by SCIN and SAK and leukocyte inhibition and lysis by CHIPS, hemolysins and leukocidin. In addition to hindering phagocytosis with its capsule and the nonopsonic binding of IgG by surface bound protein A, *S. aureus* has multiple means to deflect the deleterious effects of ROS, including carotenoid pigments and catalase. (Reproduced with permission from Elsevier and The Journal of Allergy and Clinical Immunology). (Nizet, 2007)

complement components (155). Collectively, these staphylococcal proteins reduce neutrophil recruitment and block phagocytosis (155).

Leukocytes are the fundamental effector cells of the immune system; *S. aureus* therefore has multiple approaches to reduce their function (155). The chemotaxis inhibitory proteins of staphylococci (CHIPS) are small proteins synthesized and secreted by *S. aureus* (42). CHIPS specifically bind to the formyl peptide receptor and the C5a receptor on leukocytes, thereby obstructing leukocyte activation and migration (42). Additionally, *S. aureus* secretes various hemolysins and leuckocidins that target leukocytes, resulting in their lysis (155).

Although *S. aureus* has multiple mechanisms to impede immune cell migration and activation, the host immune system is efficient at opsinization and phagocytosis of bacterial pathogens. In order to hinder phagocytosis, most strains of *S. aureus* express a polysaccharide capsule (223). Moreover, cell wall anchored protein A contains numerous domains that bind the antibody, IgG, in an orientation that cannot be recognized by phagocytes (96). In spite of these various strategies to prevent phagocytosis, *S. aureus* may still be engulfed by an immune cell. As a result, the bacterium has further methods to aid in its survival in this environment. For instance, cationic antimicrobial defensin peptides are exuded into the phagosome by the immune cell (171). To protect itself from these molecules, *S. aureus* modifies cell wall phospholipids and teichoic acids to increase positive charges on its surface, thereby enhancing electrostatic repulsion of the defensin peptides (171). Phagocytic cells also release a large amount of reactive oxygen species (ROS) during the respiratory burst to

kill ingested pathogens. Staphyloxanthin, the carotenoid pigment responsible for the golden coloration of *S. aureus*, is a cell wall anchored molecule that acts as a protective shield against these ROS by quenching singlet oxygen species (123). In addition to staphyloxanthin, *S. aureus* also produces catalase and superoxide dismutase to protect it from hydrogen peroxide and superoxide radicals, respectively (132).

Bacterial cells encased in a biofilm matrix experience increased protection from the host immune response and antibiotic therapy as compared to planktonic cells (89). *S. aureus* is an efficient biofilm former. Biofilm formation by this organism is a leading cause of medical device-associated infections (45) and has been speculated to be involved in the colonization of the gastrointestinal tract (27).

2.5 ADHESINS OF *S. AUREUS*

The process of either colonization or infection requires the adhesion of a bacterial cell to host components. *S. aureus* has multiple adhesion factors collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (166). In most cases, these MSCRAMMs are cell wall anchored proteins expressed on the surface of the bacterial cell. MSCRAMMs include fibronectin-binding protein A and B (FnbpA and FnbpB), clumping factor A and B (ClfA and ClfB), elastin-binding protein (EbpS), collagen-binding protein (Cna), and Protein A (Spa) (Fig. 2.2) (59). The names of the majority of these MSCRAMMs indicate which host component they bind; for example, fibronectin, elastin, and collagen. Protein A binds von Willebrand factor and clumping factor A and B bind fibrinogen (59). With the exception of Cna, these

MSCRAMMs are expressed in the majority of *S. aureus* strains (59). FnbpA and FnbpB are responsible for bacterial adhesion to immobilized fibronectin, contributing to the adherence of *S. aureus* to blood clots and serum-coated biomaterials (59). Additionally, Cna is essential for the adherence of *S. aureus* to collagenous tissues and cartilage (243). Staphylococcal MSCRAMMs are absolutely critical in adhesion to host extracellular matrix materials.

Though once considered a predominately extracellular pathogen, ample evidence now suggests that *S. aureus* is a facultative intracellular bacterium. *In vitro*, *S. aureus* has been reported to invade a wide variety of nonprofessional phagocytic cell types including bovine epithelial cells (5), mouse fibroblasts (60), human tracheal epithelium (94), human keratinocytes (141), human umbilical vein endothelial cells (135), and human colonic epithelium (81). The most well characterized invasion mechanism is MSCRAMM-mediated involving fibronectin-binding proteins A and B. Fibronectin serves as a bridge between *S. aureus* FnbpA or FnbpB and the host cell receptor $\alpha5\beta1$ integrins, which, when bound, leads to signal transduction events and cytoskeltal rearrangements, resulting endocytosis of the bacteria (196). *S. aureus* possesses many enzymes, including proteases, lipases, nucleases, hyaluronate lyase, phospohlipase, and elastase, that are implicated in its dissemination through a host (70).

2.6 TOXINS OF *S. AUREUS*

S. aureus has the capacity to produce over 40 different extracellular proteins that directly correlate to its virulence potential (8, 87). There is a great deal of discrepancy among strains, however, pathogenic isolates are able to generate various combinations of these toxins (87). The toxins of *S. aureus* can be characterized into three main groups: pyrogenic toxin superantigens (PTSAgs), membrane damaging toxins, and epidermolytic toxins (87) (Fig. 2.2).

PTSAgs include staphylococcal enterotoxins and toxic shock syndrome toxin (TSST), which are responsible for staphylococcal food poisoning and toxic shock syndrome. These toxins are classified together because of their superantigen activity. A PTSAg does not undergo antigen processing; it interacts directly and nonspecifically binding MHC class II sites on antigen presenting cells with T cell receptors (18). The interaction results in polyclonal T cell activation with up to 20% of T cells being activated at once, leading to a cytokine storm potentially ending in lethal shock (191). There are currently 22 known members of the staphylococcal enterotoxins (SEs) and enterotoxin-like (SE*l*) family (8). These SEs are strong emetic gastrointestinal exotoxins. In addition to their superantigen activity, they stimulate the vagus nerve, which results in nausea and vomiting (8, 18). These PTSAgs are mobile and highly stable, as well as resistant to heat and acid. Therefore, it is the toxins and not necessarily the presence of the bacteria that results in disease (49, 50, 190).

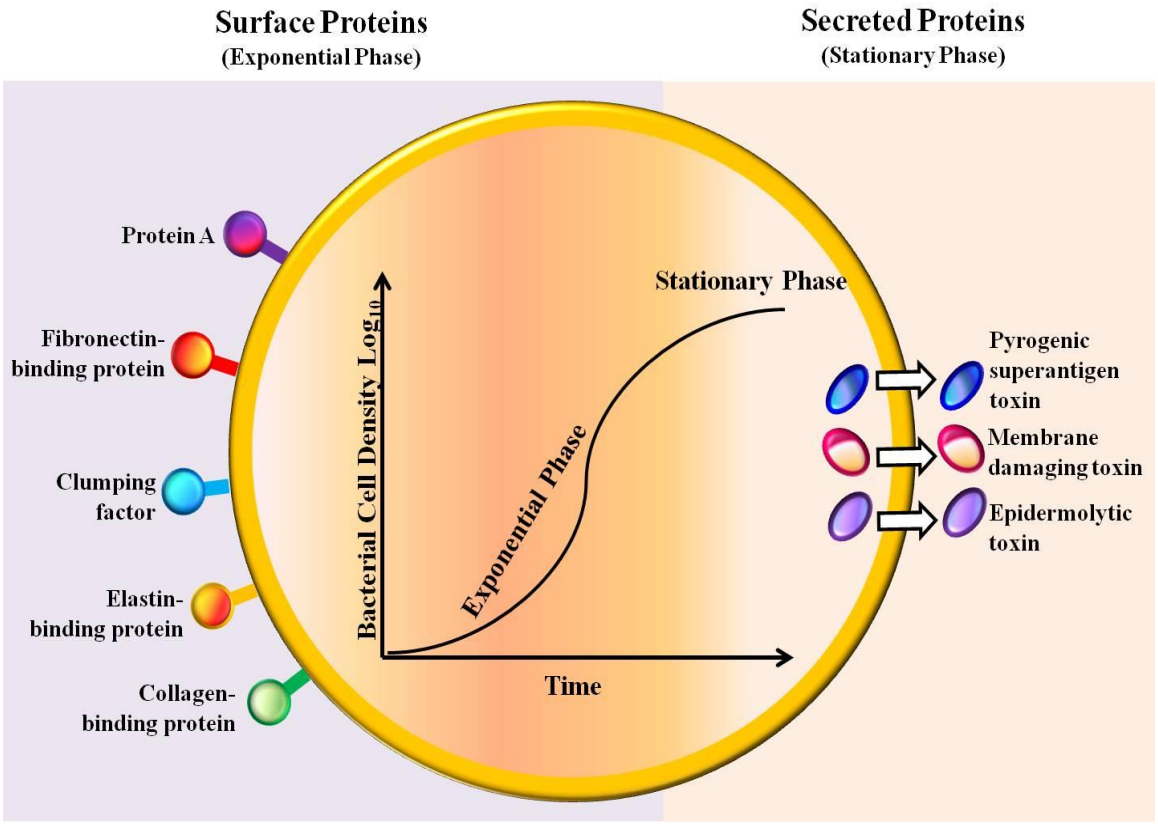


FIGURE 2.2. MSCRAMM Adhesins and Toxin Classes of *Staphylococcus aureus* Regulated in a Growth Phase-Dependent Manner. Protein A, fibronectin-binding protein, clumping factor, elastin-binding protein, and collagen-binding protein make up the MSCRAMMs of *S. aureus* and allow binding of the bacterium to host factors. These adhesins are preferentially expressed during exponential growth. The toxins of *S. aureus* are categorized into three classes based on their function, pyrogenic superantigen toxins, membrane damaging toxins, and epidermolytic toxins, and are expressed primarily during stationary growth phase. (Modified from Lowy, 1998).

S. aureus can produce hemolysins and leukocidin that damage host cell membranes (229). Of the four types of staphylococcal hemolysins, alpha-hemolysin has been the most thoroughly characterized. A high percentage of *S. aureus* strains synthesize alpha-hemolysin, and it is toxogenic to a variety of mammalian cells (44). Heptamers of monomeric alpha-hemolysin subunits assemble in eukaryotic cell membranes, resulting in the formation of 1 to 2 nanometer pores (12, 212). The presence of the pores results in an efflux of potassium as well as an influx of sodium, calcium, and other small molecules. This osmotic swelling ultimately leads to cell lysis (44).

Beta- and delta-hemolysins are also produced by *S. aureus* and lyse erythrocytes and other mammalian cells in a similar fashion to alpha-hemolysin (68, 103). Gamma-hemolysin and various leukocidins are two-component membrane-damaging toxins produced by *S. aureus* (71, 232). These pore-forming toxins target neutrophils, monocytes, and macrophages, and either alter the normal function of the cell or lead to cell lysis (215). Gamma-hemolysin is synthesized by most *S. aureus* strains (44), while Panton-Valentine leukocidin, highly associated with increased virulence, is only produced by 2 to 3% of strains (44, 120).

The epidermolytic toxins of *S. aureus* include exfoliative toxin A and B (ETA and ETB) and are responsible for staphylococcal scalded-skin syndrome (SSSS) and its localized form, bullous impentigo (7, 113). Both toxins act in an identical fashion by cleaving a desmosomal adhesion molecule, resulting in blister formation and sloughing of the skin (7).

2.7 VIRULENCE REGULATION IN *S. AUREUS*

There are in excess of 50 transcripts coding for the adhesins, invasins, and toxins that make up the virulon of *S. aureus*. For the most part, these genes are unlinked and require coordinate regulation, often in a temporal manner. Computational analysis of the *S. aureus* genome has revealed the presence of 16 two-component regulatory systems, many of which have been described in the regulation of staphylococcal pathogenesis (110). In addition to these two-component systems, *S. aureus* virulence is also regulated by the alternative sigma factor, σ^B (SigB) (31, 108, 109), DNA transcription factors (47), and regulatory RNAs (64, 157).

Of the 16 predicted two-component systems, the accessory gene regulator (*agr*), largely regulates the *S. aureus* virulon and is the most well understood (170, 179). Regulation by *agr* is density-dependent, and thus, this quorum-sensing system is responsible for the regulation of 138 transcripts, including the suppression of MSCRAMM adhesins and the up-regulation of invasins and toxins that occurs during the switch from exponential to stationary phase (47) depicted in Figure 2.2. The *agr* locus is comprised of two divergent transcripts under the control of two distinct promoters, P2and P3 (170). As depicted in Figure 2.3, P2 transcribes AgrB, AgrD, AgrC, and AgrA. These proteins are necessary for both P2 transcription and initiation of P3 (156, 157). The *agrD* gene codes for a propeptide that is processed by the protein product of *agrB*. The result of the processed AgrD is the autoinducing peptide (AIP) (237). The AIP is secreted from the bacterial cell and binds a histidine kinase receptor on the surface of the

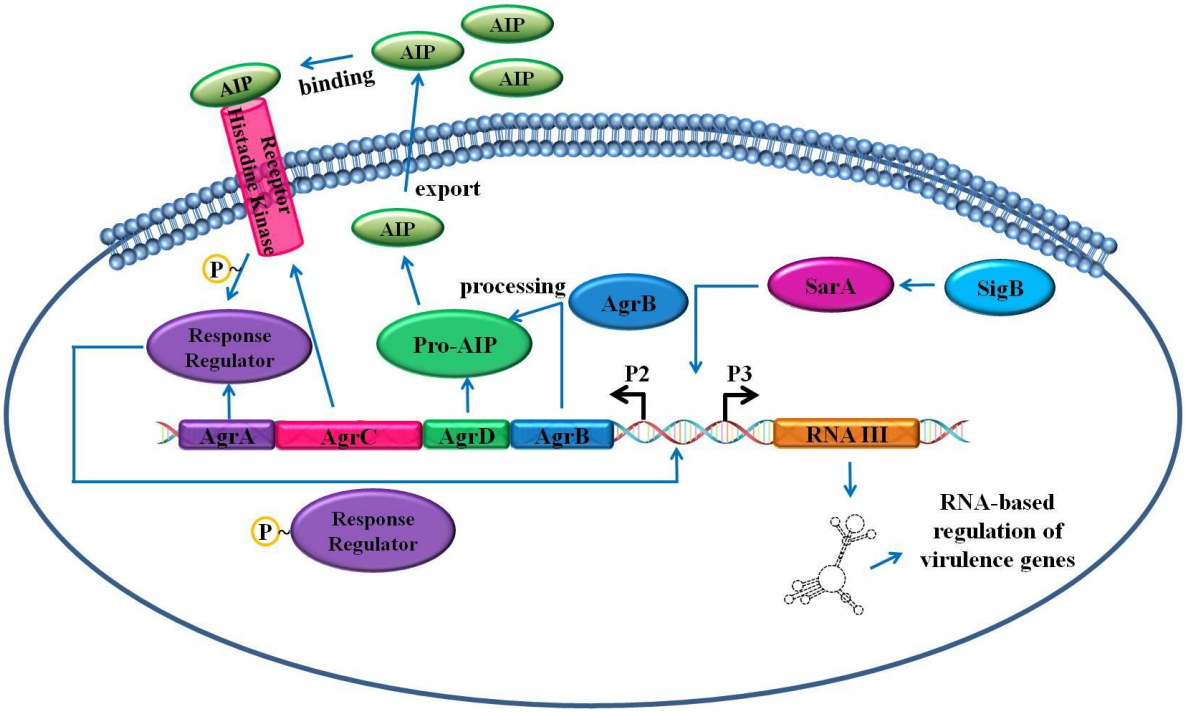


FIGURE 2.3. The Regulatory Circuit of the *agr* Quorum-Sensing System in Association with other Staphylococcal Regulators within the Bacterial Cell. The open reading frame of the *agrACDB* operon and RNAIII, transcribed from divergent promoters P2 and P3, are shown in colored boxes with their protein products in accordingly colored ovals. In this two-component system, *agrD* encodes a propeptide that is processed by the product of *agrB* into the autoinducing peptide (AIP). Cell-density-dependent accumulation of AIP is sensed via its binding to the transmembrane receptor histadine kinase product of *agrC*. The signal is transmitted through a phosphorylation cascade to the response regulator transcribed from *agrA*. The response regulator can now serve as a transcriptional activator of the P2 and P3 promoters. Additionally, the staphylococcal accessory regulator (SarA) can initiate expression from P2 and P3. The alternative sigma factor B (SigB) is also implicated in regulating both levels of SarA and RNAIII.

cell encoded by *agrC* (119). This binding leads to a conformational change in the AgrC receptor that results in a phosphorlyation cascade whereby a phosphate is transferred to the AgrA response regulator protein resulting in its activation (119). The now active response regulator is able to initiate transcription from P2 and P3 (156). Transcription from the P3 yields RNAIII, which has a high degree of secondary structure and is bifunctional in that its 5' end contains the open reading frame (ORF) for delta-hemolysin and its 3' end is a non-coding regulator (86). RNAIII is the regulatory effector molecule of the *agr* system. As AIP levels increase with growth, its expression reaches a critical threshold upon entering stationary phase that corresponds to increased RNAIII levels (214). At this point, RNAIII inhibits transcription of many MSCRAMM adhesins through antisense base pairing with these mRNAs and blocking the ribosomal binding site (86). In contrast, RNAIII binding to the mRNA of various toxins results in a freed ribosomal binding site and, consequently, their up-regulation (145). This finely tuned process is coordinated by the *agr* system via other staphylococcal regulators and promotes colonization of *S. aureus* during exponential growth phase and an increased potential for virulence during stationary growth phase to ensure its survival.

Numerous global regulatory proteins have been identified in *S. aureus* that function in both an *agr*-dependent and independent manner (17, 32, 47). For example, the DNA binding protein, SarA, is involved in the regulation of 120 transcripts, including many involved in staphylococcal pathogenesis (47). Furthermore, SarA is a transcriptional activator of both the P2 and P3 promoters of *agr* (30). In addition to the classical sigma factor σ^A, *S. aureus* possesses three alternative sigma factors (154). SigB

is up-regulated in response to environmental stressors such as acid and thermal stress and upon entrance into stationary phase (109). The SigB regulon in *S. aureus* consists of approximately 200 ORFs that encode virulence components, membrane transport processes, and proteins involved in cell wall metabolism (154). Many of these genes have a SigB consensus sequence in their promoter region. However, not all genes described as regulated by SigB have the consensus sequence, and numerous genes are negatively regulated by SigB, suggesting that they are affected indirectly by SigB, possibly in cooperation with another regulator (154). Interestingly, SigB has been connected to the increased expression of SarA and concurrently decreasing RNAIII levels revealing the interconnectedness of these three major regulators in *S. aureus* (17).

The role of RNAIII as a regulatory molecule regulating virulence in *S. aureus* has been understood for almost two decades (157). However, other regulatory RNAs have only recently been investigated in relation to the regulation of virulence. Regulation by these RNAs occurs either through interactions with proteins or base pairing with mRNAs to prevent or activate function (56). In contrast to previously characterized small non-coding RNAs in Gram-negatives, the regulatory RNAs thus far described in *S. aureus* are neither uniformly small nor non-coding (56). Many of these RNA molecules are longer than traditional small RNAs (sRNA), some in excess of 500 nucleotides, most likely to ensure efficient antisense base pairing with the mRNA they are regulating due to the low GC content of *S. aureus* (56). Additionally, it is often the high degree of secondary structure that regulates by either blocking or freeing ribosome binding sites (56). Furthermore, ORFs are frequently found in the 5' region of these RNAs that code for a

protein whereas the 3' untranslated region (UTR) is most often associated with regulation. In excess of 200 regulatory RNAs have been computationally predicted, many of which have been experimentally validated (56). In addition to RNAIII, whose regulation is well documented, SprD RNA operates in virulence regulation through antisense pairing to an immune evasion molecule and blocking translation initiation (29). Also of interest are the numerous hypothetical and experimentally confirmed members of the Rsa regulatory RNA family (64). For example, RsaE is conserved among *S. aureus* strains and is involved in regulating metabolic processes (20). Interestingly, many of the Rsa RNAs have typical SigB promoters and are themselves under its regulation (64). The regulatory RNA chaperone Hfq is necessary for the function of many sRNAs in *E. coli* and *S.* Typhimurium that govern virulence (107, 197); the role of Hfq in *S. aureus* is currently under debate, as Hfq is not expressed in all strains. When present, the deletion of *hfq* in the strains, *S. aureus* RN6390, Newman, and COL, was found to have no effect on the physiology or virulence of the organism (19). In direct contrast, however, its deletion from *S. aureus* 8325 resulted in decreased toxicity and virulence, revealing that in some *S. aureus* strains, Hfq is a global regulator governing pathogenesis (124). More recently, Hfq in *S. aureus* N315 has been associated with the organism's response to environmental shear levels (27).

Virulence regulation in *S. aureus* is extremely complex. *S. aureus* utilizes multiple two-component systems to sense and respond accordingly to its environment. Numerous DNA transcription factors, the alternative sigma factor SigB, and many regulatory RNAs, in addition to the various two-component systems, function to regulate

virulence factors in *S. aureus*. Moreover, these assorted mechanisms of regulation are integrated and intertwined. While there is substantial genetic diversity among *S. aureus* strains, the assimilation of these regulatory networks allows for compensation of virulence regulation if a factor is not expressed or becomes inhibited.

2.8 Response of *S. aureus* to Shear Force

During the lifecycle of *S. aureus*, it must survive in, and adapt to, a multitude of varying environmental conditions as it moves from host to host. Moreover, once associated with a host, the site of colonization and/or infection further increases the range of environmental stimuli experienced by the bacterium. Therefore, *S. aureus* has evolved many mechanisms that provide it with the ability to sense and respond to these contrasting environmental conditions, thus affording its long term persistence in a host and/or the environment.

Fluctuations in fluid shear are encountered by *S. aureus* throughout the course of infection and/or colonization (27). The high-fluid-shear levels within the arterial blood vessels have been well documented and range between 10 to 70 dynes/cm^2 (131). As a leading cause of sepsis, *S. aureus* experiences these high-fluid-shear levels and has the capability to sense and respond accordingly (61, 230). Investigations using flow chambers to reproduce shear rates comparable to the blood flow have demonstrated that staphylococcal MSCRAMMs function in a shear-dependent manner (158, 167). Specifically, these studies have established a correlation between fluid shear force and *S. aureus* adherence in which the efficiency of binding via protein A and clumping factor A

to fibrinogen and von Willebrand factor-coated surfaces peaks as the fluid shear is decreased (158, 167). This inverse relationship holds for *S. aureus* binding to platelets as well. As the fluid shear increases, the binding efficiency decreases (158, 167).

In addition to MSCRAMM-mediated responses to fluid flux, *S. aureus* is also able to alter its biofilm architecture according to variations in fluid shear (102, 186). The characterization of staphylococcal biofilms formed under varying degrees of physiological oscillatory shear stress revealed that the most turbulent flow conditions corresponded to the thickest biofilm morphology as well as the highest level of antibiotic resistance (102). Furthermore, as a consequence of fluid shear, the viscoelastic nature of *S. aureus* biofilms allows them to undergo rolling migration, while remaining attached, thus prompting bacterial dissemination while preserving the protection by the biofilm state (186).

Cells, both host epithelium and inhabiting microbes, within the intestine experience the fluid-shear variances that are characteristic of this site (9). High levels of shear are created by a bolus passing through the intestinal lumen, while the shear rate decreases with proximity toward the intestinal wall. The mucus layer provides a physical barrier that further serves to reduce the shear experienced by the underlying cells (168). Additionally, the viscoelasticity of the mucus provides protection to objects within it from high-shear stress (11). It has been postulated that, if the intestinal epithelium lacked microvilli, they would experience shear levels of approximately 5 dynes/cm^2; however due to the presence of microvilli the shear rate present between this brush border of intestinal epithelial cells is less than 1 dyne/cm^2 (76). Therefore, as an intestinal

colonizer (1, 16, 43, 181) that adheres to intestinal mucus (217), *S. aureus* experiences the low-fluid-shear levels imparted by this site.

The response of *S. aureus* to low-fluid-shear levels consistent with that found within the intestines has been documented through its culture in the RWV bioreactors (27, 184, 221). No differences in growth or MICs of various antibiotics were observed among three clinical isolates of *S. aureus* cultured in a rich media under low-shear conditions (184). However, these strains collectively demonstrated decreased carotenoid production, an altered ability to lyse red blood cells, and the differential expression of two genes involved in metabolism and transport (184). In contrast, separate analysis of a different *S. aureus* strain cultured in a diluted rich media under low-shear conditions suggested increased growth and increased membrane integrity (221). Although these observations have recently begun to describe the effect of low-shear culture on *S. aureus*, the role of this environmental parameter on biofilm formation and other down-stream staphylococcal responses remains unclear.

A caveat of pathogenic microorganisms is their inherent capability to adjust their virulence characteristics in response to ever changing, often extreme, environmental parameters, including low-fluid-shear, thus ensuring their survival. Investigations into the response of *S. aureus* pertaining to environmental conditions it encounters while *in vivo,* where it rarely causes disease (such as the nasal pharynx, unbroken skin surface, and intestine) are lacking. Investigating the response of a bacterium to these varied environmental factors will provide insight into the potential role of these conditions

triggering either colonization or an infectious phenotype and the associated mechanism(s).

METHODS

Chapter 3: Materials and Methods

3.1 Bacterial Strains and Growth Conditions

Methicillin-resistant *S. aureus* N315 (kindly provided by Dr. Mark Shirtliff, University of Maryland Dental School) was used for all studies. Methicillin-sensitive *S. aureus* 8325 (kindly provided by Dr. Naomi Balaban, Tufts University) was used in experiments as mentioned. Prior to every experiment, *S. aureus* was taken from a frozen stock, inoculated into tryptic soy broth (TSB), and grown statically, overnight. Aliquots of the culture were diluted (1:200) in fresh TSB and loaded into 50 ml RCCS-1 RWV bioreactors (Synthecon, Houston, TX) in both the LSMMG orientation (Fig. 1B) and control orientations (Fig. 1B). Care was taken to ensure that no bubbles were present within the reactors that could prevent disruption of the low-shear condition. The bioreactors were placed in a 37 °C incubator and operated at 25 revolutions/minute for 20 hours, at which point stationary phase was achieved.

3.2 Growth Profiles

S. aureus cultures were initiated in the RWV as described above. Following 2, 4, 6, 8, and 10 hours of growth, respectively, a 1.5 ml aliquot was removed, using a 3 ml

luer lock syringe, from sampling ports on the face of the RWV. For all time points greater than 10 hours, the entire RWV culture volume was used for enumerating samples by removing and vigorously vortexing the contents of the vessels, to ensure dispersion of any aggregates that formed during culture. Homogeneous dispersion was confirmed using light microscopy. For all time points, samples were enumerated using serial dilution with phosphate buffered saline (PBS) and plated on tryptic soy agar (TSA) followed by overnight culture at 37 °C. Growth curves were determined using a minimum of three separate cultures for each time point.

3.3 Environmental Scanning Electron Microscopy (ESEM) Imaging

Following 20 hours of growth in the RWV, cultures of *S. aureus* N315 in the LSMMG orientation resulted in the presence of visible aggregates in the fluid phase of the vessel. The aggregates, as well as samples from the control vessel, were carefully removed from only the fluid phase of each vessel and fixed with 2.5% gluteraldehyde and 1% formaldehyde for 30 minutes at room temperature. The samples were washed three times with filter-sterilized, deionized water by gentle inversion then loaded onto silicon wafer chips on T stubs. Samples were then dried in the chamber of the electron microscope and imaged using a Philips XL 30 ESEM (FEI Co. Hillsboro, OR).

3.4 Antibiotic Resistance Assay

Antibiotic resistance assays were performed using identical cultures of *S. aureus* N315 in four RWVs, two of which were operated in the LSMMG orientation and two in

the control orientation (Fig. 1B). After 20 hours of culture, one vessel from the LSMMG and one from the control orientation were used to determine the initial cell concentrations, defined as 100% survival, using direct plating as described above. The two remaining vessels were carefully removed from their rotation base units to retain the aggregate structure, and 2.5 ml of the media was replaced with 2.5 ml of ciprofloxacin to a final concentration of 25 μg/ml, corresponding to 50 times the MIC. To ensure that fluid shear force was no longer a variable, the vessels were allowed to sit statically at room temperature for 24 hours, at which point cell concentration was determined by serial dilution and plating as described above. Antibiotic resistance levels were achieved using three independent biological samples.

3.5 CAROTENOID EXTRACTION

Quantitative comparisons of carotenoid levels between LSMMG and control cultures of *S. aureus* N315 were achieved spectrophotometrically as previously described by Marshall, et al. (133). Briefly, based on growth curve estimates at 20 hours, approximately 3.0×10^7 cells were removed from both LSMMG and control cultures. Samples were pelleted by centrifugation at 10,000 × *g* for 3 minutes, then washed three times with PBS and resuspended in 500 μl of methanol, followed by a 5 minute incubation in a 55°C water bath. The cells were then pelleted again and the supernatants recovered. *Staphylococcus aureus* 8325, which has a naturally occurring mutation in a positive regulator of sigma factor B resulting an unpigmented phenotype (108), was extracted as above and used to establish a baseline absorbance. The absorbance of the

resulting supernatant, containing the extracted carotenoid pigments, was recorded at 460 nm. The carotenoid extraction protocol was followed for samples from three separate cultures.

3.6 OXIDATIVE STRESS ASSAY

The susceptibility to oxidative stress was determined for both *S. aureus* N315 and 8325. After 20 hours of culture, the contents of the reactors were removed, placed into sterile containers, and vigorously vortexed for 30 seconds to disband biofilms. A concentrated stock solution of hydrogen peroxide (H_2O_2) was immediately added to approximately 30 ml of both LSMMG and control cultures, as cell numbers evaluated were equalized based on growth curves, bringing the overall H_2O_2 concentration to 30 mM. Viable microbial concentrations were determined every 15 minutes using serial plate counts as described above.

To determine the duration of the LSMMG effect, the oxidative stress assay was repeated. *S. aureus* N315 was cultured for 20 hours, at which point the RWV bioreactors were stopped and the contents removed and immediately placed into sterile containers where they were allowed to sit statically. At various kinetic time points the oxidative stress assay was performed on these samples. All oxidative stress assays were preformed in triplicate.

3.7 ACID STRESS ASSAY

The susceptibility to acid stress was determined for both *S. aureus* N315 and 8325. After 20 hours of culture, the contents of the reactors were removed, placed into sterile containers, and vigorously vortexed for 30 seconds to disband biofilms. A concentrated citrate buffer adjusted to pH 3.0 was immediately added to approximately 30 ml of both LSMMG and control cultures. A sample was instantly removed, time zero, and every 15 minutes over a 60 minute time course. Viable microbial concentrations were determined using serial plate counts as previously described.

3.8 THERMAL STRESS ASSAY

The susceptibility to thermal stress was determined for both *S. aureus* N315 and 8325. After 20 hours of culture, the contents of the reactors were removed, placed into sterile containers, and vigorously vortexed for 30 seconds to disband biofilms. A 1 ml aliquot was transferred from the sterile container to a microcentrifuge tube and placed in a pre-warmed heat block set at 51°C. 51°C was empirically determined to be the temperature required to document *S. aureus* killing over a 60 minute time course. Samples were enumerated for survival every 15 minutes over a 60 minute time course using serial plate counts as previously described.

3.9 WHOLE BLOOD KILLING ASSAY

All relevant blood assays were reviewed and approved by the Committee for the Protection of Human Subjects at the NASA Johnson Space Center prior to implementation. After 20 hours of culture, *S. aureus* N315 from the LSMMG or control RWV was placed in sterile containers, and vigorously vortexed for 30 seconds to disband biofilms. Cell concentrations were adjusted to approximately 1×10^4 cells in 100 µl of PBS. Blood was collected from healthy human subjects in heparinized tubes to coincide with the 20 hour culture time point. 900 µl of blood was immediately added to the PBS bacterial solution. The infected blood was placed in a 37 °C incubator with agitation for 4 hours. Samples were removed at predetermined time points and viable microbial concentration was determined by serial dilution as described above. The whole blood killing assay was performed with three technical replicates and repeated in duplicate with blood from two separate subjects.

3.10 RNA ISOLATION

Samples of LSMMG- and control-cultured *S. aureus* N315 were immediately incubated with a 2:1 volume of RNAprotect (Qiagen, Valencia, CA) at room temperature for 10 minutes with occasional mixing to allow for RNA stabilization. Samples were centrifuged at 5,000 × *g* for 5 minutes resulting in pellets that were stored at -70°C until RNA isolation was performed. The pellets were resuspended in 350 µl of RLT lysis buffer (Qiagen) containing 3.5 µl of 2-mercaptoethanol. The suspension was added to

approximately 250 µl of ice cold Zirconia beads (Ambion, Austin, TX) in screw cap tubes. Cells were lysed by bead beating at maximum speed for 5 minutes using the Mini-Beadbeater-8 (Biospec Products, Bartlesville, OK). The lysed cell suspension was added to QIAshredder columns (Qiagen) and centrifuged at 10,000 × *g* for 1 minute. To the homogenized lysate, 250 µl of 200 proof ethanol was added; this mixture was applied to an RNeasy mini column (Qiagen) and the standard protocol for the RNeasy mini kit (Qiagen) was followed according to the manufacturer's instructions. Contaminating genomic DNA was removed with TURBO DNA-free (Ambion). Denaturing agarose gel electrophoresis was used to assess the quality of RNA by visualization of intact 16S and 23S rRNA bands. RNA quantity was measured with a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Purified RNA was stored at -70°C until use in downstream applications.

3.11 MICROARRAY ANALYSIS

Gene expression profiling was achieved using the Affymetrix (Santa Clara, CA) GeneChip *S. aureus* genome array containing probe sets for over 3,300 open reading frames based off sequence information from four *S. aureus* strains, including N315. Total RNA was isolated as described above. Quality of the RNA was evaluated with an Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA). GeneChip processing was performed as previously described by Dunman, et al. (47). Briefly, 25 µg of total RNA was reverse transcribed and the resulting cDNA was labeled and

fragmented to produce oligonucleotide probes in accordance with the manufacturer's instructions (Affymetrix) for antisense prokaryotic arrays. The probes were then hybridized to *S. aureus* specific GeneChips using a GeneChip Hybridization Oven 640 (Affymetrix). The chips were then washed and stained using a GeneChip Fluidics Station 400 (Affymetrix). The chips were scanned and data analyzed using an Affymetrix GeneArray Scanner (Affymetrix). To accurately compare expression patterns, each GeneChip was hybridized with LSMMG- and control-cultured cDNA from the same date of culture. Each experiment was performed in duplicate from independent biological samples. Analysis of the data by GCOS (Affymetrix) and ANOVA, filtered with a twofold cutoff limit and p-value of < 0.05, was used to generate a list of genes that demonstrated differential expression profiles in response to LSMMG culture.

3.12 QRT-PCR

Total RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) into cDNA as per the manufacturer's instructions. Primers to these genes (Table 2) were designed and purchased from Invitrogen (Carlsbad, CA) using their OligoPerfect™ Designer. Standard curves were generated by absolute quantification of targets for each primer pair using the QuantiFast SYBR Green PCR Kit (Qiagen) and 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) to verify primer efficiencies as well as the absence of dimers and nonspecific products via dissociation curve production. PCR products were also assessed for the correct amplicon size via agarose gel electrophoresis. The QuantiFast SYBR Green PCR Kit (Qiagen) and

Table 2. Primer Pairs used in this study

Primer	N315 ORF	Sequence 5' → 3'
16SF	SArRNA01	ACCGTGAGGTCAAGCAAATC
16SR	SArRNA01	GTACAAGACCCGGGAACGTA
hfqF	SA1145	CGAAAACATCCAAGACAAAGC
hfqR	SA1145	AAGTGCTGATCGCATGTTTG
cspAF	SA1234	GGTTTAACGCTGAAAAAGGATTCG
cspAR	SA1234	TAACAACGTTTGCAGCTTGTGGAC
sigBF	SA1869	GAAATTGGGCCAAGAATCAA
sigBR	SA1869	TTTGTCCCATTTCCATTGCT
rexF	SA1851	TGACTCGGCAACAATTCGTCGT
rexR	SA1851	GGTTCCCAACTCCGACAATTGCGA
asp23F	SA1984	TCGCTGCACGTGAAGTTAAA
as23R	SA1984	CAGCAGCTTGTTTTTCACCA
uspF	SA1532	GCTTAAATGCCGTGGAAAGA
uspR	SA1532	GTGGTTGGAAGTCTGCTGGT
tdcFF	SA0455	AGATTACCGGAAGCACTTGG
tdcFR	SA0455	CAGCGCTTACGATATGTCCA
mvaSF	SA2334	CTTTTGCACGTTGCTTTGAA
mvaSR	SA2334	TGTGCAATAACCATCGCAAC
mvaAF	SA2333	CTCGTTTCCGAAGGTATCCA
mvaAR	SA2333	GCGCAATTTCATCACCTTTT
mvaK1F	SA0547	TTATATGATGCGCCTGACCA
mvaK1R	SA0547	GCTGCACTCGATCCTAATCC
mvaK2F	SA0549	CAAAAGATGGTGCGTCAGAA
mvaK2R	SA0549	ACCAGCGCCTGATGTTTTAG
mvaDF	SA0548	TGTCGTTGACACGAAACACA
mvaDR	SA0548	GGTGTTGATCCCAGATTCGT
fniF	SA2136	TAATGCAATGACGGGTGGTA
fniR	SA2136	CATGCGTGGATTTCTCAATG
ispAF	SA1532	GGGCAGCGATCTTGAAAATA
ispAR	SA1532	CCACTGCTGCGTCTCTATGA
crtMF	SA2349	CGTAGAATCATGATGGCGCTTCAG
crtMR	SA2349	TCACCTACTGTACCAGCAACACCA
crtNF	SA2348	AGCCCGTATTGCTTCTCAAGGTCA
crtNR	SA2348	TGGCATCATGACAATTGTGGGACC
crtPF	SA2351	AAGACGTGTGACGGGTGTCAGATT
crtPR	SA2351	TGCCGGCTCAAATTCCCTCTCTAA
crtQF	SA2350	AGCACGTTCATATGGTGCGACTGT
crtQR	SA2350	CGATTCGTACATGCATGCGTCACA
crtOF	SA2352	AGAGACAAAGAGGGCAGAGTTG
crtOR	SA2352	CTCGGTCGATTATAGCGTTGCACA

7900HT Fast Real-Time PCR System (Applied Biosystems) were again used to establish expression levels. Relative expression values were determined by using the ΔΔCt method via SDS 2.2.2 software by means of relative quantification using 16S rRNA as the endogenous reference gene (Applied Biosystems). All quantitative PCR evaluations were repeated in triplicate from three independent experiments.

3.13 CELL CULTURE

3.13.1 Epithelial Cell Lines

The human colonic adenocarcinoma epithelial cells lines HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-37) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). HT-29 cells were cultured as monolayers in 75-cm^2 T-flasks in McCoy's 5A medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic solution containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Caco-2 cells were cultured in Minimal Essential Media (MEM) (Gibco) containing 20% FBS and 1% penicillin/streptomycin solution (Gibco) in 75-cm^2 T-flasks. Both cell lines were cultured to 80% confluency in a 37 °C, 5% CO_2 incubator. Upon reaching the desired confluency, the HT-29 and Caco-2 cells were liberated with 3 mls of 0.25% Trypsin-EDTA (Gibco), collected by centrifugation, and resuspended in the appropriate complete culture medium. Cells were then seeded into 24-well plates and placed in the 37 °C, 5% CO_2 incubator for downstream infection assays. Three wells were used for cell

enumeration via the Trypan Blue (Life Technologies) exclusion method on the day of infection to ensure an accurate multiplicity of infection (MOI).

3.13.2 Macrophage Cell Line

The murine macrophage cell line J774 (ATTC TIB-67) was obtained from ATCC. J774 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin solution (Gibco) in 75-cm^2 T-flasks. At 80% confluency, J774 macrophages were dislodged by cell scraping in the presence of prewarmed PBS (Gibco). Macrophages were collected by centrifugation and resuspended in complete growth media. Cells were seeded into 24-well plates and placed in the 37 °C, 5% CO_2 incubator for downstream infection assays. Three wells were used for cell enumeration via the Trypan Blue exclusion method on the day of infection to ensure an accurate MOI.

3.14 Adhesion Assay

RWV bioreactors were stopped after 20 hours of *S. aureus* culture. Low-shear- and control-cultured bacteria were collected by brief centrifugation, washed, and resuspended in either McCoy's 5A or MEM cell culture medium at concentrations to provide a MOI of 20. Monolayers of HT-29 and Caco-2 epithelial cells at approximately 90-95% confluency in 24-well plates were washed three times with warm PBS. The *S. aureus* suspensions in McCoy's 5A or MEM cell culture medium were added to HT-29 or Caco-2 cells, respectively. The 24-well plates were centrifuged at 200 × *g* for three

minutes to allow bacteria to interact with the cells. Afterward, the infected monolayers were placed in the 37 °C, 5% CO_2 incubator for one hour. After one hour, the media was removed from all infected wells, and the monolayers were washed three times with warm PBS. To determine the number of adherent bacteria, the cells were lysed with ice cold 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and the bacteria were enumerated by serial dilution with PBS and plating on TSA followed by overnight culture at 37 °C.

3.15 INVASION ASSAY

Invasion rates of low-shear- and control-cultured *S. aureus* were determined by the gentamicin and lysostaphin protection assay as previously described (2). HT-29 and Caco-2 monolayers were infected with low-shear- and control-cultured *S. aureus* for the adhesion assay with the addition of 0.1% FBS in the cell culture media. After a three minute centrifugation at 200 × *g*, the infected monolayers were placed into a 37 °C, 5% CO_2 incubator for 90 minutes, allowing for *S. aureus* invasion. After 90 minutes, the media was removed and the monolayers were washed three times with warm PBS. HT-29 cells received fresh McCoy's 5A media supplemented with 10% FBS and 50 μg/ml gentamicin plus 20 μg/ml lysostaphin and Caco-2 monolayers were replenished with MEM plus 20% FBS in addition to 50 μg/ml gentamicin (Sigma) and 20 μg/ml lysostaphin (Sigma) to kill adherent and extracellular bacteria. The cells were placed back into the incubator for the selected time points. To determine the number of intracellular bacteria, the cells were lysed with ice cold 0.1% Triton X-100, and the

bacteria were enumerated by serial dilution with PBS and plating on TSA followed by overnight culture at 37 °C.

3.16 Intracellular Survival Assay

Low-shear- and control-cultured *S. aureus* were collected by brief centrifugation, washed and resuspended in DMEM containing 0.1% FBS at an MOI of 20. Monolayers of J774 macrophages in 24-well plates were washed three times with warm PBS and challenged with the infected media. The plates were centrifuged at 200 × *g* for three minutes to allow bacteria to interact with the macrophages and placed in the 37 °C, 5% CO_2 incubator for 60 minutes to allow for phagocytosis. After 60 minutes, the media was removed from the wells, the macrophages were washed three times with warm PBS and given fresh DMEM supplemented with 10% FBS and 25 μg/ml gentamicin and returned to the incubator for a specified time. To determine the number of surviving intracellular bacteria, the cells were lysed with ice cold 0.1% Triton X-100, and the bacteria were enumerated by serial dilution with PBS and plating on TSA followed by overnight culture at 37 °C.

3.17 Statistical Analysis

Student's *t* tests were used to determine significance between control and LSMMG samples. A p-value of < 0.05 was considered statistically significant and is denoted by an asterisks (*) in the relevant figures.

RESULTS AND DISCUSSION

Chapter 4: Phenotypic Adaptations of *Staphylococcus aureus* in Response to Low-Fluid-Shear Culture

4.1 INTRODUCTION

A wide variety of pathogens, including *S. aureus*, have developed phenotypic adaptations that lead to their prolonged survival in the changing environmental conditions they encounter in human hosts (6, 69, 138, 210, 236). A classic example is the emergence of small colony variants (SCVs) of *S. aureus* from patients with recurrent infections or after culture within certain cell types (209). SCVs differ from their wild-type counterpart in that they display decreased pigmentation, are nonhemolytic, grow extremely slowly on complex media, often exhibit increased antibiotic resistance (172), and are significantly less virulent upon animal infection (195). In addition to SCVs, numerous reports describe phenotypic alterations in *S. aureus* after exposure to various environmental stressors (28, 31, 199). For example, heat-stressed *S. aureus* cells display an increased susceptibility to osmotic stress (199), while exposure of *S. aureus* to a sublethal pH 4.0 increases its survival when exposed to a lethal pH of 2.0 (31). A similar preadaptation to oxidative and thermal stress has also been documented in *S. aureus* (28).

As previously discussed in chapter two, staphylococcal adhesion, biofilm architecture, pigmentation, growth rate, and hemolytic ability are altered in response to changes in fluid-shear (27, 102, 158, 167, 184). As *S. aureus* may encounter low-fluid-shear conditions during colonization, an assessment of the alterations in phenotypic characteristics of *S. aureus* culture in this environment, as well as comparisons to other bacteria, will provide greater understanding of the role of low-fluid-shear as an environmental cue dictating colonization and virulence.

4.2 RESULTS

4.2.1 Formation of Attachment-Independent Biofilms by *S. aureus* in Response to Low-Fluid-Shear Culture

Cultures of *S. aureus* N315 were grown in both the LSMMG and control orientations described in Chapter 3. After approximately 18 hours, the LSMMG culture of *S. aureus* N315 resulted in the formation of visible aggregates in the fluid phase. After 20 hours, rotation was stopped, the vessels were carefully taken from their bases, and the large filling port on the face of the RWV was gently removed to allow recovery of the intact bacterial aggregates. Samples were immediately placed in a glutaraldehyde and formaldehyde fixative for imaging using scanning electron microscopy. Evaluation of these images indicated that the LSMMG cultures produced significantly higher amounts of extracellular polymeric substance (EPS) that enveloped the low-shear-cultured cells to

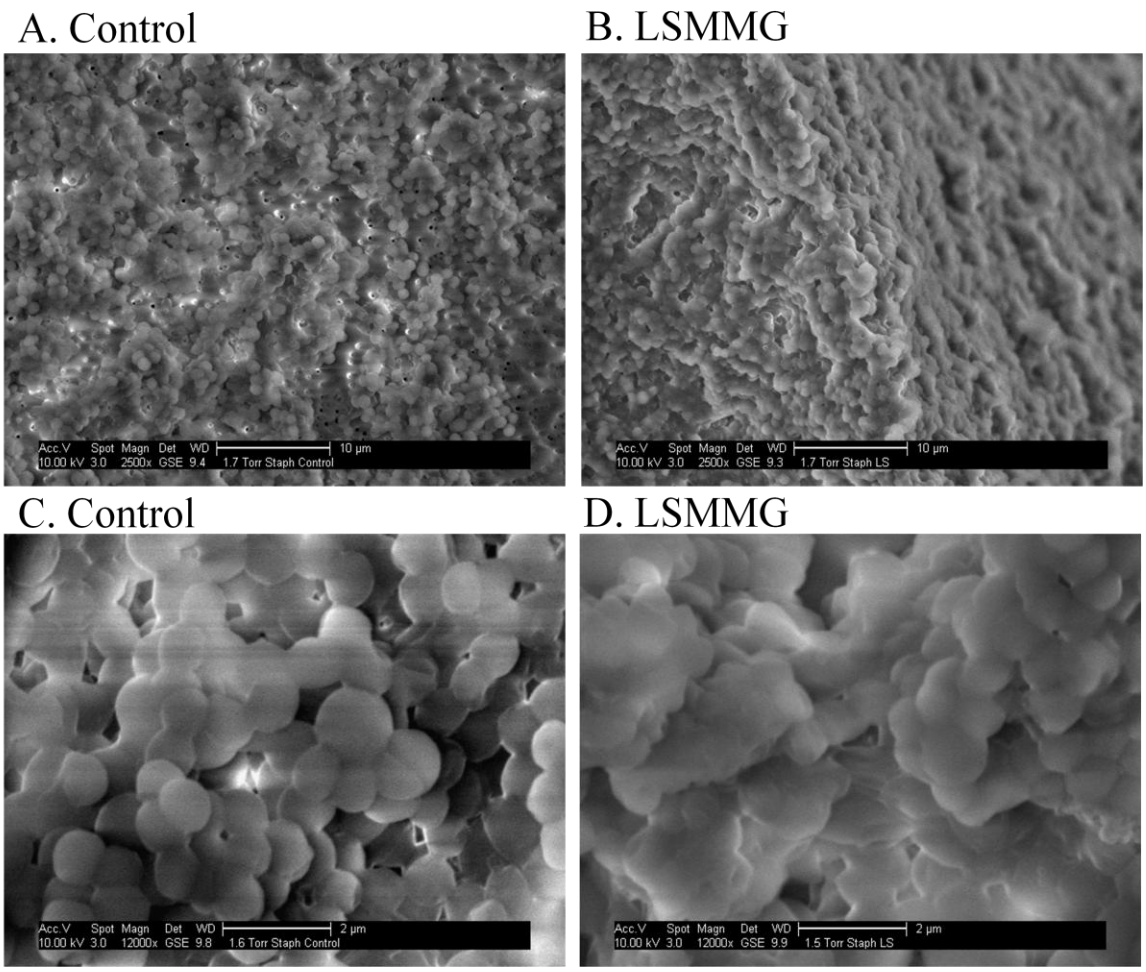


FIGURE 4.1. Environmental Scanning Electron Microscopy Images of Control- and Low-Fluid-Shear-Cultured *Staphylococcus aureus* N315. Control-cultured *S. aureus* at 2,500× (A) and 10,000× (C) magnification demonstrated that individual staphylococcal cells were clearly visible. *S. aureus* cultured under LSMMG conditions at 2,500× (B) and 10,000× (D) confirmed that the cells were much less visible and completely encased in an EPS matrix. (Castro, 2011)

a much greater extent than the control cultures (Fig. 4.1) (27). The production and subsequent encasement of *S. aureus* by EPS is characteristic of a biofilm.

4.2.2 Low-Fluid-Shear-Induced *S. aureus* Aggregates Display Increased Antibiotic Resistance

Supportive evidence for the development of a biofilm phenotype is an enhanced resistant to antibiotic stress when compared to planktonic cultures (193). As the LSMMG environment confers a visibly higher degree of EPS production, the low-shear aggregates were assessed for alterations in ciprofloxacin resistance as compared to cell clusters from control cultures.

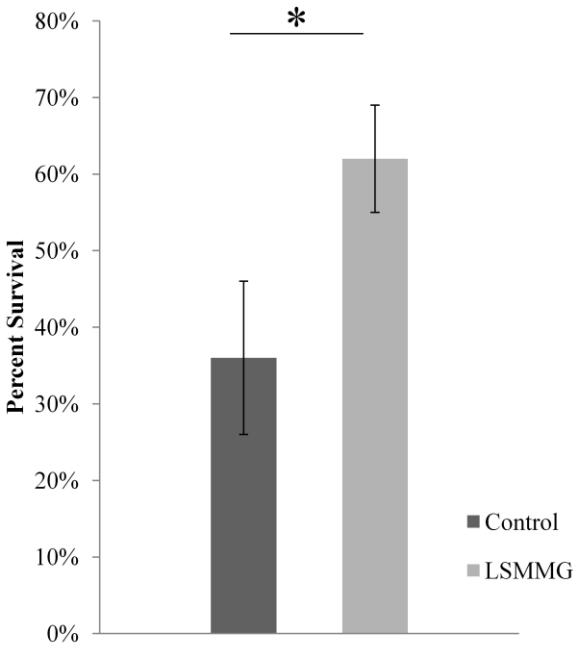


FIGURE 4.2. Levels of Antibiotic Resistance for Control- and LSMMG-Cultured *Staphylococcus aureus* N315. Low-fluid-shear-induced bacterial aggregates were 1.72-fold more resistant to ciprofloxacin as compared to bacteria cultured in the control orientated RWV. ($*$, $P < 0.05$). (Castro, 2011)

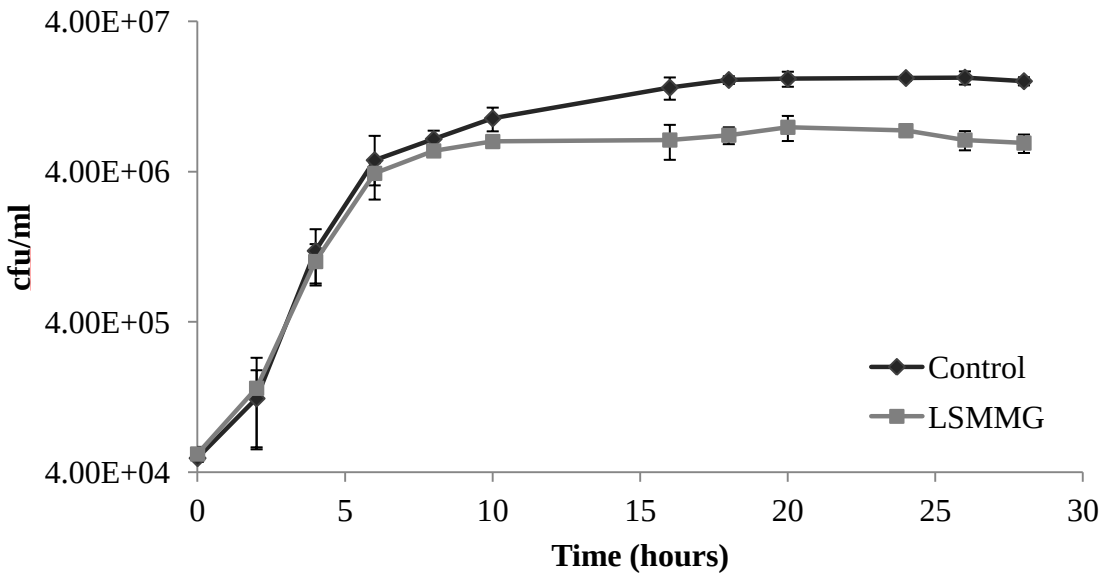
After 20 hours of culture, rotation was stopped, and the vessels were carefully taken from their bases. Care was taken as to not disrupt the structure of the low-shear-induced aggregates. Ciprofloxacin was injected directly into the statically resting vessels. After 24 hours of exposure to the antibiotic, the low-shear-cultured aggregates were 1.72-fold more resistant to ciprofloxacin as compared to control-cultured bacteria (Fig. 4.2) (27). This increase in antibiotic resistance is consistent with characteristics of surface-attached bacterial biofilms (193).

4.2.3 Low-Fluid-Shear Culture Results in Diminished Final Cell Concentrations of *S. aureus*

Previous research indicates that biofilm conditions affect bacterial metabolism and growth (193) and various microorganisms, including *S. aureus*, have demonstrated altered generation times and overall growth profiles in response to a low-shear culture environment (211, 221, 227); therefore, growth profiles were constructed for *S. aureus* during LSMMG culture. Previous investigations describing *S. aureus* growth in the RWV reported both no change in the growth of three clinical isolates (RF1, RF6, and RF11) (184) and increased growth in the strain *S. aureus* 25923 (221). To determine if the observed alterations were a result of a strain-specific effect, the growth profiles of both *S. aureus* N315 and 8325 were evaluated.

Growth curves comparing cell concentration in the LSMMG and control orientations were generated by collecting samples from the vessels every two hours after inoculation. For hours 2 through 10, samples were collected via the attachment of a

A.



B.

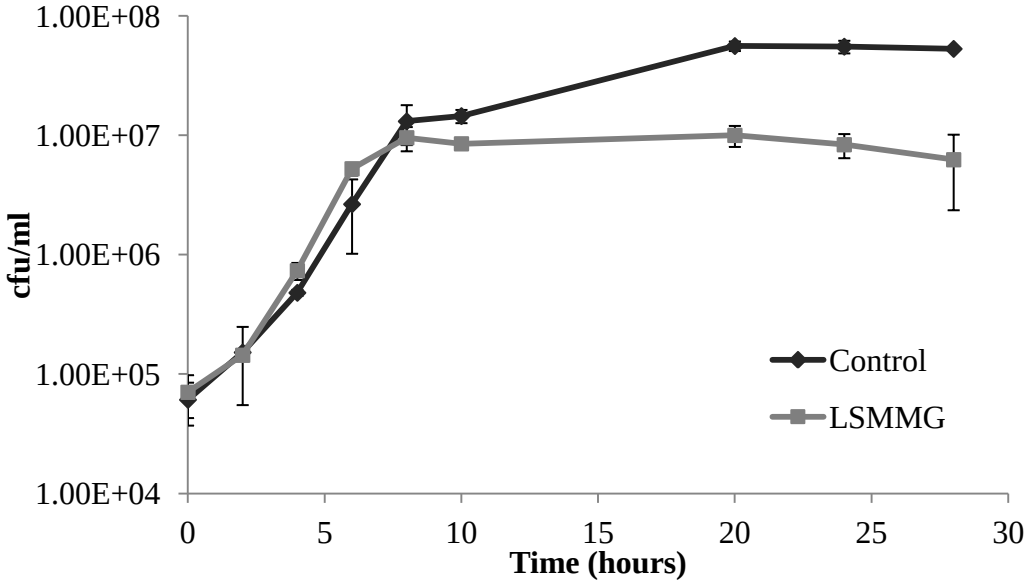


FIGURE 4.3 Comparative Growth Curves of *Staphylococcus aureus* Cultured in either the LSMMG or Control Oriented Rotating-Wall Vessel. Bacteria cultured in the low-shear environment resulted in a 2.9-fold lower total cell density for *S. aureus* N315 (A) and a 5.6-fold lower total cell density for *S. aureus* 8325 (B) in cell concentrations as compared to respective control cultures. (Castro, 2011)

sterile luer-lock syringe on the sampling ports and the removal of a 1 ml aliquot of the vessel contents. For all time points greater than 10 hours, the entire contents of the vessel were removed and vigorously vortexed prior to enumeration for both samples and strains to ensure homogenous cell distribution. Equivalent results were obtained with both *S. aureus* strains, N315 and 8325, as control cultures reached significantly higher concentrations than LSMMG cultures. After 20 hours of culture, cell concentrations were $1.86 \times 10^7 \pm 2.6 \times 10^6$ cfu/ml versus $6.27 \times 10^6 \pm 1.32 \times 10^6$ cfu/ml (means ± standard deviations) for control- and low-shear-cultured *S. aureus* N315, respectively (Fig. 4.3A), demonstrating a 2.9-fold greater cell concentration in the control orientation (27). *S. aureus* 8325 paralleled the trend of this response. Following 20 hours of culture, cell concentrations were $5.6 \times 10^7 \pm 7.12 \times 10^6$ cfu/ml versus $1.0 \times 10^7 \pm 2.0 \times 10^5$ cfu/ml for control and LSMMG cultures, respectively (Figure 4.3B), demonstrating a 5.6-fold greater cell concentration in the control orientation (27).

4.2.4 Low-Fluid-Shear Culture of *S. aureus* Represses Carotenoid Production

To further analyze the low-fluid-shear response of individual *S. aureus* cells within the biofilm matrix, additional assays were conducted after dispersal of LSMMG-cultured *S. aureus* cells using a vortex. A characteristic of most *S. aureus* strains is their golden yellow color arising from the production of the primary carotenoid pigment, staphyloxanthin, expressed during the stationary phase of growth. *S. aureus* N315 regularly produces this yellow pigment. After biofilm disbandment and upon

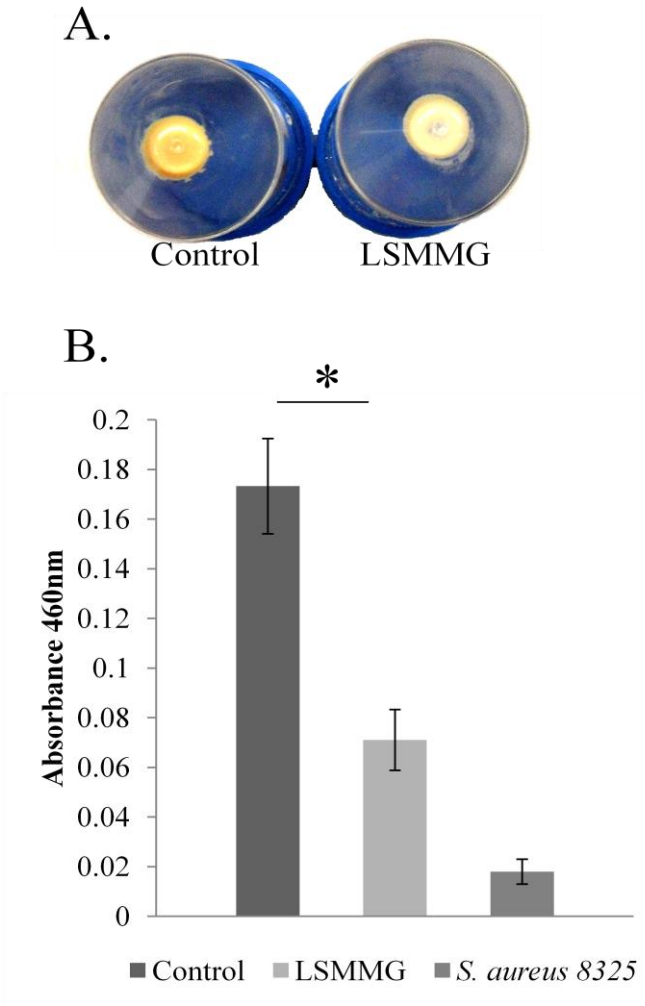


FIGURE 4.4. Decreased Carotenoid Production of *Staphylococcus aureus* N315 in Response to LSMMG Culture. (A) Pellets of control- and low-shear-cultured *S. aureus* revealing a visual difference in pigmentation of the cells. (B) The quantitative difference in pigmentation between control- and LSMMG-cultured *S. aureus* determined by carotenoid extraction and measurement spectrophotometrically at 460 nm. There was a significant reduction in the absorbance of low-shear-cultured bacteria as compared to the control ($*$, $P < 0.0001$). *S. aureus* 8325, which does not produce carotenoids, was used as a negative control for comparison. (Castro, 2011)

concentration of the LSMMG- and control-cultured cells by centrifugation, it was visibly evident that bacteria cultured under low-shear conditions were less pigmented than respective control cultures (Fig. 4.4A) (27). To quantify the observed pigmentation difference, the carotenoid pigments of both LSMMG- and control-cultured *S. aureus* cells were isolated from the cell wall by methanol extraction and their absorbance at 460 nm was measured, as previously described by Marshall, et al. (133). The absorbance of low-fluid-shear-cultured *S. aureus* was significantly lower than that of control cultures (Fig. 4.4B), although not as low as unpigmented *S. aureus* 8325 (27). This quantitative confirmation of the visual results indicates a reduction in staphyloxanthin production in *S. aureus* N315.

4.2.5 Low-Fluid-Shear Culture Increases the Susceptibility of *S. aureus* to Oxidative Stress and is a Transient Effect

As low-fluid-shear culture has been shown to alter the stress response of multiple microorganisms (4, 37, 127, 152) and as carotenoids are known to have protective antioxidant properties (123), LSMMG-cultured *S. aureus* N315 was evaluated for oxidative stress survival. As *S. aureus* 8325 does not produce carotenoid pigments and was, therefore, unaffected in terms of pigmentation by low-shear conditions, it was also evaluated for susceptibility to oxidative stress. Following 20 hours of culture, the entire contents of the RWV vessels were removed, vigorously vortexed, exposed to 30 mM hydrogen peroxide for 60 minutes, and assessed for survival. Low-shear-cultured *S. aureus* N315 displayed a 4-fold greater sensitivity to hydrogen peroxide as compared to

controls (Fig. 4.5) (27). As expected, there was not a significant difference between LSMMG and control cultures of the unpigmented *S. aureus* 8325 (Fig. 4.6) (27). Collectively, these results reinforce that a decrease in carotenoid production can be associated with the increased oxidative susceptibility.

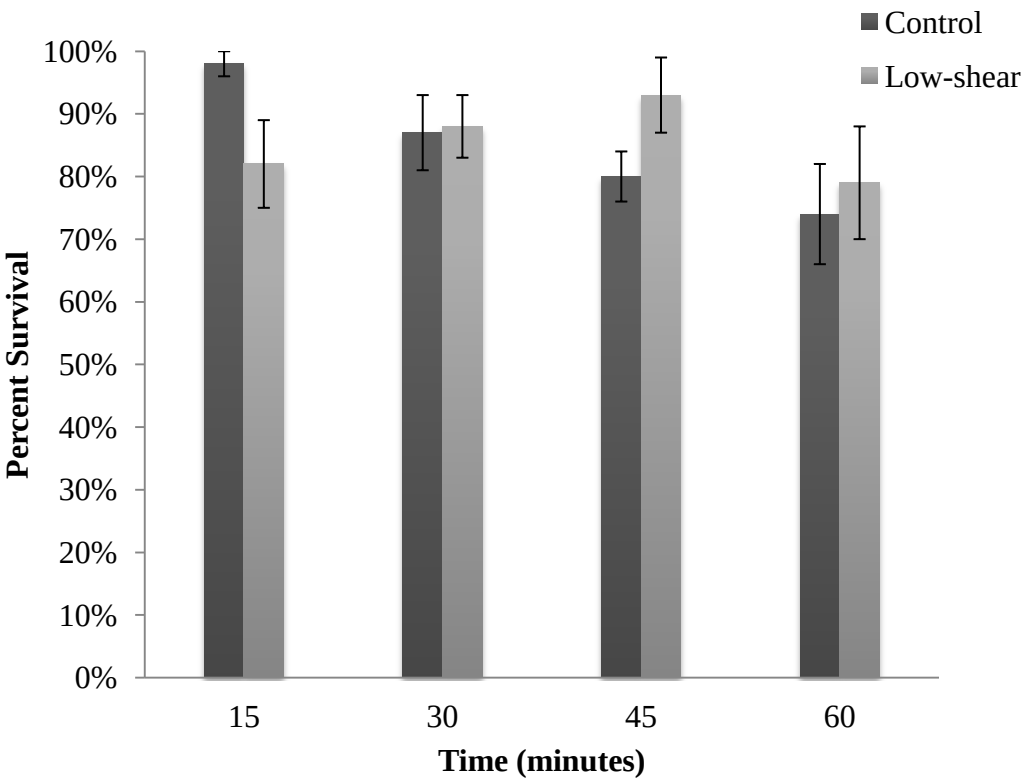


FIGURE 4.5. LSMMG Culture of *Staphylococcus aureus* 8325 Does Not Alter Susceptibility to Oxidative Stress. Exposure of low-shear-cultured *S. aureus* 8325 to H_2O_2 stress over a 60 minute time course resulted in similar survival rates as compared to control cultures.

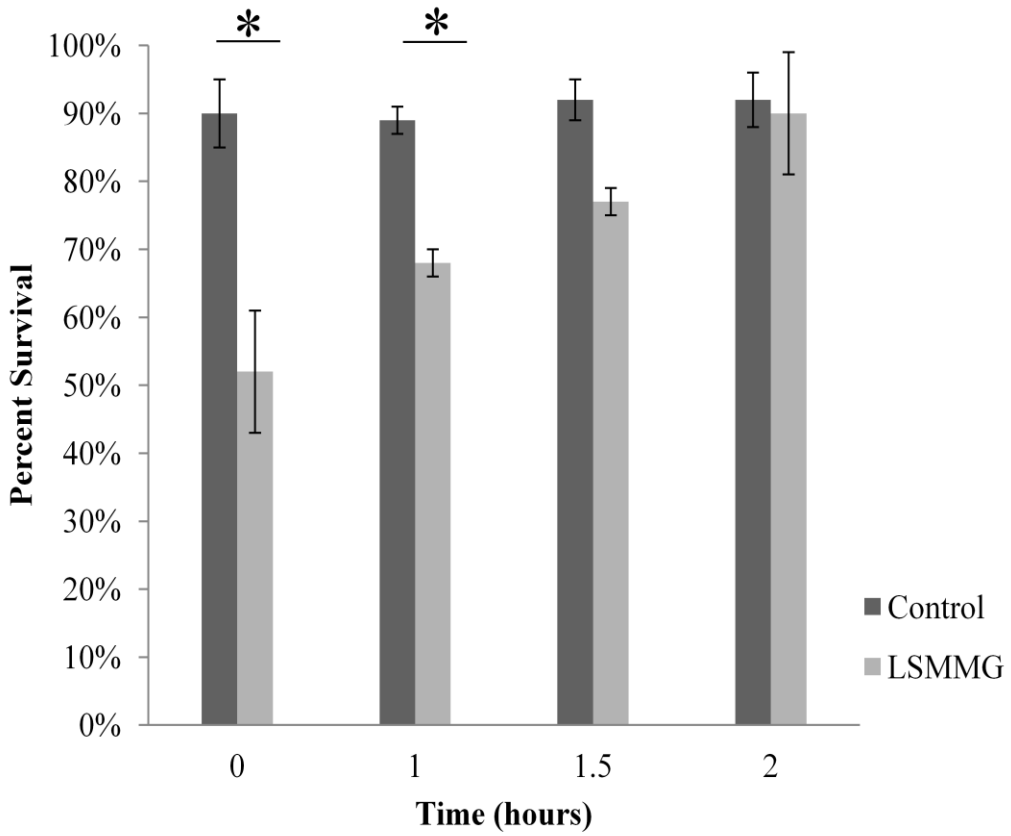


FIGURE 4.6. LSMMG-Cultured *Staphylococcus aureus* N315 is More Susceptible to Killing by Oxidative Stress and is a Transient Effect. After 60 minutes (time zero) of exposure to H_2O_2 50% of the low-fluid-shear-cultured *S. aureus* had succumbed to damage, whereas the control cultures did not fall below 90% (*, $P < 0.05$). To assess the duration of the LSMMG effect, samples of both low-shear- and control-cultured bacteria were removed from the vessels and allowed to sit statically for a period of time (1, 1.5, and 2 hours) and then subjected to 60 minutes of oxidative stress. At 1.5 hours there failed to be a significant difference (*, $P < 0.05$) in the susceptibility of low-shear- and control-cultured *S. aureus,* at which point it was determined the low-shear effect had dissipated. (Castro, 2011)

To determine if the alteration in oxidative stress sensitivity is transient after the cells are removed from the RWV, LSMMG- and control-cultured *S. aureus* N315 were removed from the vessels after 20 hours of culture, vortexed, and placed into sterile containers where they remained in a static environment. At 30 minute intervals, samples were removed from the static containers and subjected to the oxidative stress assay. When the difference in survival between the LSMMG- and control-cultured bacteria failed to show statistical significance, it was determined that the low-shear effect had dissipated (Fig. 4.5) (27). Using linear regression analysis, it was concluded that the half-life of the oxidative stress effect of LSMMG-cultured *S. aureus* was 68.3 ± 1.3 minutes (Fig. 4.7). Based on the data from these assays, the low-fluid-shear effect on *S. aureus* is transient, lasting less than 1.5 hours once removed from the LSMMG environment.

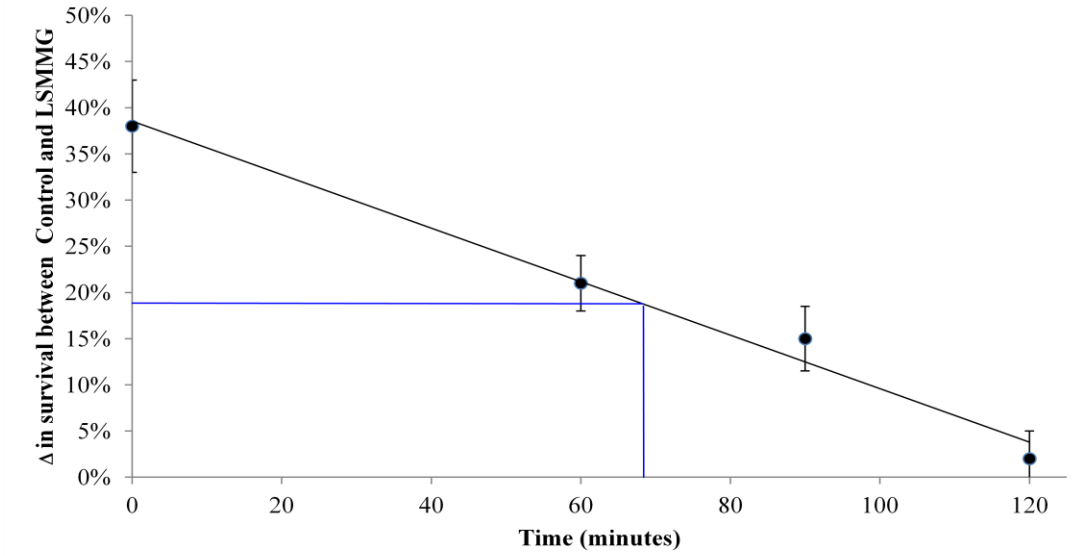


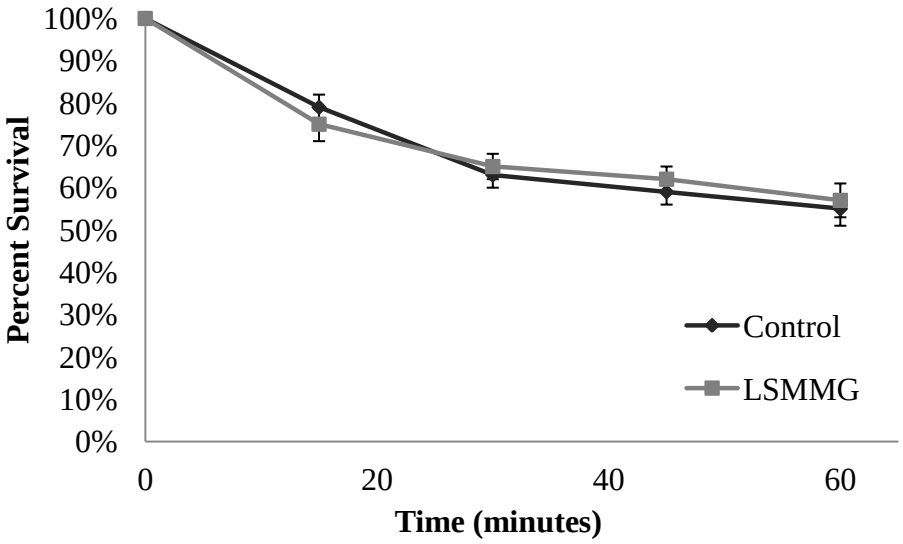
FIGURE 4.7. The Half-Life of the Oxidative Stress Effect. Plotting the difference in survival rates between control- and LSMMG-cultured *S. aureus* N315 to oxidative stress the half-life of the effect was determined by linear regression analysis to be 68.3 ± 1.3 minutes.

4.2.6 Low-Fluid-Shear Culture of *S. aureus* Does Not Alter Acid or Thermal Stress Responses

Low-shear culture conditions induce adaptations in *S.* Typhimurium, *P. aeruginosa*, and *E. coli* that result in their increased resistance to either acid or thermal stress (4, 37, 152). Therefore, LSMMG-cultured *S. aureus* N315 and 8325 were assessed for an alteration in survival when subjected to these environmental stressors. Acid stress assays were conducted comparing survival rates of LSMMG and control cultures for *S. aureus* N315 and 8325. After 20 hours of culture, the entire contents of the RWV vessels were removed, vortexed, added to a concentrated citrate buffer that exposed the bacteria to a pH of 3.0, and assessed for survival in 15 minute intervals over a 60 minute time course. There was not a significant difference between LSMMG and control cultures, as revealed by comparison of the kill curves for either *S. aureus* N315 (Fig. 4.8A) and 8325 (Fig. 4.8B).

The thermal stress response of low-shear-cultured *S. aureus* was evaluated by subjecting the bacteria to an experimentally determined temperature of 51°C as described in Chapter 3.8. Following 20 hours of culture, the contents of the RWV vessels were removed and vortexed. Aliquots of the inoculum were put into microcentrifuge tubes which were placed into a 51°C pre-warmed heat block and evaluated for survival every 15 minutes over a 60 minute time course. A significant difference was not observed between LSMMG and control cultures for *S. aureus* N315 (Fig. 4.9A) or 8325 (Fig. 4.9B).

A.



B.

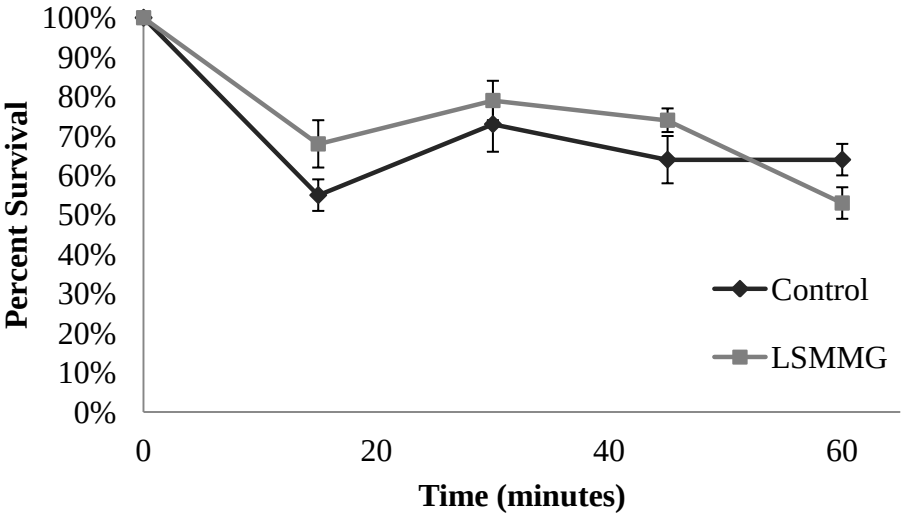
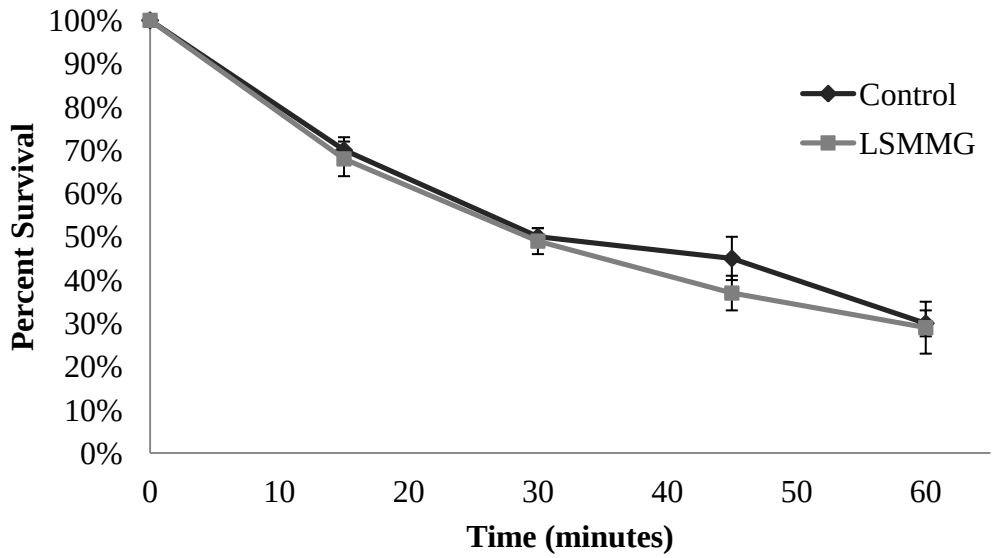


FIGURE 4.8. A Low-Fluid-Shear Culture Environment Does Not Alter the Susceptibility of *Staphylococcus aureus* to Acid Stress. Comparison of survival of LSMMG- and control-cultured bacteria subjected to acid stress at pH 3.0. There was no significant difference in the susceptibility of low-fluid-shear-cultured *S. aureus* N315 (A) or *S. aureus* 8325 (B) to acid stress as compared to controls.

A.



B.

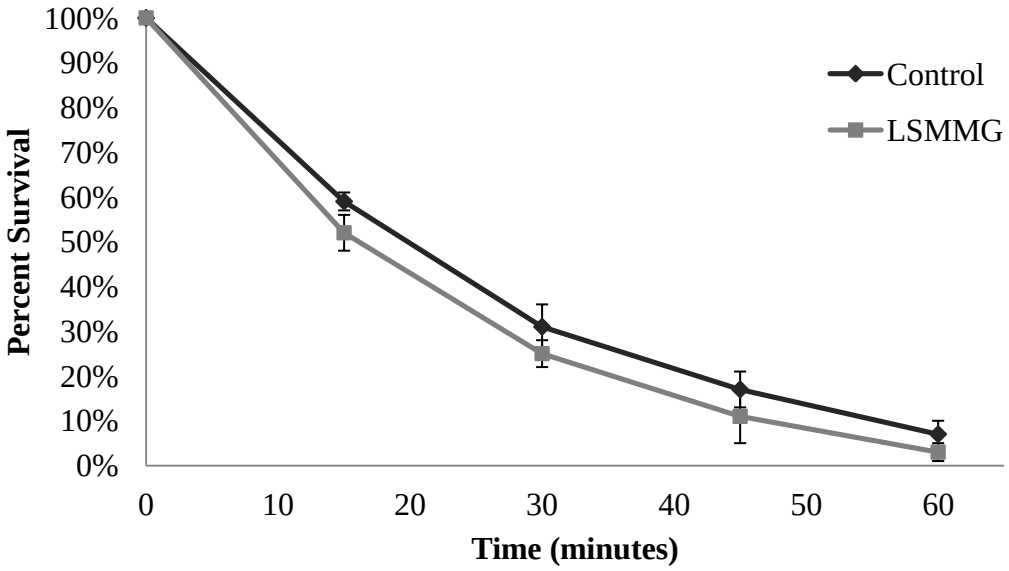


FIGURE 4.9. A Low-Fluid-Shear Culture Environment Does Not Alter the Susceptibility of *Staphylococcus aureus* to Thermal Stress. Comparison of the survival of LSMMG- and control-cultured bacteria subjected to thermal stress at 51°C. There was no significant difference in the susceptibility of low-fluid-shear-cultured *S. aureus* N315 (A) or *S. aureus* 8325 (B) to thermal stress as compared to controls.

4.2.7 Low-Fluid-Shear Culture Enhances the Susceptibility of *S. aureus* to Human Whole Blood

The decreased resistance of *S. aureus* to oxidative stress conferred by low-shear conditions could correlate with decreased survival against immune cells. Therefore, the ability of LSMMG-cultured *S. aureus* N315 to survive the immune components present in human whole blood was investigated. This testing was accomplished by scheduling human subjects for blood draws at precisely 20 hours post-inoculation of the RWVs with *S. aureus*. Following the blood draw, the whole blood was immediately returned to the lab for challenge with either LSMMG- or control-cultured *S. aureus* N315. The bacteria and blood was incubated together for 4 hours at which point bacterial survival was assessed. Low-fluid-shear-cultured *S. aureus* N315 was significantly more susceptible to killing by whole blood as compared to controls (Fig. 4.10) (27). Interestingly, the difference in survival between LSMMG and control cultures in two samples from different subjects was approximately 30%, demonstrating a consistent trend among low-fluid-shear-cultured *S. aureus* N315.

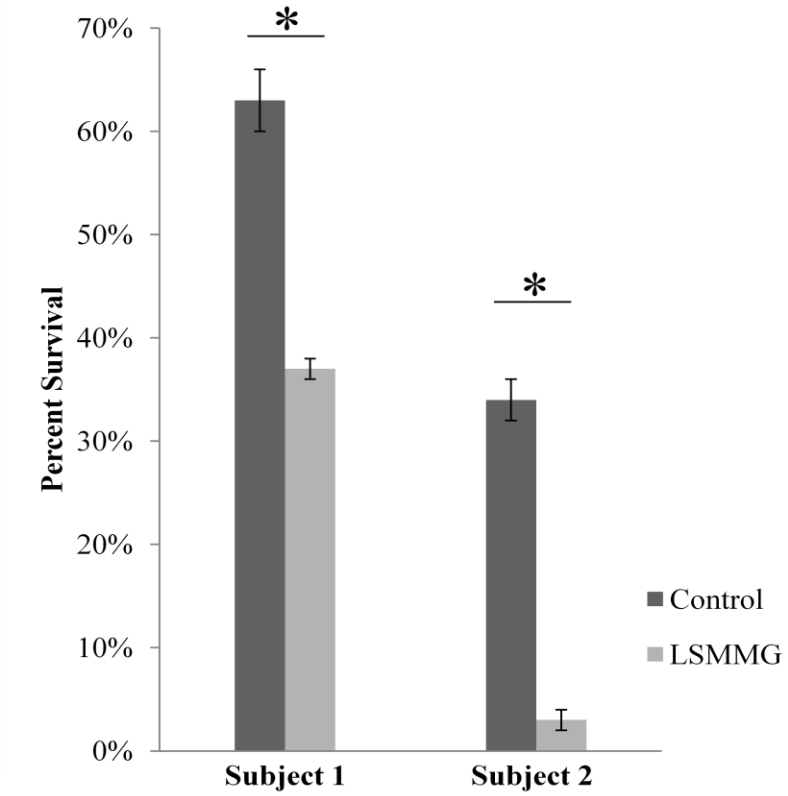


FIGURE 4.10. Low-Shear-Cultured *Staphylococcus aureus* N315 is More Susceptible to the Immune Components Present in Whole Blood. LSMMG- and control-cultured *S. aureus* was challenged with freshly-drawn human whole blood after removal from the RWVs. After 4 hours, the low-shear-cultured *S. aureus* displayed a significantly reduced ability to survive in the whole blood as compared to control cultures (*, $P < 0.05$). (Castro, 2011)

4.3 DISCUSSION

S. aureus is one of many microorganisms that can be classified as both a commensal and pathogen in the human host (126). It has been isolated from multiple locations in humans, including the gastrointestinal tract (1, 40, 126). Indeed, the mucus layer of the intestine has been demonstrated to be a key niche facilitating intestinal colonization of *S. aureus* (73). The presence of the bacteria in the intestine results in its acquisition of antibiotic resistance genes (178, 188) and leads to its further spread and carriage via a fecal-environmental route of infection (16, 22). *S. aureus* is responsible for high incidences of food poisoning; however, these cases of toxigenic gastroenteritis are due to ingestion of staphylococcal enterotoxins produced prior to ingestion of contaminated food and not the presence of the organism itself (44). There is very little evidence associating the intestinal presence of *S. aureus* with disease and, therefore, very little research has been performed to understand how this environment impacts the organism. While *S. aureus* has the capability to be dangerously pathogenic, its nonpathogenic phenotype within the intestines of a human host suggests that factors present at this site predispose the bacterium to a colonization phenotype. However, the role of the low-fluid-shear levels present at this site has not been considered as an impacting factor on the lifecycle of *S. aureus*.

The environment created within the RWV bioreactor is characterized by very low levels of fluid shear that are physiologically relevant to those experienced by intestinal epithelial cells (76). In response to the very low shear forces in the RWV, *S. aureus*

N315 formed large cellular aggregates in the fluid phase of the vessel, which were unattached to any surface and completely encased by an extracellular polymeric substance that conferred antibiotic resistance (27). Collectively, this evidence indicates that, when exposed to a low-shear environment, *S. aureus* N315 produces attachment-independent biofilms (27). This response of *S. aureus* N315 to low-shear aligns well with previously documented reports of alterations in biofilm growth and architecture (36, 37, 128). For example, *E. coli* attached to glass microcarrier beads in the RWV formed thicker and more resistant biofilms when cultured under low-shear conditions (128). Additionally, *P. aeruginosa* cultured in the LSMMG environment resulted a phenotype comparable to reports of cystic fibrosis pathology in the lung in which the bacterial cells self-aggregated and produced increased amounts of alginate (36). The existence of a bacterium in a biofilm phenotype is conducive to colonization as the biofilm imparts many advantages to the bacteria, such as persistence in a favorable environment, protection from environmental stressors and antibiotics, host immunity, communal metabolism, and an increased probability of transformation and transduction (89, 193). Low-fluid-shear levels consistent with areas within the intestines induce *S. aureus* biofilm formation (27); as an intestinal colonizer (1, 16, 43, 181) and as biofilms are beneficial to colonizing bacteria, it logically follows that it could exhibit a biofilm phenotype while colonizing this host site.

For bacteria thus far examined, the low-fluid-shear effect on microbial growth kinetics is varied, including reports of the LSMMG environment having no effect on growth or resulting in a higher or lower cell density as compared to controls (4, 152, 184,

211, 227). The growth profile of *S.* Typhimurium cultured in a nutrient-rich broth under low-shear conditions nearly paralleled that of control cultures through mid-exponential growth phase (153). Interestingly, when cultured in a minimal salts media under LSMMG conditions, the same strain displayed significantly higher cell concentrations as compared to controls (227). The growth of numerous strains of *E. coli* are unaltered when cultured in minimal media under low-shear conditions (4, 127, 211, 224), yet, when cultured in a rich media, have displayed lower total cell concentrations (211). Investigators comparing three *S. aureus* isolates, RF1, RF6, and RF11, cultured in a complex media, reported no difference between the growth profiles of LSMMG- and control-cultured bacteria (184). In contrast to these findings, this work showed that there was an approximately 3-fold decrease in final cell concentrations of *S. aureus* N315 and more than a 5-fold decrease in final cell concentrations of *S. aureus* 8325 cultured in a nutrient-rich media under low-shear conditions, as compared to controls (27). Further complicating comparisons with other studies, researchers assessing *S. aureus* 25293 in a complex media reported an increase in total cell concentration in response to low-shear conditions (221).

To better visualize the impact of RWV culture on microorganisms commonly associated with the intestinal tract, a summary of growth responses and media was compiled (Table 3). Trends from this evaluation suggest that pathogenic bacteria, *S.* Typhimurium, *E. coli* 86-24, and *E. coli* 2348/69, either increase or do not alter their growth (26, 227), whereas opportunistic pathogens and possible commensals, *S. aureus*

Table 3. Comparison of the LSMMG Growth Response of Potential Intestinal-Associated Bacteria

	Growth Media		
	Complex	Minimal	Reference
Increased Cell Concentration in Response to LSMMG	*S. aureus* 25923*	*S.* Typhimurium χ^{3339}	Wilson, 2002[227] Vukanti, 2012[221]
Decreased Cell Concentration in Response to LSMMG	*S. aureus* 8325 *S. aureus* N315 *E. coli* MG1655		Castro, 2011[27] Tucker, 2007[211]
No Change in Cell Concentration in Response to LSMMG	*S.* Typhimurium χ^{3339} *S. aureus* RF1 *S. aureus* RF6 *S. aureus* RF11 *E. coli* 86-24 *E. coli* 2348/69	 *E. coli* O83:H1 *E. coli* MG1655 *E. coli* AMS6	Nickerson, 2000[152] Rosado, 2010[184] Carvalho, 2005[26] Allen, 2008[4] Tucker, 2007[211] Lynch, 2004[127]

*All cell density data was collected between 4-24 hours except *S. aureus* 25923 in which the difference reported was obtained after 40 hours of LSMMG culture.

N315, *S. aureus* 8325, *S. aureus* RF1,RF6, RF11, and *E. coli* MG1655, *E. coli* O83:H1, and *E. coli* AMS6, either decrease or do not alter their growth (4, 27, 127, 184, 211). The reported increase in cell density of *S. aureus* 25293 was not noted until deep into stationary growth phase after 40 hours of culture in the RWV (221). All other organisms thus far assessed in terms of growth have been analyzed during mid-logarithmic (4-12 hours) and stationary (12-24 hours) phases of growth, dependent on the organism (26, 27, 127, 184, 211). Therefore, the increase noted by the investigators may not be solely

based on growth characteristics in low-fluid-shear culture but may instead be based on the enhanced survival characteristics associated with changes in nutrient depletion and/or waste build-up. Collectively, Table 3 indicates that the growth response of a microorganism to low-fluid-shear culture occurs on a strain-specific basis with the composition of the media serving as an influencing factor. Interestingly, the pathogenic or non-pathogenic nature of the bacterium at low-shear sites in the intestines appears to correlate with the organism's growth response in the LSMMG environment.

Visual inspection of LSMMG-cultured *S. aureus* N315 revealed a decrease in the pigmentation of bacterial pellets (27). The carotenoid pigment, staphyloxanthin, is responsible for the golden coloration of *S. aureus*. Extraction, measurement, and comparison of the carotenoids from low-shear-cultured *S. aureus* N315 to unpigmented *S. aureus* 8325 quantified the significant decrease in pigmentation (27). However, the levels of pigmentation of LSMMG-cultured *S. aureus* N315 were not as low as 8325. The enzymatic process of carotenoid production involves the formation of increasingly pigmented intermediates, suggesting that an early intermediate of staphyloxanthin may be produced (27). A reduction in *S. aureus* carotenoid production in response to low-shear culture has also been documented for strains RF1, RF6, and RF11 (184). Collectively, all pigmented *S. aureus* strains that have been assessed for alterations in carotenoid expression in response to LSMMG have displayed a reduction in staphyloxanthin production, indicating a common response among strains to this environmental parameter (27, 184).

Staphyloxanthin is a virulence component that serves as a shield protecting the bacterial cell from the toxic effects of reactive oxygen species associated with host immunity (123). It was therefore suspected that the reduction in pigmentation of *S. aureus* N315 would be associated with increased susceptibility to oxidative stress. Oxidative stress analysis confirmed this for *S. aureus* N315 and, as expected, low-shear-cultured, unpigmented *S. aureus* 8325 demonstrated similar levels of resistance as compared to controls (27). The duration of the low-shear effect in *S. aureus* N315 was investigated by allowing both LSMMG and control cultures to rest statically for a period of time prior to the oxidative stress assay. At a time greater than 1 hour but less than 1.5 hours, there was no longer a significant difference between the percent survival of low-shear- and control-cultured *S. aureus* N315 (27). As this response is not a result of the emergence of staphyloxanthin, it is probable that both LSMMG and control cultures are responding to the new static environment, and the production of other compensatory mechanisms, such as catalase and/or superoxide dismutase, are similar. Based on this data, the half-life of the observed oxidative sensitivity is 68.3 ± 1.3 minutes; however, it is important to note that this finding can only be applied to this specific characteristic. It is highly possible that other phenotypic characteristics, as well as molecular expression and virulence characteristics, respond in different ways.

The LSMMG environment predisposes *S.* Typhimurium and various *E. coli* strains with an increased ability to withstand acid and thermal stress (4, 127, 152, 227), while the survival rates of low-shear-cultured *S. aureus* N315 and 8325 were unaltered upon challenge to acid and heat as compared to controls. Enteric pathogens, *S.*

Typhimurium and pathogenic *E. coli*, are among the most significant causes of bacterial gastroenteritis (140). It follows that environmental factors, such as low-fluid-shear, present in the intestine trigger adaptations in the bacteria resulting in their prolonged survival upon encountering immune stressors such as elevated host temperature or acidic conditions arising from phagosome-lysosome fusion. As the presence of *S. aureus* in the intestine is not often associated with disease, there would be little evolutionary advantage to increasing survival mechanisms directed toward host immunity. Furthermore, *S. aureus* is an adaptable and resilient pathogen. In order to construct kill curves, the microorganism was subjected to a pH of 3.0 and a temperature of 51°C, which are beyond the conditions typically employed by the immune system.

The phenotypic adaptations of *S. aureus* imparted by a low-fluid-shear culture environment include the formation of attachment-independent biofilms with increased antibiotic resistance, decreased carotenoid pigmentation, increased susceptibility to oxidative stress and whole blood, as well as decreased cell concentration. As opposed to many obligate pathogens previously studied using the RWV (26, 152, 227), these alterations are consistent with a colonization phenotype in which *S. aureus* decreases virulence components, reduces its growth, and remains protected within a biofilm matrix. As an intestinal colonizer, this finding would have implications in the prevalence, persistence, and, possibly, characteristics of the intestinal microbiome. Potentially, low-fluid-shear may serve as a cue directing microorganisms toward either a colonization or infectious phenotype.

Chapter 5: Molecular Alterations of *Staphylococcus aureus* Imparted by Low-Fluid-Shear Culture

5.1 INTRODUCTION

Phenotypic adaptations are indicative of alterations occurring at the molecular level within an organism. The comparison of gene expression profiles after genetic manipulation or induction by environmental stimuli has provided fundamental insight into the molecular mechanisms driving these modifications and the downstream consequences (35, 95, 109, 222, 228). *S. aureus* has demonstrated significant phenotypic differences in response to low-fluid-shear culture (27), suggesting that gene expression levels will also be altered when compared to control cultures. Genetic expression analysis of LSMMG-cultured *S. aureus* affords a molecular picture to compare with observed phenotypic changes and may potentially lead to the identification of a molecular mechanism associated with the low-shear response. As genetic expression data from numerous microorganisms in response to low-fluid-shear culture has been previously reported (3, 37, 184, 211, 228), comparative analysis with *S. aureus* will provide an opportunity to define trends and molecularly-conserved responses to this environment.

5.2 RESULTS

5.2.1 Up-Regulation of *S. aureus* N315 Genes in Response to Low-Fluid-Shear Culture

Whole genome microarray analysis using *S. aureus* GeneChips identified 17 genes whose expression was up-regulated in response to LSMMG culture; down-regulated expression values could not be correlated to any gene or open reading frame (27). Table 4 lists the up-regulated genes, their ORF identification from the sequencing of *S. aureus* N315 (111), as well as their associated protein products and the fold change.

Table 4. Genes Up-Regulated in Response to LSMMG Culture

N315 open reading frame	Gene name	Fold change	Protein product
SA1986		2.71	Hypothetical protein
SA1985		2.65	Hypothetical protein
SA1984	*asp23*	2.26	Alkaline Shock Protein
SA0218	*pflB*	4.19	Formate acetyltransferase
SA0219	*pflA*	3.84	Formate acetyltransferase activating enzyme
SA1012	*argF*	2.35	Ornithine carbamoyltransferase
SA1013	*arcC*	2.43	Carbamate kinase
SA1532	*usp*	3.19	Probable Universal Stress Protein
SA0455		2.17	Probable TdcF Protein
SA2336	*clpL*	2.81	ATP-dependent Clp proteinase chain ClpL
SA2312	*ddh*	2.31	D-lactate dehydrogenase
SA0562	*adh1*	2.27	Alcohol dehydrogenase
SA2410	*nrdD*	2.09	Anaerobic ribonucleoside triphosphate reductase
SA0102		2.14	Myosin-cross-reactive antigen
SA0570		2.14	Hypothetical protein
SA0752		2.07	Hypothetical protein
SA2268		4.45	Hypothetical protein

The genes whose expression increased in response to low-fluid-shear culture are dispersed throughout the staphylococcal chromosome and in three instances belonged to the same operon (27). Biochemically, the protein products of many of these genes are associated with fermentative metabolism, as formate acetyltransferase, D-lactate dehydrogenase, and alcohol dehydrogenase were up-regulated in response to low-fluid-shear culture (27). In addition to increases in genes whose products are involved in pyruvate, carbohydrate, and arginine metabolism, those implicated in the response to environmental stress were also represented (27).

5.2.2 Predicted Regulatory Consensus Sequences of Genes Up-Regulated in Response to Low-Fluid-Shear Culture

The predicted regulatory regions (500 bases upstream of the start codon) of LSMMG-responsive genes were aligned and analyzed for consensus sequences. The alignment revealed the presence of two conserved consensus sequences which are listed along with their corresponding gene and position from the start codon in Table 5 (27). The first conserved motif, t/a<u>TGTGA</u>t/a$_6$<u>TCACA</u>t/a (the most common nucleotides are lowercase and the predicted binding sites are underlined) was located in the regulatory regions of *pflA*, *pflB*, encoding formate acetyltransferase and formate acetyltransferase activating enzyme (SA0218, SA0219); *adh1*, encoding alcohol dehydrogenase (SA0562); *ddh*, encoding D-lactate dehydrogenase (SA2312); and a hypothetical protein (SA2268) (27). This consensus sequence is a binding site for the Rex repressor, a regulator of the intracellular redox balance (162). In addition to the Rex binding sites, alignment analysis

also revealed the alternative sigma factor B (SigB) promoter consensus sequence, GTTTa/ta/t_{12-15} GGGa/tAa/t (67), in the regulatory regions of LSMMG-responsive transcripts. A SigB promoter was located upstream of the operon containing alkaline shock protein 23 (SA1984-SA1986); and the ORFs of the ATP-dependent protease clpL (SA2336); a myosin-cross-reactive streptococcal antigen homologue (SA0102); a probable TdcF protein (SA0455); and a hypothetical protein (SA0752) (27).

Table 5. Predicted Regulatory Consensus Sequences of LSMMG-Responsive Genes

N315 open reading frame	Gene name	Predicted regulatory consensus sequence	Position from start codon
SA1986		**GTTTA**AGAAAAAAACTGAT**GGGTAC**	-92
SA1985		**GTTTA**AGAAAAAAACTGAT**GGGTAC**	ORF 2*
SA1984	*asp23*	**GTTTA**AGAAAAAAACTGAT**GGGTAC**	ORF 3*
SA0218	*pflB*	A**TGTGA**AAAAAA**TCACA**A	-96
SA0219	*pflA*	A**TGTGA**AAAAAA**TCACA**A	ORF 2*
SA1012	*argF*		
SA1013	*arcC*		
SA1532	*usp*		
SA0455		**GTTTA**AAGCCCATGTAAAAG**GGGTAT**	-179
SA2336	*clpL*	**GTTTTA**TCACCTATTATTAGT**GGAAA**	-115
SA2312	*ddh*	T**TGTGA**TATTTT**TCACA**A	-39
SA0562	*adh1*	T**TGTGA**ATTAAT**TCACA**T	-306
SA2410	*nrdD*		
SA0102		**ATTTA**CAATTCACAAAG**GGGTAT**	-61
SA0570			
SA0752		**GTTTA**AAAGATAATGTGACG**GGGTAA**	-67
SA2268		T**TGTGA**AATACA**TCACA**A	-129

* Open reading frame position in the operon with a predicted regulatory consensus sequence

5.2.3 *hfq* Expression is Repressed in Response to Low-Fluid-Shear Culture

As regulatory proteins were either directly implicated by the alignment of LSMMG-responsive genes of *S. aureus* or have been connected to this response in Gram-negative bacteria, primers were designed to evaluate the expression of potential regulators of the low-shear response by quantitative real-time PCR. The Rex repressor is a regulator of genes whose protein products are involved in maintaining the intracellular $NADH/NAD^+$ redox balance (162), and SigB is a global regulator involved in the entrance into stationary phase and in response to multiple environmental stressors in *S. aureus* (108, 109). Moreover, CspA, the major cold shock protein in *S. aureus*, has also demonstrated its function as a molecular chaperone involved in the regulation of staphyloxanthin production (95). The translational regulator Hfq is highly involved in the stress response of numerous Gram-negative bacteria and has been directly implicated in the regulation of the response of *S.* Typhimurium and *P. aeruginosa* to LSMMG culture (37, 225). Table 6 displays the fold change levels for the potential regulators analyzed. Despite the presence of multiple conserved Rex and SigB binding sites in the regulatory regions of LSMMG-induced genes, qRT-PCR analysis revealed similar expression levels between LSMMG- and control-cultured *S. aureus* (27). Additionally, the expression of *cspA* was not significantly altered in response to the LSMMG environment (27). Interestingly, the expression of *hfq* was down-regulated 2.68-fold in response to low-fluid-shear conditions (27).

Table 6. Expression Levels of Potential Regulators of the LSMMG Response

N315 open reading frame	Gene	1/Fold Change
SA1851	*rex*	1.44 ± 0.22
SA1869	*sigB*	0.95 ± 0.23
SA1234	*cspA*	1.41 ± 0.06
SA1145	*hfq*	2.68 ± 0.14

5.2.4 Expression Analysis of the Carotenoid Operon

In response to a low-shear culture environment, *S. aureus* N315 displayed a significant reduction in carotenoid pigmentation (27). Carotenoids belong to the isoprenoid class of organic molecules. They are non-essential, secondary metabolites that are synthesized via the mevalonate biosynthetic pathway, which is directly connected to the carotenoid operon (Fig. 5.1) (10). Microarray analysis did not reveal alterations in the expression of any genes involved in mavalonate biosynthesis or carotenoid production. Therefore, primers were constructed to analyze the expression levels of the enzyme products of the genes involved in the conversion of acetyl-CoA from glycolysis all the way through staphyloxanthin production (*mvaS* – *crtO* as shown in Fig. 5.1). Using a 2-fold cutoff limit, a significant change in expression levels of these

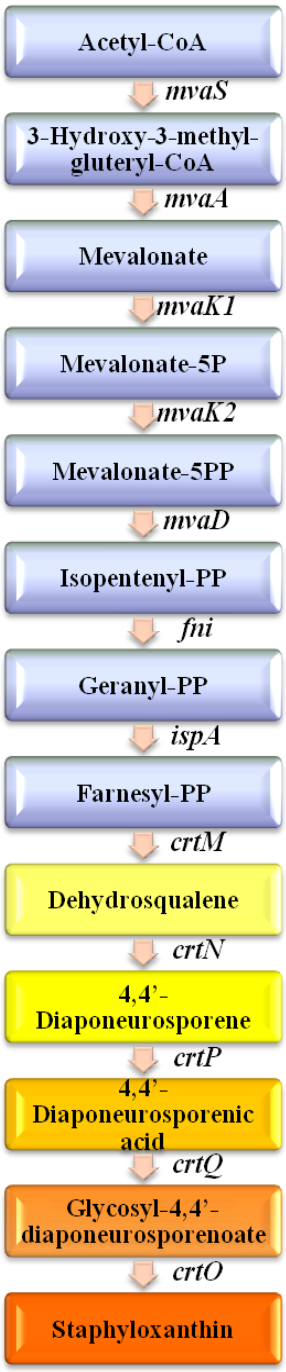


FIGURE 5.1. Products and Genes of the Mevalonate Pathway and Carotenoid Operon. In blue, acetyl-coA from glycolysis is converted by the enzyme products of the associated genes to farnesyl pyrophosphate via the mevalonate pathway. Carotenogenesis by the enzymes of the carotenoid operon (*crtM – crtO)* leads to the conversion of farnesyl pyrophosphate to the pigmented intermediates and staphyloxanthin production in *S. aureus*.

staphylococcal genes, after 20 hours of culture in the low-fluid-shear environment, was not detected, further confirming the results of the microarray analysis.

Although the golden pigmentation of *S. aureus* is not evident until stationary phase, the production of carotenoids may be initiated earlier in the growth cycle. The generation of farnesyl pyrophosphate by the mevalonate pathway creates a biochemical branching point in which this product is an essential precursor of the production of many vital *S. aureus* compounds (10). Therefore, in order to further investigate the reduction in pigmentation of LSMMG-cultured *S. aureus*, expression levels of genes composing only the carotenoid operon were evaluated after 10 and 15 hours of low-shear culture by quantitative real-time PCR. Following 10 hours of culture under low-shear conditions, there was a slight increase in the expression of *crtM*, *crtP*, and *crtQ* as compared to control cultures (Table 7). This increase in expression was far more pronounced after 15 hours of low-shear culture, with all genes in the carotenoid operon demonstrating significant up-regulation as compared to controls (Table 7).

Table 7. Expression Levels of the Carotenoid Operon Following 10 and 15 Hours of LSMMG Culture

N315 open reading frame	Gene	Fold Change	
		10 hours	15 hours
SA2349	*crtM*	2.1 ± 0.37	7.8 ± 0.26
SA2348	*crtN*	1.8 ± 0.33	7.8 ± 0.27
SA2351	*crtP*	2.7 ± 0.53	14.4 ± 0.99
SA2350	*crtQ*	2.2 ±0.52	10.8 ± 0.28
SA2352	*crtO*	1.2 ± 0.23	3.7 ± 0.45

5.3 DISCUSSION

Whole genome microarray and quantitative real-time PCR analysis provided a means of constructing a molecular picture describing the changes in gene expression levels of *S. aureus* in response to culture under low-shear conditions. The LSMMG-responsive genes, as determined by microarray analysis, are characteristic of a fermentative metabolism, with formate acetyltransferase, D-lactate dehydrogenase, and alcohol dehydrogenase being up-regulated (27). Alignment analysis of the genes up-regulated in response to low-shear culture revealed two conserved consensus sequences, both of which are binding sites for staphylococcal regulators, t/aTGTGAt/a$_6$TCACAt/a and GTTTa/ta/t$_{12\text{-}15}$ GGGa/tAa/t (27). The first is a biding site for the Rex repressor. Rex is considered a key regulator of anaerobic metabolism in *S. aureus* where it functions to maintain an intracellular balance between NAD^+ and NADH levels (162). Both NAD^+ and NADH bind Rex; however, when bound to NADH, a conformational change occurs such that Rex is no longer able to bind DNA (162). Therefore, as NADH levels within the cell rise, it outcompetes NAD^+ for Rex binding, and, thus, the derepression of genes whose protein products are involved in fermentative respiration are transcribed, regenerating the NAD^+ pool (162). As the conditions within a biofilm are associated with low oxygen tension (147), it is not surprising that microarray analysis revealed the up-regulation of genes under the control of Rex. The second conserved binding site is for the alternative sigma factor, SigB (67). SigB is a staphylococcal global regulator involved in the differential regulation of over 200 ORFs in response to entrance into

stationary phase and multiple environmental stressors (67, 108, 109). It is therefore not surprising that genes under the control of SigB were determined up-regulated in response to LSMMG culture.

Molecular characterization of the low-shear response of *S.* Typhimurium and *P. aeruginosa* has led to the association of a global regulator to this environmental parameter (37, 225). Additionally, staphylococcal regulators were directly implicated from the microarray data. For their role as potential regulators in *S. aureus*, the expression levels of *rex*, *sigB*, *cspA*, and *hfq* were determined by qRT-PCR. CspA is the major cold shock protein of *S. aureus* that has also demonstrated function as a RNA chaperone and has been connected to pigment production (95). The RNA chaperone Hfq has been directly associated with the molecular mechanism governing the low-shear response in both *S.* Typhimurium and *P. aeruginosa* (37, 225). In addition, Rex and SigB are both staphylococcal transcriptional regulators whose binding sites were located in the regulatory regions of numerous LSMMG-responsive genes. Despite the increase in the expression of multiple SigB and Rex regulated ORFs, no significant difference in the expression of these regulators was noted in response to low-shear culture conditions. This was true for CspA as well. However, in response to a low-shear environment, a 2.68-fold decrease in Hfq expression was observed (27). The contribution of Hfq to the molecular regulation of *S. aureus* is currently under much debate (19, 124); however, Liu, et al., described Hfq as a global regulator in *S. aureus* (124) based upon their investigations of an *hfq* mutant. Interestingly, a side-by-side comparison of the microarray data in these studies with the work by Liu, et al., revealed that 7 of the 17

LSMMG-up-regulated genes were also up-regulated in response to an *hfq* mutation (Table 8) (124). In addition, 9 of the 17 LSMMG-induced genes were described by Liu, et al. to bind Hfq (Table 8) (124). Moreover, the work by Liu, et al. reported that the mutation of *hfq* affected pigment production (124). Taken together, the decreased expression level of *hfq* in response to low-fluid-shear culture combined with the significant correlations of this work with that of Liu, et al. strongly suggests that Hfq is involved in the LSMMG response of *S. aureus*.

Table 8. The Association of LSMMG-Responsive Genes with Hfq

N315 open reading frame	Gene name	Protein product	Up-regulated in an *hfq* mutant[a b]	Binds Hfq[a c]
SA1986		Hypothetical protein	x	x
SA1985		Hypothetical protein	x	x
SA1984	*asp23*	Alkaline Shock Protein	x	x
SA0218	*pflB*	Formate acetyltransferase		
SA0219	*pflA*	Formate acetyltransferase activating enzyme		x
SA1012	*argF*	Ornithine carbamoyltransferase		
SA1013	*arcC*	Carbamate kinase		
SA1532	*usp*	Probable Universal Stress Protein		
SA0455		Probable TdcF Protein	x	x
SA2336	*clpL*	ATP-dependent Clp proteinase chain ClpL	x	x
SA2312	*ddh*	D-lactate dehydrogenase		
SA0562	*adh1*	Alcohol dehydrogenase		x
SA2410	*nrdD*	Anaerobic ribonucleoside triphosphate reductase	x	
SA0102		Myosin-cross-reactive antigen		
SA0570		Hypothetical protein	x	x
SA0752		Hypothetical protein		x
SA2268		Hypothetical protein		

[a] Data generated from previously published work (Liu, et al., 2010)
[b] Genes whose expression was up-regulated in response to mutation of the *hfq* gene in *S. aureus* (Liu, et al., 2010)
[c] mRNAs of genes that bind Hfq determined by immunoprecipitation (Liu, et al., 2010)

More importantly, this is the first description associating an Hfq response to a low-shear environment in a Gram-positive microorganism. This connection in *S. aureus*, as well as with previous reported responses in Gram-negative organisms, strongly suggests that the ability to sense and respond to mechanical stimuli is evolutionarily conserved among structurally diverse prokaryotes.

Examination of the microarray data failed to connect a molecular response with the vast phenotypic difference in the pigmentation of low-shear-cultured *S. aureus*. After confirmation that the expression levels of genes involved in carotenogensis were unaltered after 20 hours of LSMMG culture, levels of expression of the carotenoid operon were evaluated following 10 and 15 hours of low-shear culture under the assumption that gene expression may be occurring prior to stationary growth phase. Indeed, levels of *crtM*, *crtP*, and *crtQ* were up-regulated after 10 hours of low-shear culture, whereas expression levels of the entire operon were greatly increased following 15 hours of LSMMG culture. Interestingly, the increased expression levels of the genes involved in carotenoid biosynthesis of low-shear-cultured *S. aureus* do not correlate with the decrease in cell pigmentation. The explanation for this unexpected result is unclear, as the regulation of the carotenoid operon remains largely undefined, with the exception that SigB defective strains lack pigmentation (108, 109) and mutations in *cspA* and *hfq* affect carotenoid production (95, 124). The increased expression levels at 10 and 15 hours compared to control cultures may merely indicate that the low-shear environment is delaying the production of carotenoids or affecting their stability and/or ability to target the cell membrane, resulting in only slight levels of pigmentation after 20 hours.

However, due to the concise nature of the bacterial genome and tight metabolic regulation (200, 235), it is very possible that, while the carotenoid operon is being transcribed at higher levels under LSMMG conditions at 15 hours, post-transcriptional regulation via an RNA chaperone such as Hfq could be inhibiting translation of these proteins and thus halting carotenoid synthesis.

The LSMMG conditions within RWV bioreactors create a novel environmental parameter in which traditional mechanisms of regulation are likely employed, albeit through means not yet clearly defined. For example, *S*. Typhimurium, the microorganism whose responses to LSMMG culture are the most completely molecularly and phenotypically characterized, demonstrated a strong increase in virulence while the expression of *hfq*, an essential virulence factor of *S.* Typhimurium, was decreased (225). Likewise, the counterintuitive finding between the expression analysis of the carotenoid operon and the phenotypic properties of low-shear-cultured *S. aureus* suggest novel mechanistic responses to this environment. In response to LSMMG conditions, the microarray analysis suggested transcription factors as possible regulators and qRT-PCR data implied an RNA chaperone. However, the RNA isolation technique was not optimal for the recovery of small noncoding RNAs, the microarray chips did not contain probes to the coding sequences of such genes, and only one time point (20 hours) was assessed by microarray analysis: thus, it is possible that in addition to Hfq, other RNA chaperones and/or small RNAs, govern the LSMMG response via classical regulators, such as SigB and Rex.

Chapter 6: *In Vitro* Analysis of Low-Fluid-Shear-Cultured *Staphylococcus aureus* on the Host-Pathogen Interaction

6.1 INTRODUCTION

S. aureus has long been considered an extracellular organism maintaining presence in a host through cellular adhesion (59, 82, 158, 194, 208). However, multiple recent, independent investigations with numerous cell types have documented its ability to invade and survive within host cells, resulting in its current classification as facultative intracellular bacteria (94, 234). While its invasion and survival within epithelial and endothelial cells (94, 234), kerotinocytes (141), fibroblasts (60), enterocytes (81), and osteoblasts (48) has been demonstrated, the mechanism involved in the invasion process is only partially understood (196). Furthermore, the events and/or stimuli that lead from colonization to an invasive phenotype are understudied and unclear.

While many aspects of the colonization and infection processes are not clearly understood, previously reported *in vitro* analysis suggests that those *S. aureus* strains with an enhanced capability to invade are not only able to cross cellular barriers, but also to avoid host immunity and cause chronic infection (172, 195). These findings are further corroborated by *in vivo* reports describing biopsies in which *S. aureus* within epithelial cells was linked with recurrent patient disease (63). Conversely, multiple assessments of nasal and intestinal carriage in healthy human and animal *S. aureus* carriers has revealed the adherence of the microorganism to the epithelium and/or mucin with few to no

reports of the organism within host cells (217). Although requiring greater experimental validation, the trends from these observations suggest that the degree of cellular invasion by *S. aureus* is correlated with the incidence of colonization and infection.

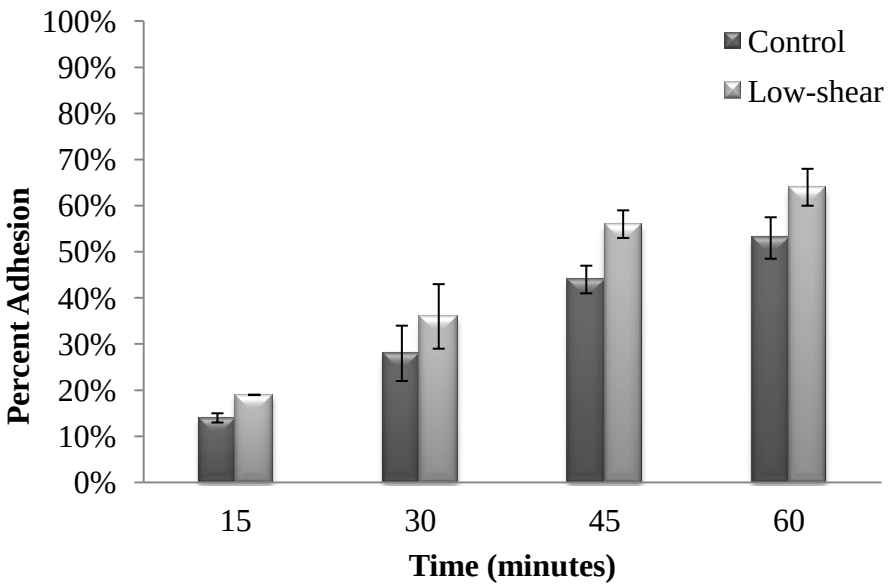
6.2 RESULTS

6.2.1 Adhesion to Epithelial Cells by *S. aureus* is Not Affected by Low-Fluid-Shear Culture

Adhesion to the host is an essential precursor in both staphylococcal colonization and infection (166). As MSCRAMMs, which are responsible for the ability of *S. aureus* to bind host factors, have been shown to function in a shear-dependent manner (158, 167), and as LSMMG culture has resulted in the increased adhesion of *E. coli* to host cells (4), the adhesion kinetics of low-shear-cultured *S. aureus* to epithelial cells was evaluated. Monolayers of the human colonic epithelial cell lines HT-29 and Caco-2 were chosen for these assays, as they have been extensively used and characterized as models for the intestinal barrier and have maintained differentiation traits characteristic of mature enterocytes (116, 189).

Immediately following 20 hours of culture in the LSMMG or control orientations of the RWV, *S. aureus* was added at an MOI of 20 to HT-29 or Caco-2 monolayers and assessed for adhesion every 15 minutes over a 60 minute time course. Adhesion occurred rapidly with approximately 15% and 35% of the bacteria adhering to the HT-29 and

A.



B.

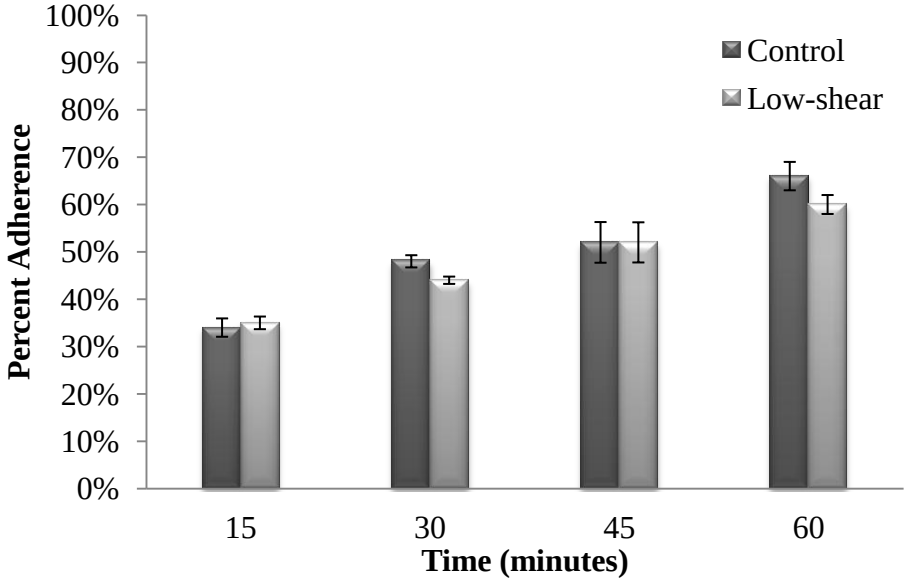


FIGURE 6.1. Low-Shear Culture Does Not Alter the Adhesion Rates of *S. aureus* to Human Epithelial Monolayers. LSMMG-cultured *S. aureus* N315 added at an MOI of 20 to HT-29 (A) and Caco-2 (B) monolayers did not display an altered ability to adhere as compared to controls over a 60 minute time course. Data is expressed as an average of three wells plus standard deviation and is representative of three independent biological replicates.

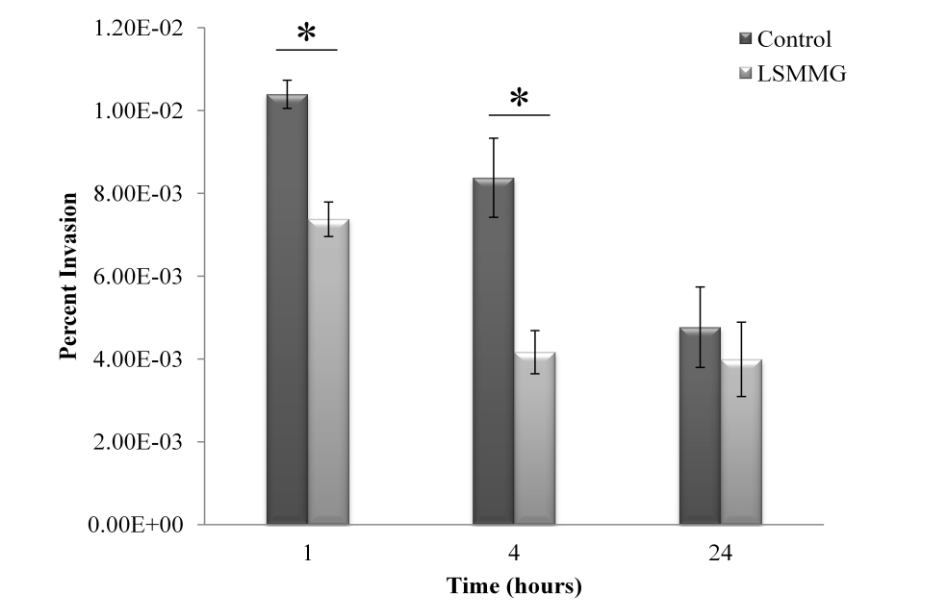
Caco-2 monolayers, respectively, within the first 15 minutes (Fig. 6.2A and B). Adhesion rates peaked between 55% and 70% at 60 minutes for both cell lines (Fig. 6.1A and B). There was no significant difference ($P < 0.05$) at any time point in the adhesion rates of low-shear-cultured *S. aureus* as compared to controls using either HT-29 or Caco-2 epithelial cells (Fig. 6.1A and B).

6.2.2 Invasion of Epithelial Cells by *S. aureus* is Decreased by Low-Fluid-Shear Culture

S. aureus is considered a facultative intracellular bacterium, as it invades a range of cell types (59, 82, 158, 194, 208). Furthermore, invasion of host cells by *S. aureus* likely plays an important role in recurring infections (173, 174, 192, 216, 218, 219). The invasion of HT-29 and Caco-2 monolayers by LSMMG-cultured *S. aureus* was assessed by exploiting a gentamicin and lysostaphin protection assay. As with the adhesion assay, *S. aureus* was added at an MOI of 20 to either HT-29 or Caco-2 monolayers following 20 hours of culture in the LSMMG- or control-orientated RWV. The bacteria were allowed to invade for 90 minutes at which point the cells were washed and lysed to determine the number of bacteria present at that point or given fresh cell culture media containing gentamicin and lysostaphin to kill extracellular bacteria, as described in Chapter 3. Invasion of the cells was evaluated at 1, 4, and 24 hour time points after the addition of gentamicin and lysostaphin.

Although adhesion rates of low-shear culture *S. aureus* were unaltered compared to rates from control orientation culture, the percentage of invading LSMMG-cultured

A.



B.

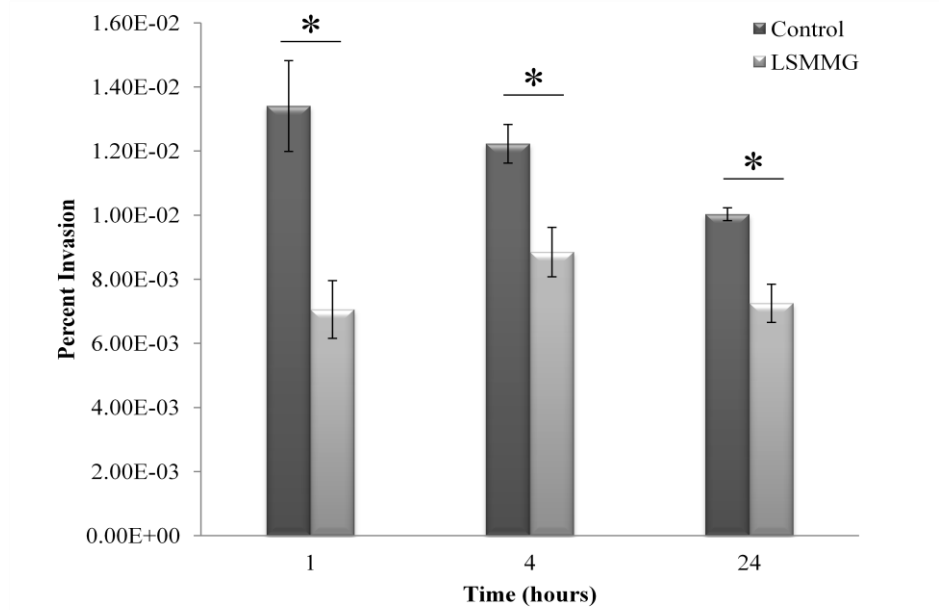


FIGURE 6.2. Low-Fluid-Shear Culture Results in Decreased *Staphylococcus aureus* Epithelial Cell Invasion. The percent invasion of LSMMG-cultured *S. aureus* is significantly reduced (*, $P < 0.05$) upon infection of HT-29 monolayers (A) and Caco-2 monolayers (B) post gentamicin and lysostaphin treatment as compared to control-cultured bacteria. Data is expressed as an average of three wells plus standard deviation and is representative of three independent biological replicates.

bacteria was significantly lower than the corresponding control cultures upon infection of either HT-29 or Caco-2 monolayers (Fig. 6.2A and B). The decreased percentage of invasion by low-fluid-shear-cultured bacteria as compared to controls within the HT-29 monolayers was statistically significant ($*$, $P < 0.05$) at 1 and 4 hours (Fig. 6.2A) and at 1, 4 and 24 hours within Caco-2 monolayers (Fig. 6.2B).

6.2.3 Low-Fluid-Shear Culture of *S. aureus* Results in Increased Susceptibility to Macrophage Clearance

Macrophages are key components of the host immune response to invading pathogens such as *S. aureus*, which has developed numerous resistance mechanisms against the deleterious effects of these cells (106). *S. aureus* is able to survive within macrophages for days before causing the immune cell to lyse (106). This persistence within macrophages may facilitate the dissemination of this organism through a host (106). One example of a resistance mechanism is the production of carotenoid pigments that supplement the organism's antioxidant properties (123). As carotenoid production is decreased in response to low-fluid-shear culture conditions (27), and as this environment has been shown to increase *S.* Typhimurium's ability to survive within macrophages, the survival of LSMMG-cultured *S. aureus* within macrophages was assessed.

To determine if the low-shear culture environment resulted in a difference in susceptibility to macrophage killing, the intracellular survival of LSMMG- and control-cultured *S. aureus* within the murine macrophage cell line J774 was measured.

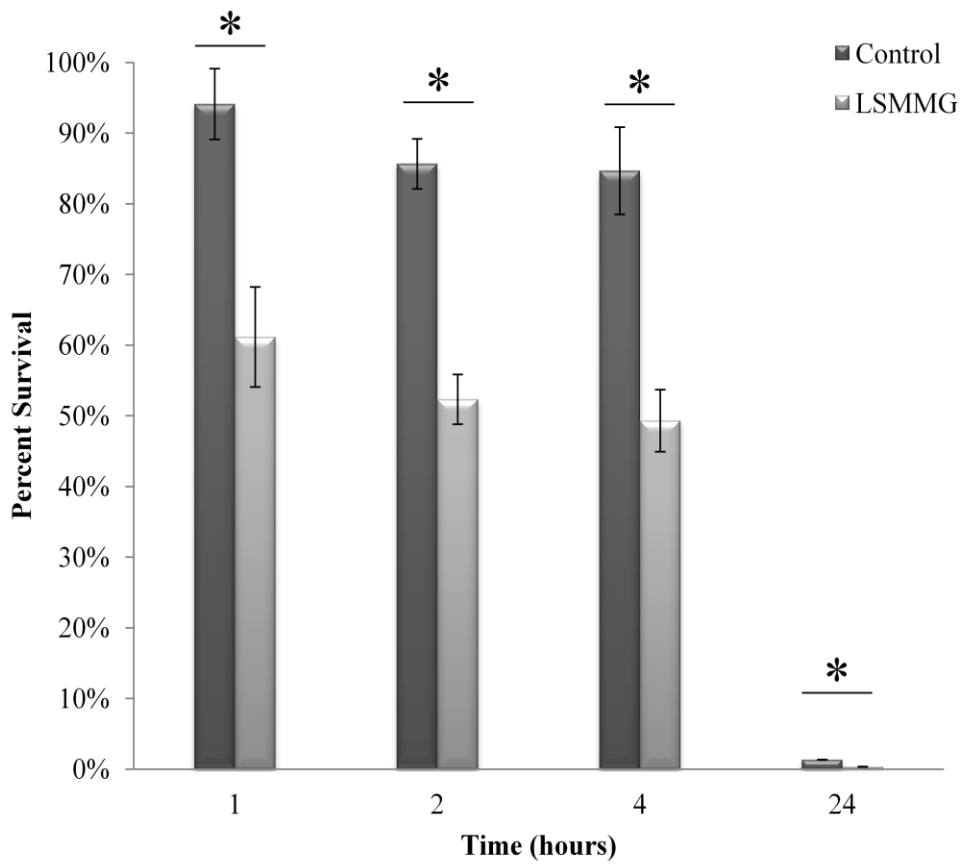


FIGURE 6.3. Low-Fluid-Shear Culture of *Staphylococcus aureus* Results in Increased Susceptibility to Macrophage Killing. The percent survival of LSMMG- and control-cultured *S. aureus* within J774 macrophages as determined after 1, 2, 4 and 24 hours after internalization. The low-shear-cultured *S. aureus* was significantly (*, $P < 0.05$) more susceptible to macrophage clearance as compared to controls at all time points assessed. Data is expressed as an average of three wells plus standard deviation and is representative of three independent biological replicates.

Bacteria were incubated with the J774 monolayers for 60 minutes, at which point the cells were washed and lysed to determine the number of bacteria present at that point or given fresh cell culture media containing gentamicin kill extracellular bacteria, as described in Chapter 3. Macrophage survival was evaluated 1, 2, 4, and 24 hours after the addition of gentamicin. The percent of intracellular bacteria present after 60 minutes incubation was similar between the LSMMG- and control-cultured *S. aureus*, with 71% and 76% of the initial inoculum internalized, respectively. While there was not a significant difference in the number of ingested bacteria, low-shear-cultured *S. aureus* was significantly more susceptible to macrophage clearance as compared to controls for all time points evaluated (Fig. 6.3). After 24 hours within the macrophages, the number of surviving bacteria was greatly reduced; however, the survival disadvantage imparted by LSMMG culture persisted through this time point, as these bacteria were found in significantly lower numbers compared to control culture bacteria.

6.3 DISCUSSION

Critical to *S. aureus* maintaining a presence within a host is its ability to adhere to host factors (166). Once established, the microorganism may become internalized within a host cell either (a) by binding to host cell receptors, resulting in a cascade that ends with its invasion or (b) by engulfment from professional phagocytic cells (106, 196). While the adhesion of *S. aureus* to host epithelium has often been connected to colonization (166), invasion, both bacteria-mediated invasion and phagocytosis, has been associated with an infectious phenotype (173, 174, 192, 216, 218, 219). Though these correlations

have been noted (154), the conditions that influence *S. aureus* adhesion to and invasion of host cells has not been investigated.

The region between the brush border and within the mucin of intestinal epithelial cells has been characterized as a low-fluid-shear environment (76). Therefore, many studies investigating the impact of low-shear microbial culture on host-pathogen interaction have used intestinal epithelium models (4, 33, 152, 177). Amid the intestinal-associated bacteria thus far examined in response to low-shear culture, *S*. Typhimurium has demonstrated increased cellular invasion (33) as well as increased survival within macrophages (152). Similar investigations of *E. coli* revealed increased adhesion to epithelial cells (4). As the ability of *S. aureus* to adhere to, invade, and survive within host cells is characteristic of its relationship with a host, the effect of a low-shear environment on these parameters was examined.

Adhesion rates were determined by infecting HT-29 or Caco-2 monolayers, commonly used as intestinal epithelium models (116, 189), with LSMMG- or control-cultured *S. aureus*. The adhesion rates of LSMMG-cultured *S. aureus* to HT-29 and Caco-2 monolayers were not significantly different than those of control cultures. As previously mentioned in Chapter 2, *S. aureus* has numerous cell wall-associated proteins that function in binding host factors, collectively known as MSCRAMMs (166). While MSCRAMM expression peaks during exponential phase to increase binding to host components (166), they are expressed continually throughout the life of the bacterium, as attachment and persistence is central in the lifecycle of *S. aureus*. Additionally, the shear-dependent behavior of MSCRAMM-host binding has been documented when both

the host cell and the bacteria were allowed to interact under shear flow conditions (65, 102, 144, 158, 167). The finding that the adhesion rates of low-shear-cultured *S. aureus* paralleled those of control cultures suggests that the expression and activity of the MSCRAMMs responsible for adhesion to host epithelium are comparable after 20 hours of culture in the LSMMG and control RWVs, although more conclusive evidence would be required to confirm this premise. The binding kinetics displayed for both LSMMG and control cultures are descriptive of *S. aureus* binding to epithelium *in vitro*, as previously reported (81).

Invasion of multiple cell types by *S. aureus* is well documented, and with the exception of certain compounds, environmental stimuli that enhance or inhibit invasion have not been investigated (85). *S. aureus* was able to invade both HT-29 and Caco-2 cell lines at a rate that is consistent with previously published *S. aureus* studies (81). Interestingly, low-shear-cultured *S. aureus* displayed a reduced capacity to invade both HT-29 and Caco-2 monolayers.

The impact of low-shear culture on the adhesion and invasion characteristics of *S. aureus* in an *in vitro* interaction with epithelial monolayers is consistent with a colonization phenotype in which adhesion levels are unaffected but invasion rates of the bacterium are decreased. This finding differs from the LSMMG responses seen with potential enteric pathogens (4, 33). In response to low-shear culture, the potential intestinal pathogen, adherent-invasive *E. coli* O83:H1, demonstrated increased adhesion to Caco-2 cells (4). Additionally, an increased percentage of the enteric pathogen, *S.* Typhimurium, invaded Caco-2 monolayers after culture under low-shear conditions (33).

Taken together, in response to a low-shear culture environment, microorganisms that have been associated with intestinal disease reveal an increased ability to adhere to or invade epithelial monolayers. In contrast, *S. aureus,* as an intestinal colonizer, demonstrates an unaltered ability to adhere yet a decreased capacity to invade epithelial monolayers. Collectively, this evidence suggests that the fluid-shear conditions present at sites within the intestine are serving as an environmental trigger that drives many microorganisms toward either a colonization or infectious phenotype.

If low-shear culture of *S. aureus* initiates a phenotype enabling colonization, it would follow that cell resources would not be utilized to defend against host macrophages, an indispensable property of invading pathogens. Accordingly, low-shear-cultured *S. aureus* was significantly more susceptible to macrophage clearance as compared to control cultures. In comparison, following LSMMG culture, *S.* Typhimurium demonstrates an increased ability to survive within macrophage monolayers (152). Given that *S. aureus* is so well equipped at surviving within macrophages, the high percentage of macrophage killing of low-shear-cultured bacteria suggests that at least one of the mechanisms associated with combating macrophage destruction was decreased. As colonizing microorganisms often down-regulate dispensable virulence components in favor of essential factors supportive of this lifecycle (25, 122, 174), it is likely that the decrease in antioxidant carotenoids observed in LSMMG culture (27) increases the susceptibility to the lethal effects of reactive oxygen species produced within the macrophage. The increased susceptibility to macrophage

killing further supports adhesion and invasion data, which indicate that low-shear culture conditions induce a colonization phenotype.

CONCLUSION

Over 100 years have passed since Alexander Ogston described the role of *S. aureus* in disease and put forth the question as to how a microorganism connected with such acute pathology could be otherwise innocuous when associated with a host. While the details necessary to fully answer his question are lacking, a picture is emerging describing environmental stimuli as a driver behind the staphylococcal-host interaction.

As a colonizer and pathogen, no site on or within a host is inaccessible to *S. aureus*. One such site associated with staphylococcal colonization is the intestine. As clinical symptoms are associated with the staphylococcal production of a toxin rather than through invasion, very few investigations have focused on how this environment impacts the bacteria. Within the intestinal tract exists a vast range of microenvironments resulting from luminal flow rates, fluctuating levels of nutrients, alterations in the mucus layer, and interactions with epithelial surfaces, all of which an inhabiting microorganism must withstand (130). Prior to colonization, a bacterium must outlast the high levels of shear created by material flowing through the intestinal lumen (198). Successful colonizers convene near the intestinal barrier out of the direct luminal flow where they encounter the mucus layer, intestinal epithelium and decreased levels of fluid shear (76). At this juncture, the microorganism can persist and colonize or actively infect the epithelium. While *S. aureus* is capable of being highly pathogenic, its nonpathogenic phenotype within the intestine of a host suggests that factors at this site predispose the organism to a colonization phenotype. However, the low levels of fluid shear present at

this site have not been considered previously as a factor impacting the lifecycle of *S. aureus*. Therefore, to better understand the influence of this low-shear environment, the response of *S. aureus* was investigated by means of the physiologically relevant low-fluid-shear conditions generated by the RWV bioreactor.

Culture of *S. aureus* within the LSMMG environment initiated the formation of attachment-independent biofilms, demonstrated by the abundant production of EPS completely encasing the bacterial cells and a corresponding increase in antibiotic resistance (27). As intestinal colonization could be advantageous in the lifecycle of *S. aureus* (201), so would be the ability to form a biofilm (193). Bacterial biofilms associated with gastrointestinal mucus have been proposed and substantiated by multiple investigators (21, 129, 130, 163) and are evolutionarily beneficial as they (a) provide shelter from environmental stressors, antibiotics, and host immunity, (b) allow for persistence in a favorable environment, (c) enable communal metabolism, and (d) increase the possibility of transformation and transduction (89, 193). The association of low-shear culture and alterations in biofilm production has been previously described, as *P. aeruginosa* and *E. coli* have demonstrated increased biofilm potential following LSMMG culture (36, 128). The response of *P. aeruginosa* to low-shear conditions parallels the phenotype of the organism when isolated from the lungs of cystic fibrosis patients in which it self-aggregates and produces increased alginate (36, 37). Additionally, *E. coli* seeded on glass microcarrier beads and cultured in LSMMG conditions forms thicker, more resistant biofilms (128). In combination with the observations of this study with *S. aureus*, EPS alteration in low-shear environments

appears to occur and possibly benefit multiple microorganisms. Indeed, if the enhanced EPS production by *S. aureus* during LSMMG culture reflects an *in vivo* response in the low-fluid-shear sites of the intestinal tract, then the RWV bioreactors may serve a model of mucus-associated intestinal biofilms.

Different environmental conditions are experienced by bacterial cells within the EPS matrix of an attached biofilm, as compared to cells growing planktonically (180). The conditions within the biofilm matrix have been described as having low oxygen tension and limited nutrient availability, resulting in an altered metabolic profile of the microorganism (147, 180, 193). Through the use of whole genome microarray analysis, 17 genes were identified whose expression was increased in response to the LSMMG culture environment. Biochemically, the protein products of several of these genes are associated with fermentative metabolism, as formate acetyltransferase, D-lactate dehydrogenase, and alcohol dehydrogenase were induced by low-shear culture (27). In a search for common regulation, an alignment of the predicted regulatory regions of the low-shear-responsive genes revealed two conserved consensus sequences: t/aTGTGA t/a_6 TCACAt/a and GTTTa/ta/$t_{12\text{-}15}$ GGGa/tAa/t. The first is a binding site for the Rex repressor (162). Rex is a transcriptional repressor that functions to maintain the intracellular NADH/NAD^+ redox balance (162). As NADH within the cell increases, Rex binds the overabundant NADH, resulting in the derepression of genes whose protein products are involved in fermentative respiration, which serves to regenerate the NAD^+ pool (162). Therefore, the increased expression of genes under the control of Rex, in response to LSMMG culture, is consistent with cells surviving under the conditions

associated with a biofilm. The second conserved consensus sequence is a binding site for the alternative sigma factor, SigB (67). As SigB is a global regulator involved in the differential regulation of more than 200 staphylococcal genes in response to various environmental stressors and upon entrance into stationary phase (31, 67, 108, 109, 150, 165), it is not surprising that genes connected with its regulation were up-regulated in response to LSMMG culture. However, the role of SigB regulation in biofilm formation remains under debate (176, 213). Additionally, 8 of the 17 low-shear-responsive genes have been previously reported as being up-regulated in *S. aureus* biofilms as compared to planktonic cultures (180). Taken together, the microarray analysis data correlates with the phenotypic data that *S. aureus* responds to low-fluid-shear by initiating a biofilm phenotype.

Further characterization of the staphylococcal cells within the LSMMG-induced biofilms revealed a phenotype consistent with colonization, in which decreased growth and carotenoid pigmentation, increased susceptibility to hydrogen peroxide, killing by whole blood, and macrophage clearance, as well as a decreased ability to invade epithelial monolayers, was demonstrated. The yellow coloration of *S. aureus* is a result of carotenoids anchored to the cell membrane (133, 134). These compounds serve as a protective coat shielding the cell from reactive oxygen species (105, 123, 164). The reduction in carotenoid production in response to low-shear culture amounts to a reduction in antioxidant protection which could account, in part, for the increased susceptibility to hydrogen peroxide, killing by whole blood, and macrophage clearance. When subjected to a hydrogen peroxide stress assay, a higher percentage of LSMMG-

cultured *S. aureus* succumbed to oxidant attack. However, when allowed to sit statically, out of the low-shear environment, the difference in susceptibility to hydrogen peroxide between the low-shear- and control-cultured *S. aureus* diminished after approximately 1.5 hours (27). Interestingly, when challenged with the immune components present in whole blood, in which neutrophils are present and use ROS to kill ingested pathogens, LSMMG-cultured *S. aureus* was significantly more susceptible to killing after 4 hours of co-incubation. Additionally, when phagocytized by macrophage monolayers, LSMMG-cultured *S. aureus* was significantly more susceptible to clearance 24 hours after infection. While the increased susceptibility to oxidative stress induced by low-shear culture is transient, the increased susceptibility to more complex, *in vitro* interactions was found to be prolonged, lasting up to 24 hours. This type of response is not unprecedented as it is analogous to previous reports of increased virulence in *S.* Typhimurium. In those studies, significantly decreased mouse survival rates were observed days after infection by LSMMG-cultured *Salmonella* as compared to controls (152). It remains unclear whether the adaptation of the bacterial cells in response to low-shear culture is slower to transition back to the control phenotype in a host or cell culture or their response is more persistent in this environment. Regardless, the low-shear-induced effect is indeed extended in the host or host cells and requires further examination to fully understand this host-pathogen interaction.

S. Typhimurium and pathogenic *E. coli* are among the most commonly associated enteric pathogens with bacterial gastroenteritis, due, in part, to their ability to traverse the mucus layer and interact with the underlying epithelium (140). Furthermore, both have

the capability to actively invade intestinal epithelial cells, providing themselves with a protected site for replication (57, 58, 140). In response to low-shear culture conditions, the potential pathogen *E. coli* 083:H1 has demonstrated an increased ability to adhere (4) and *S.* Typhimurium has displayed increased invasiveness to epithelial monolayers (33). In direct contrast, LSMMG-cultured *S. aureus* revealed a decreased ability to invade host epithelium *in vitro*. As adherence levels were unaffected by low-shear culture and as mediators of invasion were not found to be differentially expressed by molecular methods, the reason for decreased cellular invasion is unclear. Some insight might be gained from patients on statin regimens, who are at a decreased risk of developing bacterial sepsis (85). Research indicates that the treatment of endothelial cells with statin drugs results in the reduced ability of *S. aureus* to invade host cells (85). These investigations have focused on the effects of the drug on the host cell, in which the statin blocks steps in the cholesterol biosynthesis pathway, including depleting host cell isoprenoid intermediates, which resulted in reduced *S. aureus* invasion due to defects within host cell endocytosis (85). Interestingly, these same drugs inhibit *S. aureus* staphyloxanthin production. As the presence of staphyloxanthin alters the bacterial membrane structure (104, 169), it is possible that its absence may affect the ability of *S. aureus* to initiate classical cellular invasion cascades. While the mechanism behind the reduced invasive capability of LSMMG-cultured *S. aureus* warrants further investigation, it follows that, while *S. aureus* does not benefit from invasion of the intestinal epithelium, upon sensing the low-shear conditions in the mucosa near the intestinal cell surface, *S. aureus* preferentially establishes a biofilm/colonization phenotype.

While the molecular expression data generated through microarray analysis correlated with the phenotypic finding of low-shear-cultured *S. aureus* existing in a biofilm, it did not afford any evidence as to the lack of pigmentation. Investigation of changes in gene expression of the carotenoid biosynthesis process following 20 hours of low-shear culture failed to reveal differential expression levels that could account for the difference in carotenoid concentration. Therefore, levels of expression of the carotenoid operon were evaluated following 10 and 15 hours of low-shear culture under the assumption that gene expression may be occurring prior to stationary growth phase. Interestingly, expression levels of members of the carotenoid operon were found up-regulated after 10 hours of LSMMG culture, with the entire operon revealing significantly increased expression levels after 15 hours. Unexpectedly, this increase in expression does not correspond with the decrease in carotenoid pigmentation demonstrated by LSMMG-grown *S. aureus*. The regulation of the carotenoid operon is essentially unknown with the exception that strains lacking SigB are unpigmented (107, 132) and mutations in *cspA* and *hfq* alter carotenoid production (94, 122). Based on qRT-PCR analysis, expression levels of *sigB* and *cspA* were unaltered in response to low-shear culture at the 20 hour time point analyzed. While SigB and CspA may be involved in the low-shear response prior to the time point assessed, there is compelling evidence to associate a regulatory role for Hfq with this response, as expression levels of *hfq* were decreased. In addition to its decreased expression and possible involvement in carotenoid synthesis, the case for Hfq as a regulator in *S. aureus* in the low-shear environment is strengthened by a comparison of the microarray data with the work of Liu, et al. (122).

Of the 17 LSMMG-induced up-regulated genes, 10 have been shown to be either up-regulated in an *hfq* mutant or to bind Hfq (122). Even more compelling is the mechanistic connection of Hfq to the low-shear response of *S.* Typhimurium and *P. aeruginosa* (37, 222). This is the first description associating an Hfq response to the low-fluid-shear environment in a Gram-positive bacterium. The correlation of Hfq to the low-shear response of *S. aureus*, in addition to the previously documented responses in Gram-negatives, strongly suggests that the ability to sense and respond to mechanical stimuli is evolutionarily conserved among structurally diverse prokaryotes.

The potentially evolutionarily-conserved responses of *S. aureus* to low-shear culture share common mechanistic characteristics with Gram-negative organisms, such as the involvement of Hfq; however, a fundamental difference in how these organisms alter virulence characteristics exists. This difference consistently appears to be based on the benefit toward each microorganism's proliferation and perseverance capabilities in this environment. Contrary to previous reports of enhanced virulence and/or virulence properties of *S.* Typhimurium and the potential pathogen adherent-invasive *E. coli* (4, 152), *S. aureus* appears to favor a phenotype consistent with colonization, in which it forms a biofilm, slows its growth, and down-regulates virulence characteristics. This response potentially benefits *S. aureus* within the gastrointestinal tract and possibly other mucosal host sites where low-fluid-shear levels may be encountered. These findings clearly define a role for fluid-shear as an environmental cue that dictates the nature of the host-pathogen interaction in *S. aureus* and directs this organism toward a colonization phenotype in low-fluid-shear.

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Nickerson, C.A., Ott C.M., **Castro S.L.**, Garcia V.M., Molian, T.C., Brigger J.T., Pitt A.L., Tavano J.J., Byram J.K., Barrila J., Nickerson M.A. 2011. Evaluation of Microorganisms Cultured from Injured and Repressed Tissue Regeneration Site in Endangered Giant Aquatic Ozark Hellbender Salamanders. PLoS ONE. 6(12):e28906.

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