Long-term Stationary Phase Behavior of *Streptococcus pyogenes* Biofilms

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By
Gregory R. Steinberg
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Thesis Advisory Committee:

Bettina Buttaro, PhD., Thesis Advisor, Microbiology & Immunology
Patrick Piggot, PhD., Microbiology & Immunology
Çagla Tükel, PhD., Microbiology & Immunology

ABSTRACT

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Department of Microbiology and Immunology

Streptococcus pyogenes is the etiological agent of many human diseases ranging from mild superficial skin infections and pharyngitis to life-threatening necrotizing fasciitis. There can be several complications as a result of *S. pyogenes* infection including post-streptococcal glomerulonephritis and rheumatic fever, which leads to rheumatic heart disease. Despite the significant virulence associated with the pathogen, the bacteria can also persist asymptomatically in human host carriers. *S. pyogenes* is characterized by significant strain-to-strain variation with many single nucleotide polymorphisms and differences in genetic content of up to 33% of the genome. Active infection is associated with the rapid growth of the pathogen, whereas survival or carriage is associated with slow growth.

Our laboratory has demonstrated that during survival in long-term stationary phase cultures and in eukaryotic cells, *S. pyogenes* diversifies into a mixed population. Isolates from this population show diversification in their proteome, in metabolism, and in virulence factor transcription patterns. These are stable, heritable changes with unique mutations in global gene regulators in some isolates, suggesting that an accumulation of genetic mutations leads to diversification. There are two proposed modes of survival in the human host; by taking residence intracellularly in host cells and as biofilms. Previous studies showed that isolates surviving within eukaryotic cells acquire heritable changes in metabolism and virulence factor expression.

Biofilms are highly organized structures formed by many bacteria, which provide resiliency to harsh environmental conditions. It has been demonstrated that *S. pyogenes* form biofilms in vivo and in vitro, and up to 90% of clinical isolates can form biofilms. Considering the resiliency of biofilms, and the organized roles played by individual cells in biofilms, we hypothesized that biofilms may provide *S. pyogenes* with a niche for persistence and diversification. Despite the capacity for survival of planktonic cells, we have found that viable cells could not be isolated from static biofilms after 10 days. No metabolic variants were found among biofilm isolates prior to loss of biofilm viability. Biofilm structure was examined using confocal microscopy to image cells after LiveDead® staining. These experiments revealed that the biofilms lost viability rapidly, and also appeared to disperse. Dispersion of 2-day old biofilms could be induced with culture supernatants collected from 7-day old planktonic cells. Overall, the results of these studies suggest that secreted factors from late stationary phase cultures induce biofilm dispersion and biofilms do not serve as a niche for long-term survival and diversification of *S. pyogenes*. Therefore, *S. pyogenes* biofilms may be more critical for initial colonization of the oropharynx. These studies may provide a valuable insight to the role of biofilms in *S. pyogenes* infections.

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ABBREVIATIONS

®	trademark
APC	antigen presenting cell
ATP	adenosine triphosphate
C	celsius
CFU	colony forming unit
dH2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonulclease
eDNA	extracellular deoxyribonucleic acid
GAS	group A streptococcus
h	hour
LTA	lipoteichoic acid
m	minutes
mg	milligram
MHC	major histocompatibility complex
ml	milliliter

MLST	multi-locus sequence typing
mm	millimeter
NAD+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogen
n.d.	not detected
NIH	National Institutes of Health
nm	nanometer
OD	optical density
PBS	phosphate buffered saline
PI	propidium iodide
RBC	red blood cell
s	seconds
SNP	single nucleotide polymorphism
SpeB	streptococcal pyrogenic exotoxin B
STSS	streptococcal toxic shock syndrome
TCR	T cell receptor
T_H	helper T cell

TH	Todd Hewitt
TSLS	toxic shock like syndrome
μg	microgram
μl	microliter
μm	micrometer

CHAPTER 1

INTRODUCTION

1.1 Overview

Streptococcus pyogenes is a pathogen specific to the human host, and is capable of causing a wide array of diseases. The pathogen is a Gram-positive non-motile coccus which grows in chains. *S. pyogenes* is classified as a Group A Streptococci (GAS) on the basis of its Lancefield Antigen, which is a cell wall antigen. GAS are beta-hemolytic, meaning they produce hemolysins which lead to complete destruction of red blood cells (RBC) on 5% blood-agar plates. The organism is homofermentative producing lactic acid from glycolysis during exponential growth. Upon reaching stationary phase, the organism can produce a variety of other end product metabolites, including formate, ethanol and acetate. *S. pyogenes* is a common human pathogen, causing up to 700 million infections yearly with a mortality of 500,000 cases (Carapetis et al., 2005). *S. pyogenes* causes infections in a variety of niches, including relatively mild, superficial infections such as skin infections and tonsillitis to severe systemic diseases such as necrotizing fasciitis and toxic shock syndrome. In addition to causing disease, *S. pyogenes* can also exist in an asymptomatic carriage state, where it can survive in the host and cause recurrent infections.

1.2 Diseases caused by *S. pyogenes*

There are several diseases associated with *S. pyogenes*. These diseases either can be the result of an acute infection or mediated by the host immune system in response to *S. pyogenes* infection.

Pharyngitis

S. pyogenes is the most common cause of bacterial pharyngitis in children (Shaikh et al., 2010). The disease is characterized by the sudden onset of symptoms, which include sore throat, fever, headache, and malaise. There appears to be little correlation, if any, with prevalence and M type, although M protein has a significant role in virulence (Bisno et al., 2003). This suggests the different serotypes of M protein do not vary significantly in their biological activities despite their antigenic variation. During infection, the organism remains outside of the host cells, and produces a variety of toxins and proteases to fuel rapid growth (Ahmad, 2010). While streptococcal pharyngitis is typically treated with beta-lactam antibiotics, such as penicillin, the treatment comes with an unusually high failure rate (Brandt et al., 2001). More puzzling is the absence of any penicillin-resistant clinical strains. While non-compliance to antibiotic treatment may account for some treatment failures, it has been suggested that there may be alternative mechanisms for antibiotic failure, such as biofilm formation and intracellular invasion of epitheliocytes (Podbielski and Kreikemeyer, 2001, Molinari and Chhatwal, 1999).

In some patients, tonsillar biopsies show the presence of *S. pyogenes* intracellularly, although the organism is considered an extracellular pathogen (Osterlund and Engstrand, 1997). In vitro *S. pyogenes* has been shown to invade epithelial cell lines and primary tonsillar epithelial cells (Molinari and Chhatwal, 1999, Osterlund and Engstrand, 1995, LaPenta et al., 1994). This finding suggests that the organism can reside inside of host cells, allowing for the successful evasion of immune defenses during

clearance. This intracellular residence would allow the pathogen to escape treatment by penicillin, which is unable to cross the cell membrane and may account for some cases of antibiotic treatment failure where the patient has been compliant with treatment courses. Supporting this hypothesis is the clinical success of treatments using antibiotics that can cross the cell membrane, such as macrolides (Passali et al., 2007). Intracellular residence and re-emergence may also account for recurrent tonsillitis, although not all cases of recurrent tonsillitis are caused by the same strain, based on MLST typing or M-type (Podbielski et al., 2003, Chang et al., 2011). Some of this variation may be due to diversification during intracellular survival (see below).

In addition, over 90 percent of *S. pyogenes* strains can form biofilms to varying degrees (Baldassarri et al., 2006). Biofilms are layers of live cells embedded in an extracellular matrix that may be attached to biotic or abiotic surfaces. These layers are intrinsically resistant to antibiotic treatment and to host immune defenses by preventing phagocytosis. Biofilms are a mechanism of survival for other pathogens, such as *Escherichia coli* (Lewis, 2008).

Impetigo

Impetigo is a localized skin infection with *S. pyogenes* commonly affecting children. While the skin normally acts as an innate barrier, the bacteria can establish infection in the skin via small breaks in the skin caused by trauma, animal bites, and mosquitoes (Ahmad, 2010). There is a strong serotype correlation with impetigo, specifically serotype M49 (Bisno and Stevens, 1996). There is also a strong association between impetigo infection and post-streptococcal glomerulonephritis, but not rheumatic

fever. Impetigo is a chronic condition and probably not associated with rapid growth of the bacteria.

Necrotizing fasciitis

Perhaps the most infamous disease associated with *S. pyogenes* is necrotizing fasciitis, also called flesh-eating disease, although this is a misnomer as the bacteria do not feed on the flesh. Instead, necrotizing fasciitis is the fulminant destruction of the cutaneous connective tissues (fascia) facilitated by the secretion of pyogenic exotoxins and possibly other unidentified virulence factors (Olsen and Musser, 2010). While it is a rare disease, necrotizing fasciitis is deadly with a lethality rate of 50 percent in the United States (Trent and Kirsner, 2002, Carapetis et al., 2005). Surviving patients usually have significant complications including amputation and severe scarring. Treatment consists of debridement of the affected area to remove exotoxin-producing bacteria (Trent and Kirsner, 2002, Olsen and Musser, 2010). The reasons behind the development of necrotizing fasciitis are not clear and there is not a correlation between strains isolated from the infections or known host factors and the disease.

Streptococcal Toxic Shock Syndrome

Toxic shock syndrome is a serious, sometimes fatal disease caused by *Staphylococcus aure*us and *S. pyogenes*. When *S. pyogenes* is the causative agent the disease is referred to as Streptococcal Toxic Shock Syndrome (STSS), or Toxic Shock-Like Syndrome (TSLS) (Ahmad, 2010). STSS has a very high mortality rate of over 65% (Huang et al., 2001). STSS differs from the aforementioned disease presentations, as it is a systemic toxemia with *S. pyogenes* (Lappin and Ferguson, 2009). The disease is the

result of toxin secretion by *S. pyogenes*, which act as superantigens. Superantigens activate T cells via a distinct mechanism. Typically, processed antigens are presented on antigen presenting cells (APCs) through Major Histocompatibility Complex II (MHCII). The antigen-primed MHCII can be detected by the T Cell Receptor (TCR) present on $CD3^+CD4^+$ Helper T lymphocytes (T_H cells). The T_H cells become activated, and act in a regulated fashion to secrete immune cell attracting chemokines resulting in inflammation at the site of infection. Superantigens crosslink the MHC and the TCR in an antigen-independent manner, binding outside of the normal antigen-binding pocket (Petersson et al., 2004). This leads to excessive and unregulated cytokine release, ultimately leading to multi-organ failure due to a systemic inflammatory response mimicking endotoxic shock (Martin et al., 1995, Eriksson et al., 1998). Scarlet fever is a mild form of STSS.

Post-Streptococcal Sequelae

Rheumatic fever mostly affects children and adolescents, and occurs roughly 20 days after the presence of pharyngitis or Scarlet fever. It is a Type II hypersensitivity reaction, where antibodies targeted against the M-protein cross-react with the arteriolar connective tissue in the absence of bacteria (Carapetis et al., 2005). The result is inflammatory destruction of cardiac tissue resulting in Rheumatic fever. As the reaction progresses, anatomical scarring of the heart leads to altered cardiac function, resulting in chronic rheumatic heart disease (Hilario and Terreri, 2002). Symptoms associated with rheumatic heart disease include heart arrhythmia, murmur, angina, congestive heart failure, and syncope resulting from reduced cardiac function.

Like rheumatic fever, acute glomerulonephritis is immune-mediated, however classified as a Type III Hypersensitivity (Cunningham et al., 2000, Rodriguez-Iturbe and Batsford, 2007). This type of immune dysfunction involves deposition of antigen-antibody complexes, both specific to *S. pyogenes* and non-specific, in the glomerulus of the kidneys. The immune complexes initiate complement-mediated destruction of kidneys, leading to renal impairment.

S. pyogenes Diversification during Survival

Previous work in our laboratory investigated carriage of *S. pyogenes* CS101 using an in vitro model of survival, which may be reminiscent of the environment of the physiological state of the bacteria during carriage in the host.

Survival in Stationary Phase Cultures

S. pyogenes can survive in static cultures in Todd-Hewitt (TH) medium for over 1 year (Wood et al., 2005). After inoculation of the medium, the bacteria grow quickly to achieve peak Colony Forming Units (CFUs) within 16 hours. After a brief transition to stationary phase, the bacteria enter a brief death phase until about 2 weeks post inoculation. At this point, the bacterial population stabilizes and a small subpopulation consisting of 0.01% of the exponential-phase population survives for over 1 year. Isolates from these stationary phase cultures, after about 4 weeks of incubation without the addition of fresh media, form altered colony morphologies on TH agar. The different colonies do not have significantly different numbers of CFUs/colony, however capsule expression is varied, and isogenic capsule mutants form small colonies (Leonard et al.,

1998). This suggests that differential capsule expression is responsible for the colony morphology.

During exponential growth, *S. pyogenes* is a homofermentative lactic-acid producer. When glucose is depleted the organism can utilize several stationary phase pathways for energy (Figure 1). Two of these pathways in particular start by oxidizing lactic acid produced during exponential phase back to pyruvate, which can be converted to acetyl-CoA and formate (Figure 1A). These products may be processed to yield acetate and generate additional ATP, or are used to generate ethanol to recycle NAD+. Alternatively, the bacteria can utilize amino acids as an energy source via the arginine deminase pathway yielding ATP and NH_3 (Figure 1B) or the serine utilization pathway yielding additional pyruvate and NH_3 (Figure 1B). Some stationary phase isolates, are heterofermentative during exponential growth producing varying amounts of formate, acetate and ethanol. When the proteomes of microcolonies were compared, there were significant differences in the expression of several proteins relating to metabolism, heat shock, and cell growth.

These changes in colony morphology, metabolism, and proteomes, are stable and heritable changes in the strains genome. The characteristics of the various isolates are different, suggesting random changes may be occurring. Consistent with this hypothesis the sequencing of a limited number of genes has revealed an SNP in one isolate and an insertion in a different isolate.

Surviving isolates do not survive differently than the parental strain in stationary phase in pure cultures, unless there is an overproduction of acid during exponential

growth, in which case survival is shortened. The isolates show different levels of virulence, predominantly associated with changes in expression of known virulence factors. Since survival in stationary phase does not select for some of these virulence factors, these observations further support the random nature of the mutations.

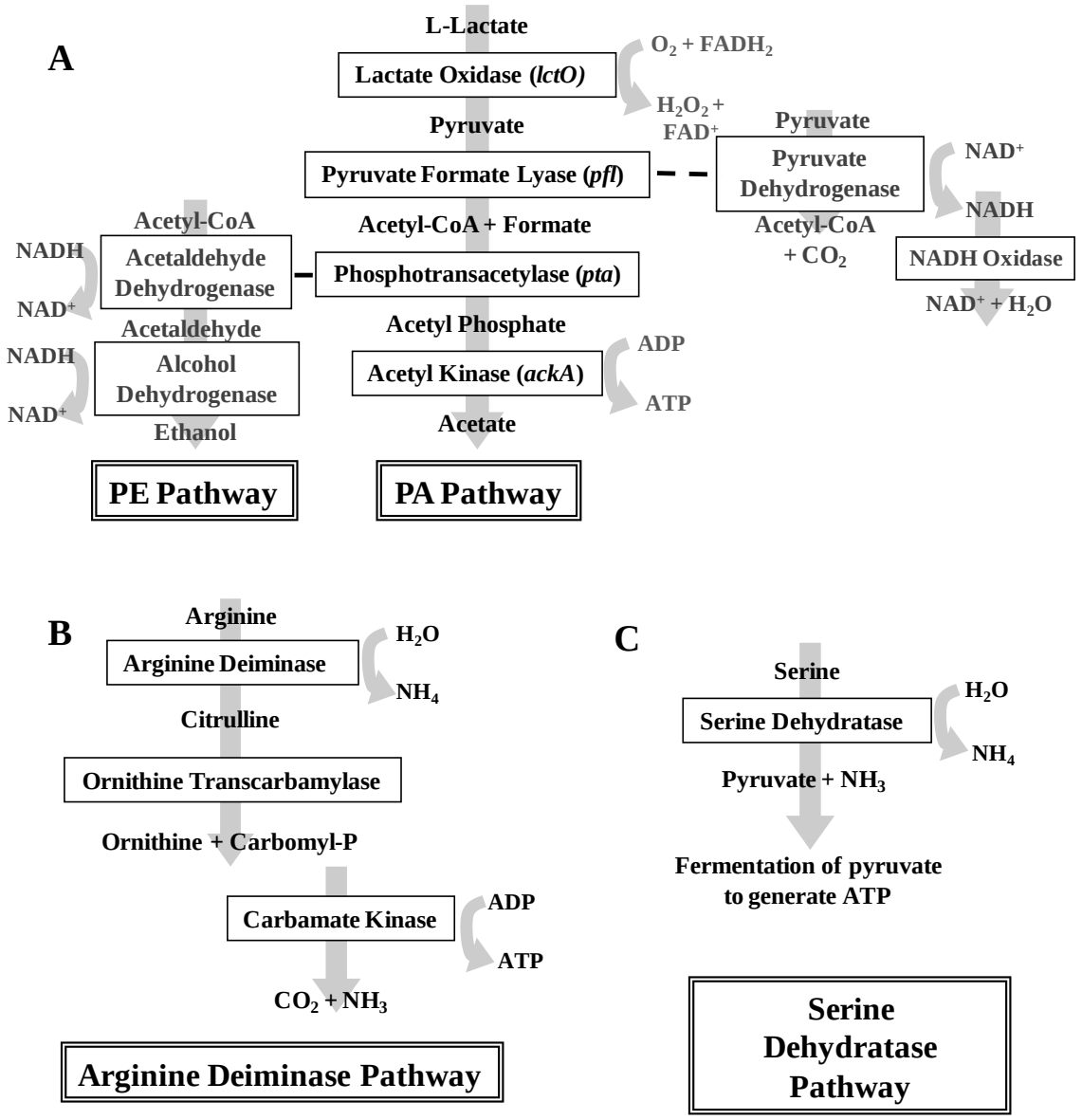

Figure 1. Metabolic pathways upregulated during long term survival

A: The Pyruvate to Acetate (PA) and the Pyruvate to Ethanol (PE) pathways generate ATP and NAD^+ by converting pyruvate to acetate and ethanol. B: The arginine deiminase pathway produces ATP and NH_3. C: The serine dehydratase pathway produces NH_3 and pyruvate, which can feed into the PA or PE pathway. (Wood et al., 2009)

Intracellular Survival

S. pyogenes is an extracellular pathogen, but is also able to invade and take up intracellular residence (Molinari and Chhatwal, 1999, Podbielski and Kreikemeyer, 2001). Previous studies from our laboratory investigated intracellular residence as a niche for diversification. As an in vitro co-culture model, A549 human lung epithelial cells were co-cultured with *S. pyogenes* CS101. Penicillin and gentamycin were added after 2 hours to destroy any remaining extracellular bacteria and fresh antibiotic-containing medium was added every 48 hours. After 5 days the eukaryotic cells were lysed and the surviving bacteria were cultured. These bacteria showed altered colony morphology as small-colony variants and a diversification similar to that of surviving stationary phase cultures; however, these changes occurred in the co-culture model at an accelerated rate. Surviving strains isolated from eukaryotic cell co-cultures invaded better, but did not have increased survival fitness. All strains produced low levels of capsule; capsule has been shown to interfere with intracellular invasion to protect the bacteria against phagocytosis (Wessels and Bronze, 1994). Once inside the cell, the bacteria likely exist in a quiescent state to evade detection. This state is likely characterized by slow growth, like that of stationary phase. Taken together, these data suggest bacteria surviving in eukaryotic cells may be natural niche in the host where diversification can occur.

S. pyogenes Biofilms

It has been estimated that over 90% of *S. pyogenes* strains can form measurable biofilms (Baldassarri et al., 2006), suggesting that biofilms may play some role in the host. *S. pyogenes* can form biofilms to varying degrees (Courtney et al., 2009, Lembke et

al., 2006, Thenmozhi et al., 2011) and some strains (known as hyper-biofilm formers) can form biofilms in vitro comparable to classical biofilm formers such as *Streptococcus mutans* (Thenmozhi et al., 2011) and *Staphylococcus epidermidis* (Lembke et al., 2006, Ogawa et al., 2011b). The ability for *S. pyogenes* to form biofilms is correlates with their M-type, although significant strain-to-strain variation remains (Lembke et al., 2006, Thenmozhi et al., 2011) possibly because of the amount of lipoteichoic acid (LTA) contributing to the hydrophobicity of the bacterial surface (Molinari and Chhatwal, 1999).

Little is known about the composition of the matrix material of the *S. pyogenes* biofilms. DNase I and Proteinase K treatment inhibits biofilm formation, indicating the importance of extracellular DNA (eDNA) and secreted proteins in the formation of biofilms (Roberts et al., 2010). In contrast to *Pseudomonas aeruginosa* and other bacteria, which rely on polysaccharides as a matrix material (Hall-Stoodley et al., 2004), metaperiodate does not inhibit biofilm formation suggesting that polysaccharides play a limited role in biofilm formation by *S. pyogenes* (Roberts et al., 2010). Mature *S. pyogenes* biofilms are also disrupted upon the addition of DNase I and Proteinase K, but not metaperiodate, providing more evidence for the role of DNA and protein in the formation of biofilms.

Transcriptome pattern analysis reveals that *S. pyogenes* biofilms have distinct gene expression, entirely distinct from exponential and stationary phase planktonic cells (Cho and Caparon, 2005). This evidence shows the inverse expression of biofilm-mediating genes with virulence, suggesting biofilms may have a limited role at the peak of infection.

The global transcription regulator Srv has been implicated in regulating formation of biofilms in *S. pyogenes* (Doern et al., 2009, Roberts et al., 2010). Srv mutants show a decrease in biofilm formation and an increase the production of the cysteine protease SpeB. Inhibition of SpeB restores biofilm formation, suggesting SpeB may have a role in disruption of biofilm formation (Doern et al., 2009). Srv does not directly regulate SpeB. However, Srv controls many genes associated with amino acid and nutrient uptake, which are downregulated upon the deletion of Srv. The genes controlling SpeB likely perceive the downregulation of the uptake machinery as a lack of available nutrients, and upregulate SpeB as a result (Chaussee et al., 2001) and inhibit biofilm formation. Srv deletion mutants are less virulent and mice have an increased survival rate compared to those infected with wild-type *S. pyogenes* (Reid et al., 2004). The observed decreased virulence of Srv mutants could be attributed to a loss of nutrient scavenging genes, dereases in biofilm formation, and/or silencing of other Srv-regulated genes (Reid et al., 2006).

Other virulence regulators also have been shown to affect biofilm formation in *S. pyogenes*. Mutants lacking the genes *mga* and *covR*, which encode regulators of virulence factors such as SpeB, fail to form biofilms (Cho and Caparon, 2005), suggesting that biofilm formation, although inversely regulated, is controlled by the same global regulators as major virulence factors. These studies also show that SpeB is expressed in remarkably high amounts in biofilms, even when compared to the already robust expression during stationary phase. These findings, that SpeB is expressed in high levels in biofilms are counter-intuitive when considering the experiments of Doern et al., which show that inhibition of SpeB restores biofilms in Srv mutants (Doern et al., 2009). Taken

together this may suggest that biofilms, or certain members within, produce SpeB in sufficient quantities to induce their own dispersion. Further studies have shown that biofilm deficient strains (Δ*srv*) are not readily cleared in an otitis media model of infection (Connolly et al., 2011). Although quorum sensing models show a density-dependent biofilm-regulating system independent of SpeB (Chang et al., 2011), it is likely that the regulatory network is much more complex than current descriptions, and the presence of SpeB may be important in determining the dispersion of *S. pyogenes* biofilms.

Despite the ubiquitous ability of *S. pyogenes* to form biofilms, little is known about their role during infection of the host. However, transcriptome patterns from a mouse wound model used to mimic necrotizing fasciitis show many biofilm-regulated genes are not expressed in the wounds, suggesting that biofilms are not important in tissue destruction (Kreth et al., 2011). Biofilms have been implicated in antibiotic tolerance of *S. pyogenes*, allowing the organism to escape treatment with penicillin (Conley et al., 2003, Ogawa et al., 2011a, Baldassarri et al., 2006). These studies also show that host immune defense peptides, such as LL-37 and β-defensin were able to inhibit biofilms, reflecting an evolved key host-pathogen relationship relating to biofilms (Ogawa et al., 2011a).

Biofilms in Pathogen Survival.

The protection conferred by biofilms lead towards a distinct advantage in survival. The ubiquitous nature and conserved maturation and structure suggest that the adaptation of biofilms occurred early on, providing a distinct survival advantage (Hall-

Stoodley et al., 2004). Biofilms are also implicated in pathogenesis; the NIH estimates that 80% of chronic infections are biofilm related. Biofilms fundamentally exist separate from planktonic cells, and have distinct gene expression in comparison to planktonic cells (Stoodley et al., 2002). Biofilms offer physical protection against desiccation and oxidative stress. Bacteria in biofilms often differentiate into one or more altered phenotype states, which may be reminiscent of a slow-growth state and allow the bacteria to survive in nutrient limited conditions. In the human host, biofilms offer protection against host-mediated phagocytosis by both macrophages and neutrophils (Cerca et al., 2006, Jesaitis et al., 2003, Leid et al., 2005). These studies show that biofilm matrix interferes with the ability of phagocytes to engulf *P. aeruginosa* and *S. epidermidis*. The feature to evade predation is common even in non-pathogenic bacteria, which can evade amoeboid phagocytosis in the environment (Matz et al., 2008b, Matz et al., 2008a). In these studies, mixed culture biofilms secreted a potent antiprotozoan compound, which induced apoptosis in feeding protozoa.

In the pathogen *P. aeruginosa*, planktonic growth of the pathogen is indicative of acute infection while biofilms are associated with chronic infection. *P. aeruginosa* is a major opportunistic pathogen in immunocompromised patients and a source of nosocomial infections, specifically in patients with cystic fibrosis. Cystic fibrosis patients are unable to effectively clear mucous from the lungs, allowing the accumulation and growth of bacteria. Upon initial colonization, *P. aeruginosa* is non-mucoidal (Govan and Deretic, 1996) and does not form robust biofilms. However, there is a sudden switch to a mucoidal phenotype via the accumulation of mutations (Govan and Deretic, 1996, Koch and Hoiby, 1993) suggesting for selection for the mucoidal phenotype in the lungs. These

mucoidal mutants are more resistant to dehydration, antibiotics, and nutrient limitation (May et al., 1991). Most interestingly, these mutants are resistant to activated peripheral blood polynuclear leukocytes and oxidative stress (Mathee et al., 1999). Researchers have hypothesized that the accumulation of random mutations and subsequent selection allows for the appearance of a mucoid phenotype, which may be more suited to exist as biofilms, providing resistance against host immune defenses and antibiotics (Donlan and Costerton, 2002).

Biofilms as a niche for diversification

The highly organized and diverse structure of biofilms creates a variety of altered microenvironments. For example, cells located within internal structures of the biofilm experience distinct conditions from a cell located on external structures. The presence of chemical gradients across a biofilm produces a litany of environmental niches, inducing the development of a community of cells with adapted phenotypes suitable for their specific niche. This diversity in sub-populations allows the bacteria to adapt rapidly to a sudden change in environmental conditions. Due to their closeness, sedentary, and protected environments, biofilms offer bacteria an ideal environment to engage in gene transfer.

Transformation is the uptake of free DNA into bacteria and the subsequent incorporation of that DNA into the genome. Transformation in biofilms may occur at an accelerated frequency. In biofilms, *S. mutans* transformation can occur at a rate 10 to 600 fold higher than in planktonic cultures (Cvitkovitch and Hamilton, 1994). The peak

transformation frequencies occurred 8-16 hours into the growth of the biofilm, and a sub-population of biofilm cells are identified to be perpetually competent.

Conjugation is essentially the mating of bacteria, where some of the DNA of one bacterium is transferred to the recipient bacterium. The opportunist *Enterococcus faecalis* uses a pheromone-based quorum sensing system for conjugation of the pCF10 plasmid. Conjugation between the OG1SSp donor strain of the plasmid and the OG1RF recipient strain occurred at an accelerated rate in biofilms (Kristich et al., 2004). Moreover, the OG1SSp donor strain was capable of forming a biofilm nearly twice as robust as the OG1RF recipient strain, suggesting that the plasmid may play a role in enhanced biofilm formation and bacterial conjugation. This is specifically important because of the ability of *E. faecalis* to harbor antibiotic resistance genes.

CHAPTER 2

PROJECT AIMS

Previous studies in the laboratory show that *Streptococcus pyogenes* diversifies in respect to metabolism, virulence factor expression, proteome, and global gene regulator expression during late stationary phase and in eukaryotic co-cultures. Considering the ability of biofilms to form diverse microenvironments, and their given roles in survival and generating diversity in other bacteria, we initially hypothesized that biofilms offer a niche for survival and diversification of *S. pyogenes*. To test this hypothesis, the first aim of this proposal was to determine the survival of the biofilm-former JRS4 in static biofilms. The second aim was to determine whether diversification was occurring during survival use formate production during exponential growth and colony morphology as markers of bacterial diversification. The goal of these studies was to investigate the long-term stationary phase behavior of *S. pyogenes* biofilms, which may provide valuable insight in the role of biofilms in *S. pyogenes* pathogenesis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains

S. pyogenes M6 JRS4 (Scott et al., 1986) was the parental strain of all strains used in this study. Freezer stocks of strains were maintained in 30% glycerol/70% TH stocks at -80^{o}C. *S. pyogenes* were cultured in Todd Hewitt (TH) broth (Oxoid, Hampshire, UK). All growth was carried out at 37^{o}C with 5% CO_2.

Strain	Strain Characteristics	Reference
JRS4	Serotype M6	(Scott et al., 1986)
F1.1	JRS4 biofilm derived[a]	This study
F1.2	JRS4 biofilm derived[a]	This study
F1.3	JRS4 biofilm derived[a]	This study
F1.4	JRS4 biofilm derived[a]	This study
F1.5	JRS4 biofilm derived[a]	This study
F3.1	JRS4 biofilm derived[b]	This study
F3.2	JRS4 biofilm derived[b]	This study
F3.4	JRS4 biofilm derived[b]	This study
F3.5	JRS4 biofilm derived[b]	This study
F6.1	JRS4 biofilm derived[c]	This study
F6.2	JRS4 biofilm derived[c]	This study
F6.3	JRS4 biofilm derived[c]	This study
F6.4	JRS4 biofilm derived[c]	This study
F6.5	JRS4 biofilm derived[c]	This study
F10.1	JRS4 biofilm derived[d]	This study
F10.2	JRS4 biofilm derived[d]	This study
F10.3	JRS4 biofilm derived[d]	This study
F10.4	JRS4 biofilm derived[d]	This study
F10.5	JRS4 biofilm derived[d]	This study

[a]Isolated from a 1-day old biofilm culture
[b]Isolated from a 3-day old biofilm culture
[c]Isolated from a 6-day old biofilm culture
[d]Isolated from a 10-day old biofilm culture

Table 1. *S. pyogenes* strains

3.2 Biofilm Formation

Static biofilms were grown on fibrinogen-coated coverslips. A stock solution of 0.5 mg/ml human fibrinogen (Calbiotech, Spring Valley, CA) in sterile PBS was filter-sterilized and stored at -20^{o}C. Borosilicate glass coverslips (8 mm diameter) (Fisher Scientific, Pittsburgh, PA) were soaked in ethanol, flame sterilized and transferred by forceps into the petri dish containing 20 ml solution of 50 μg/ml fibrinogen and incubated overnight at 4^{o}C refrigerator. Coverslips were rinsed three times in PBS and aseptically transferred to a sterile 24-well plate, each well containing only 1 coverslip. TH (3 ml) was added immediately following the transfer of the coverslip, not allowing the coverslip time to dry. Each well was inoculated with 40 μl of an overnight culture (<15 h) of JRS4 in TH, which had been inoculated from an isolated colony of *S. pyogenes* JRS4 grown on TH agar from the freezer stock. The 24-well plate was incubated at 37^{o}C 5% CO_2 incubator.

3.3 Biofilm Survival

Biofilms were grown on fibrinogen coated glass coverslips at 37^{o}C with 5% CO_2 for up to 21 days without the addition of fresh media. Media supernatants containing planktonic cells were collected prior to biofilm washing and serial plated on TH agar to determine CFU concentrations. Each biofilm was then gently washed with 1 ml PBS 3 times. The coverslip containing the biofilm was transferred to a 15 ml Falcon tube containing 3 ml of sterile PBS and placed on ice. Each tube was vortexed and then sonicated on ice for 20 s at 60% amplitude with a Sonic Dismembranator Model 500 (Fisher Scientific, Pittsburgh, PA). The sonicated biofilm suspension was serially

diluted in PBS and plated on TH agar. Plates were grown 1-3 days in 37^{o}C 5% CO_2 incubators. Colonies were counted and are reported as CFU/coverslip.

3.4 Biofilm Imaging

The Livedead® stain containing Syto 9 and Propidium Iodide (PI) was used per manufacturer's instructions (Invitrogen, Carlsbad, CA). Each dye was diluted 1:1000 in PBS. Biofilms were washed 3 times with 1 ml of PBS. Following the removal of the final PBS wash, 0.5 ml of prepared stain was added to the biofilm and allowed to sit at room temperature for 5 min. The biofilm was carefully inverted, then transferred to an 8-well slide containing a droplet (10 µl) of PBS. The coverslip was secured to the slide with gentle pressure from sterilized forceps, and then sealed with molten paraffin.

Livedead® analysis was done by laser scanning confocal microscopy using a Leica SP5 Confocal Microscopy (Leica Microsystems, Heidelberg, Germany) and a 63x objective. Samples were excited with an Argon laser at an excitation wavelength of 488 nm. An emission spectrum of 496-534 nm and 652-704 nm were collected for Syto 9 and PI, respectively.

3.5 Determination of Formate Production during Exponential Growth

Culture supernatants used for metabolite analysis were collected from exponential phase cultures. An overnight (<15 h) culture of *S. pyogenes* was diluted 1:100 in fresh medium. Growth of inoculated cultures was monitored by spectrometry at OD_{600} until the culture reached mid-exponential phase (OD_{600} = 0.8 ± 0.1). Culture samples were filter sterilized through a 0.22 µm syringe filter (Millipore; Billerica, MA) and subsequently heat inactivated at 90°C for five minutes before being stored at -20°C. The

samples were diluted 1:5 in dH_2O, formate concentrations were determined from culture supernatants using R-Biopharm test kits (Marshall, MI), as described by the manufacturer.

3.6 Depleted Media Dispersion

Depleted medium was generated by inoculating *S. pyogenes* JRS4 into 40 ml TH and incubating at 37^{o}C with 5% CO_2 for 7 days. Cells were removed by centrifugation at 5100 RPM/3400 xG (Fisher Scientific Model 225, Pittsburgh, PA). The supernatant was removed and filter sterilized through a 0.22 µm syringe filter (Millipore; Billerica, MA) and stored at 4^{o}C. Tubes containing either depleted medium or sterile PBS were pre-warmed in 37^{o}C with 5% CO_2 at least 6 hours before being used in dispersion experiments.

Biofilms were grown for 2 days on fibrinogen coated coverslips in a 24-well plate at 37^{o}C with 5% CO_2. Biofilms were washed 3 times with 1 ml of PBS at 37^{o}C. After the final wash, either pre-warmed depleted media or PBS (1 ml) was used to replace the biofilm supernatant. Whole supernatants were collected at indicated time points, serially diluted and plated onto TH agar and grown for 1-3 days at 37^{o}C with 5% CO_2. CFU was reported as CFU/ml.

CHAPTER 4

RESULTS

4.1 *S. pyogenes* biofilms do not survive longer than 10 days

To investigate the role of biofilms in survival, *S. pyogenes* JRS4 static biofilms were grown on fibrinogen-coated glass coverslips at 37^{o}C with 5% CO_2. Viable cells could not be harvested from biofilms past 10 days (lower limit of detection: 10^0) despite the continued survival of the planktonic cells directly above the biofilms for 21 days at which time the experiment was terminated (Fig. 2 and data not shown). The addition of mucin, a glycoprotein secreted by epithelial tissues, which increases survival of *Streptococcus mutans* biofilms did not change the growth patterns of JRS4 nor was it able to prolong survival past 10 days (data not shown).

It was noted that colonies isolated from these biofilms did not display altered colony morphology on TH agar plates and were indistinguishable from parental colonies.

4.2 Biofilms lose viability and thin over the course of 10 days

The loss of biofilm viability could be attributed to the dispersion of biofilm cells into the supernatant, the death of the biofilm cells or both. To investigate biofilm behavior on a microscopic level, confocal microscopy with LiveDead® staining was employed. Biofilms were grown to 1, 3, 6, and 10 days before being rinsed of any planktonic cells and stained with LiveDead® stain.

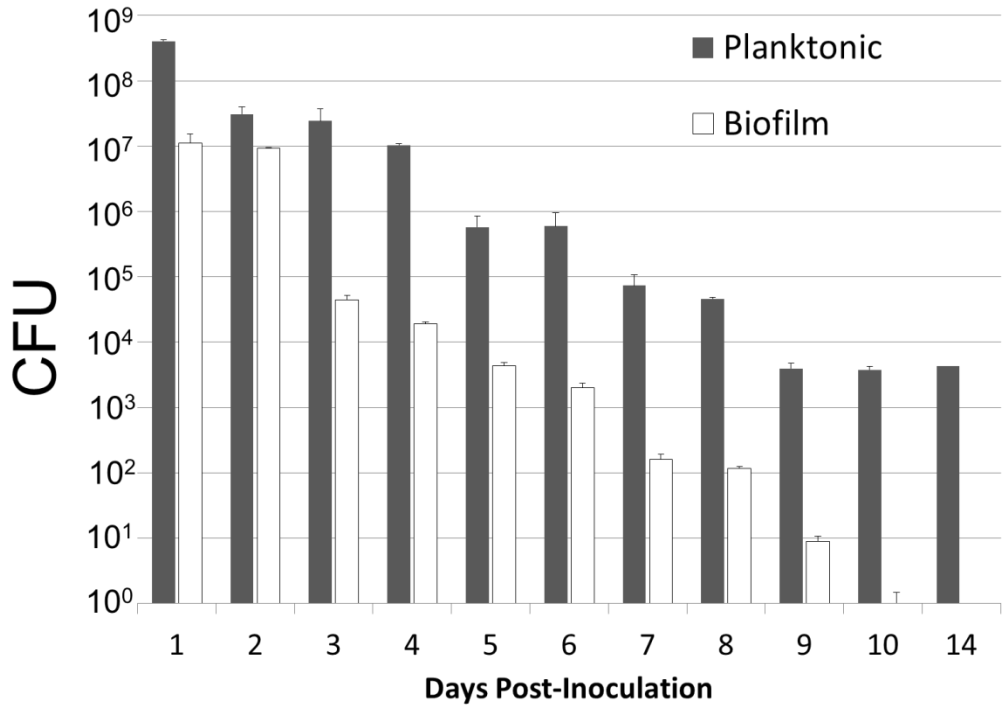

Figure 2. Survival of biofilms and their planktonic counterparts

S. pyogenes M6 JRS4 biofilms were grown on fibrinogen-coated coverslips in TH medium. Planktonic cells, present above the biofilms, were washed off with PBS and enumerated via dilution plating (Grey, CFU/mL). Glass coverslips containing biofilms were washed 3 times with 1 ml PBS and sonicated (Sonicator, FisherSci) in 3 ml PBS at 60% amplitude for 20s to disrupt the biofilm matrix. Sonicated biofilms were briefly vortexed and enumerated via dilution plating (White, CFU/coverslip).

LiveDead® staining uses two DNA-binding dyes, Syto 9 and propidium iodide (PI) which stain live and dead cells, respectively. Prior to binding DNA neither dye fluoresces. PI binds with a stronger affinity than Syto 9. Syto 9 stains all cells regardless of cell membrane condition, both live and dead. However, live cells actively pump PI out of the cells. After cell death, PI remains in the cell, displacing Syto9 and shifting the emission spectrum from green for Syto9 to red for PI. The result is that live-staining cells stain "green" and dead-staining cells stain "red". When these signals are overlapped, dead, damaged, or dying cells can turn yellow; however cells with intact cell membranes remain green staining. The number of dead cells remaining in the biofilm increases, correlating with loses in culturability (Fig. 3). At later time points a number of yellow-staining cells become evident. The conversion of *S. pyogenes* cells from green to red is slow and cells can continue to stain green after they lose culturability. These cells are thought to be dying cells, since the culture will go completely red about 2 weeks after loss of culturability (Wood et al., 2005).

Z-stack analysis was used to determine biofilm thickness (Fig. 3). The apparent decrease in the thickness and the decrease in viability were measured by LiveDead® staining and standard CFU enumeration (Fig. 4).

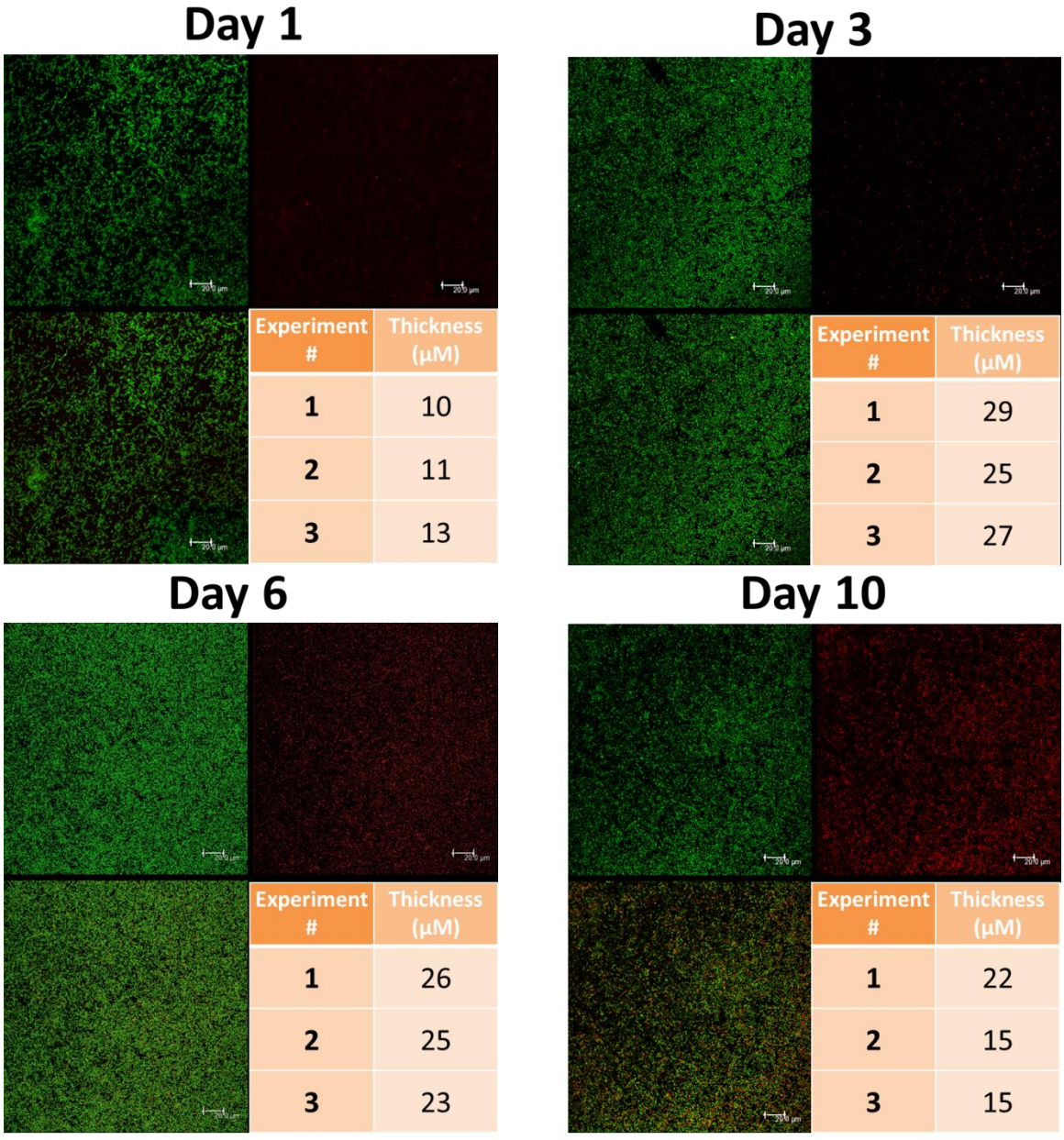

Figure 3. Confocal Microscopy with LiveDead® Staining of Total Biofilm Thickness

S. pyogenes JRS4 biofilms were grown on fibrinogen-coated glass coverslips at 37°C with 5% CO_2 for 1 (A), 3 (B), 6 (C), and 10 (D) days. Biofilms were washed with PBS to remove planktonic cells. Coverslips were then stained with LiveDead® and imaged by laser scanning confocal microscopy (Leica Sp5). The green (upper left quadrant) indicate live-staining cells whereas the red (upper right quadrant) indicates dead-staining cells. The overlay is shown in the lower left quadrant. Scale bar is 20 □m. Z-stacks were used to determine biofilm thickness (lower right quadrant). The images shown are representative of 3 independent experiments.

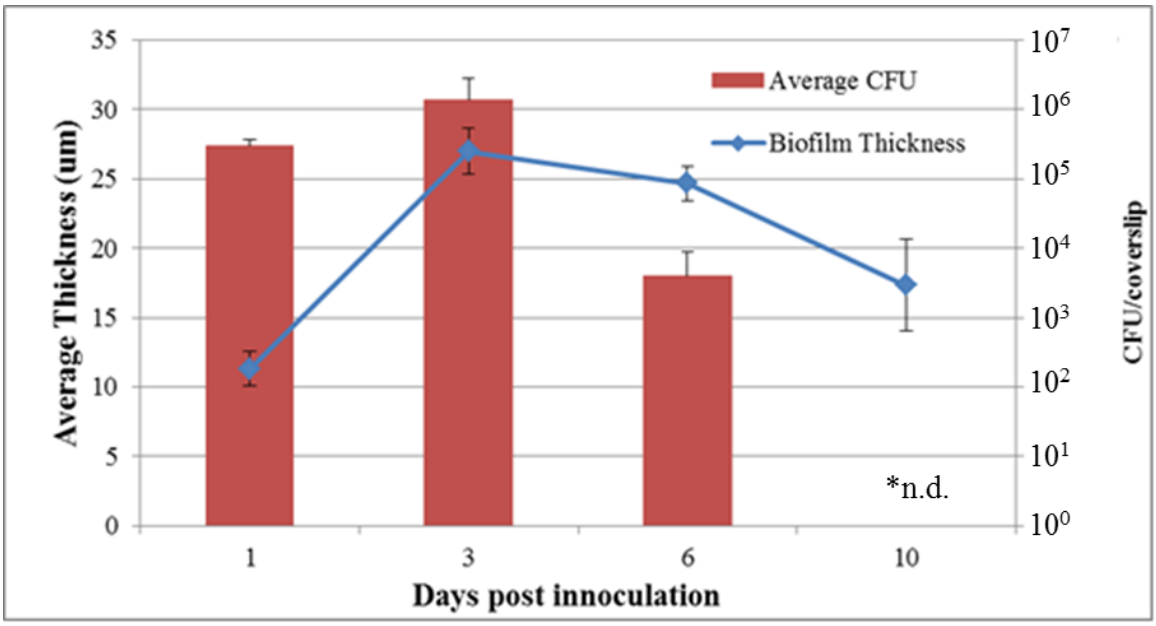

Figure 4. Decreases in biofilm growth correlate with decrease in biofilm thickness

4.3 Induction of dispersion upon media exchange

Growth curve analysis and confocal microscopy indicate a decrease in viability and biofilm density over time. One explanation for the apparent loss of biofilm thickness is the dispersion of viable cells from the biofilm matrix. The apparent decrease in signal density and thickness could be contributed to the shedding of live cells in an active dispersion process, or the simple sloughing of dead cells from the biofilm surface. To test the hypothesis that the accumulation of secreted factors induces dispersion, biofilm supernatants were replaced with spent TH from 7-day-old planktonic cultures or PBS and dispersion of live cells was monitored by collecting supernatants above the biofilm at various time points and plating on TH agar (Fig. 5). The spent medium did not support growth of planktonic or biofilm cells (data not shown). After the initial 3 quick washes with PBS there were no longer any planktonic cells associated with the biofilm (<10 CFU/ml). Upon addition of PBS or spent medium, there was an initial dispersion of cell after 5 minutes (Fig. 5). Viable cells continued to accumulate in the medium above biofilms in spent medium but not PBS (Fig. 5). Taken together these data suggest there may be a population of loosely adherent cells which disperse upon addition or PBS or spent medium and then a population of cells that are dispersed over time with spent medium.

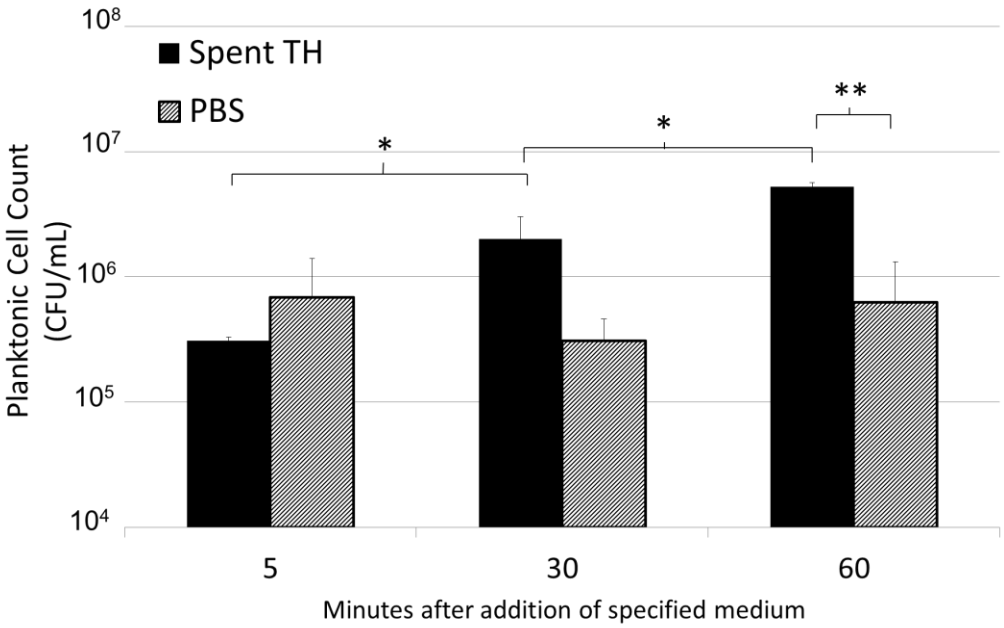

Figure 5. Dispersion after supernatant exchange with spent TH

The medium above 2 day old biofilms was removed and the biofilms were washed 3 times with PBS. The medium was then replaced with either PBS or filter sterilized spent medium from a 7-day planktonic culture. At 5, 30 and 60 minutes after addition of PBS or depleted TH a sample of supernatant was removed and cells were quantitated by dilution plating.

4.4 Metabolic diversification does not occur in biofilms

To determine if biofilms serve as a niche for metabolic diversification, isolates from biofilms were screened for an increase in formate production during exponential phase, indicating use of heterofermentation during exponential growth. In a diversifying culture, at least 75% of randomly selected isolates had increases in formate production (Wood et al., 2009). No biofilm isolates (0/19) had an increase in formate production during exponential phase when compared to the parental JRS4 strain (Fig. 6). The lack of phenotypic diversification as evident in metabolism and colony morphology suggest that biofilms are not a niche for diversification.

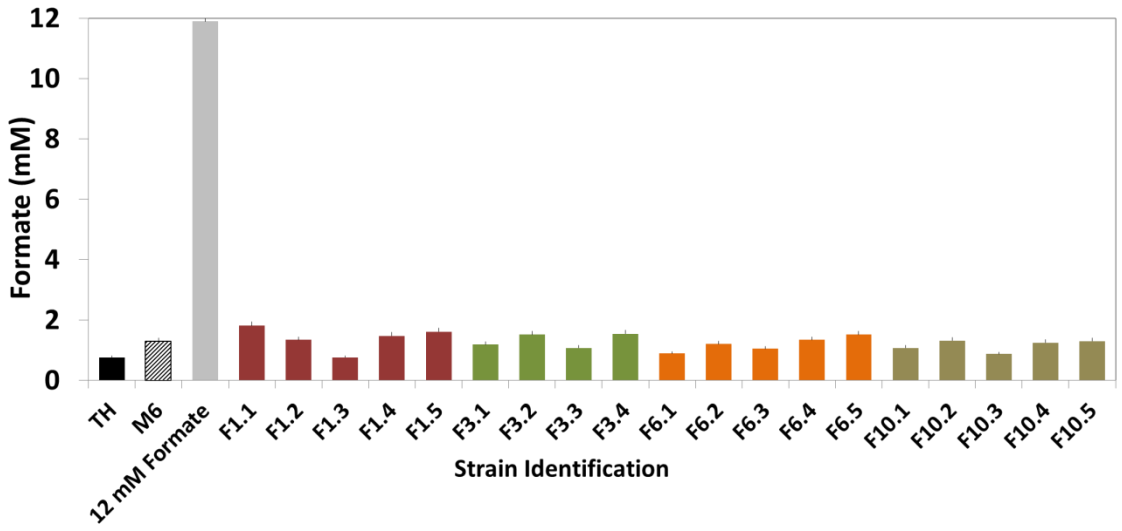

Figure 6. Formate production in exponentially growing *S. pyogenes* JRS4 (M6) and biofilm isolates from 1, 3, 6, and 10 days

Supernatants from late exponential phase ($OD_{600\ nm}$ 0.8 in TH) were filter sterilized, and formate concentrations were determined. The bar at the left represents formate concentrations present in sterile TH broth and TH containing 12 mM formate.

CHAPTER 5

DISCUSSION

5.1 Discussion Overview and Conclusions

Our laboratory investigates the survival of *S. pyogenes*. Previous models involving batch cultures show that *S. pyogenes* can survive for up to 18 months in static media conditions. During survival, the bacteria accumulate mutations leading to heritable differences in virulence factors expression, metabolism, proteomes and colony morphology in surviving isolates. The diversification exhibited in long-term stationary phase cultures can also be found in survivors from the co-culture with eukaryotic cells; intracellular survivors. The mutations appear to be random and changes between isolates vary. The purpose of the current study was to examine the survival of *S. pyogenes* in biofilms, and determine if biofilms could serve as a niche for diversification. The major conclusions of these studies were (1) biofilm cells disperse and remaining adherent cells lose viability within 11 days, (2) factors in stationary phase medium appear to mediate biofilm dispersion and (3) no diversification can be detected in biofilm cells.

5.2 *S. pyogenes* biofilms rapidly disperse and remaining cells lose culturability

S. pyogenes M6 JRS4, which is a robust biofilm former in vitro were unable to survive in biofilms on fibrinogen-coated borosilicate coverslips for more than 10 days in Todd-Hewitt broth. The loss of biofilm viability was not due to an inherently shorter survival time of JRS4 because planktonic cell concentrations from the broth directly

above the biofilms remained constant at a CFU of about 10^5 per ml, which is the same as the surviving population documented with planktonic cultures of other strains (Wood et al., 2005). Survival of planktonic cells above the biofilm was documented for up to 3 weeks before the termination of the experiment.

The loss of biofilm viability appeared to result from the death of the adherent cells as well dispersion of live cells. By day 10 was it apparent that the biofilms had lost a majority of their viability, and the remaining adherent cells were either red showing complete death or yellow-staining cells, which might be dying cells. Wood et al. reported that it took multiple days after loss of culturability for all the cells to stain red with propridium iodide contained in the LiveDead® stain (Wood et al., 2005). Dispersion is supported by loss of biofilm thickness as assessed by Z-stack analysis. Experimental evidence suggests the thinning of the biofilm could be due to the dispersion of live cells rather than collapse of the dead biofilm or the release of dead cells, because the release of live cells, which formed colonies on TH agar, could be triggered by the addition of spent medium. The biofilm appeared to be composed of two different cells types; loosely adherent cells and tightly adherent cells. Despite final washes of the biofilm containing less than 10^2 cells/mL, an initial spike of cells (10^5-10^6) was observed when changing media, independent of the medium. These are likely to be loosely adherent cells but not planktonic cells, which were removed by the initial quick PBS washes. The cells released by longer incubation in spent medium are interpreted to be tightly adherent cells being released from the biofilms.

It is currently not clear if the loosely adherent cells are part of the mature biofilm or if adherence differences between plastic and fibrinogen-coated coverslips may account

for the two populations. Because biofilms are grown on glass coverslips in polystyrene wells, the polystyrene surface also permits a biofilm to form, which may ultimately contributes to the planktonic CFU count. The coverslip, which is used to quantify biofilm CFUs, is potentially only a portion of the total biofilm. This issue could be addressed by transplanting the coverslip into a biofilm-free well, then adding PBS or spent medium and monitoring viable cell release.

The ability of depleted media to induce dispersion suggests that factors secreted from planktonic cells may act as a signal, or act directly, to induce or cause dispersion in biofilms. Virulence factor expression in *S. pyogenes* is upregulated in response to nutrient starvation and entry into stationary phase (Chaussee et al., 1997). Of the factors secreted during stationary phase, SpeB is produced in very high levels. SpeB is a cysteine protease, which acts on host and bacterial proteins (Ohara-Nemoto et al., 1994, Kapur et al., 1993). eDNA and secreted bacterial proteins are integral to the biofilm matrix of *S. pyogenes* (Doern et al., 2009). In 2009, Reid et al proposed that the timed expression of SpeB might contribute to biofilm dispersion. Their experiments show than a deletion mutant of *covR*, which encodes a negative regulator of *speB*, leads to a decreased ability to form biofilms. Biofilm formation is restored upon addition of the SpeB inhibitor E64. The authors suggest that an unknown trigger, such as nutrient deprivation, initiates the release of bacteria expressing virulence factors from the biofilm, allowing for dissemination of the bacteria in the host. The stationary phase supernatants would be likely to contain SpeB, and the enzymatic action of the pre-formed SpeB could contribute to dispersion of cells at early time points (e.g. 30 minutes). Experiments comparing biofilm dispersion after 10 days and the ability of stationary phase medium to induce

dispersion of JRS4 and an isogenic *speB* mutant could be used to examine the contribution of SpeB to biofilm dispersion.

Unlike in previously studied survival niches, biofilm cells do not diversify

Because biofilms may serve as a niche for diversification despite their inability for survival, it was necessary to investigate metabolic diversification of biofilm isolates.

In strain CS101, in late stationary phase, at least 75% of randomly selected isolates show changes in colony morphology or formate production. Since none of the biofilm isolates from 1, 3, 6, and 10 day old cultures displayed a measurable increase in formate production during exponential phase nor grew with altered colony morphology, it is unlikely significant levels of diversification were occurring.

Considering the lack of phenotypic diversification in metabolism and colony morphology, as well as the apparent inability to survive in biofilms, we suggest that biofilms are not a niche for survival and diversification. In vitro conditions, which are permissive to the survival of *S. pyogenes* for over 1 year, are niches for diversification during this time. In addition, diversification occurs rapidly inside eukaryotic cells. Therefore, invasion into eukaryotic cells may be a niche for diversification in the host, but biofilms from JRS4 display neither of these characteristics, and have a notably short life span.

Biofilms may be a Transient State used to Establish Infection

In 2009, it was hypothesized that biofilms of *S. pyogenes* are involved in establishing infection (Doern et al., 2009). These authors suggested that biofilms are not a

long-term persistence mechanism, but rather a method to ensure short-term survival inside the host. Biofilms may allow the pathogen to establish an infection in the host, providing resistance to phagocytosis and other host immune factors. Gene regulation patterns provide evidence for this hypothesis; genes for biofilm formation and other virulence factors are inversely regulated (Cho and Caparon, 2005). Biofilms form under nutrient-rich conditions, when the pathogen would have an opportunity to reach sufficient cell concentrations for a successful infection. Upon the nutrient depletion in thick biofilms, the pathogen produces virulence factors such as the cysteine protease SpeB to liberate cells and allow for dispersion and spread of the infection. We hypothesize that the accumulation of factors secreted by nutrient-deprived cells are able to induce biofilm dispersion.

The results of this study, which shows that biofilms are unable to survive for extended periods, support the hypothesis that biofilms are not a niche for long-term survival. The observation that cells from biofilms do not diversify also supports this conclusion. Finally, the ability for depleted media from planktonic cultures to initiate dispersion support the hypothesis that secreted factors accumulate during stationary phase, leading to the coordinated dispersion and dissemination of the biofilm.

REFERENCES

AHMAD, N., PLORDE, J.J., AND DREW, W.L. 2010. *Sherris Medical Microbiology*, McGraw-Hill Companies, Inc.

BALDASSARRI, L., CRETI, R., RECCHIA, S., IMPERI, M., FACINELLI, B., GIOVANETTI, E., PATARACCHIA, M., ALFARONE, G. & OREFICI, G. 2006. Therapeutic failures of antibiotics used to treat macrolide-susceptible Streptococcus pyogenes infections may be due to biofilm formation. *J Clin Microbiol,* 44**,** 2721-7.

BISNO, A. L., BRITO, M. O. & COLLINS, C. M. 2003. Molecular basis of group A streptococcal virulence. *Lancet Infect Dis,* 3**,** 191-200.

BISNO, A. L. & STEVENS, D. L. 1996. Streptococcal infections of skin and soft tissues. *N Engl J Med,* 334**,** 240-5.

BRANDT, C. M., ALLERBERGER, F., SPELLERBERG, B., HOLLAND, R., LUTTICKEN, R. & HAASE, G. 2001. Characterization of consecutive Streptococcus pyogenes isolates from patients with pharyngitis and bacteriological treatment failure: special reference to prtF1 and sic / drs. *J Infect Dis,* 183**,** 670-4.

CARAPETIS, J. R., STEER, A. C., MULHOLLAND, E. K. & WEBER, M. 2005. The global burden of group A streptococcal diseases. *Lancet Infect Dis,* 5**,** 685-94.

CERCA, N., JEFFERSON, K. K., OLIVEIRA, R., PIER, G. B. & AZEREDO, J. 2006. Comparative antibody-mediated phagocytosis of Staphylococcus epidermidis cells grown in a biofilm or in the planktonic state. *Infect Immun,* 74**,** 4849-55.

CHANG, J. C., LASARRE, B., JIMENEZ, J. C., AGGARWAL, C. & FEDERLE, M. J. 2011. Two group A streptococcal peptide pheromones act through opposing Rgg regulators to control biofilm development. *PLoS Pathog,* 7**,** e1002190.

CHAUSSEE, M. S., PHILLIPS, E. R. & FERRETTI, J. J. 1997. Temporal production of streptococcal erythrogenic toxin B (streptococcal cysteine proteinase) in response to nutrient depletion. *Infect Immun,* 65**,** 1956-9.

CHAUSSEE, M. S., WATSON, R. O., SMOOT, J. C. & MUSSER, J. M. 2001. Identification of Rgg-regulated exoproteins of Streptococcus pyogenes. *Infect Immun,* 69**,** 822-31.

CHO, K. H. & CAPARON, M. G. 2005. Patterns of virulence gene expression differ between biofilm and tissue communities of Streptococcus pyogenes. *Mol Microbiol,* 57**,** 1545-56.

CONLEY, J., OLSON, M. E., COOK, L. S., CERI, H., PHAN, V. & DAVIES, H. D. 2003. Biofilm formation by group a streptococci: is there a relationship with treatment failure? *J Clin Microbiol,* 41**,** 4043-8.

CONNOLLY, K. L., ROBERTS, A. L., HOLDER, R. C. & REID, S. D. 2011. Dispersal of Group A streptococcal biofilms by the cysteine protease SpeB leads to increased disease severity in a murine model. *PLoS One,* 6**,** e18984.

COURTNEY, H. S., OFEK, I., PENFOUND, T., NIZET, V., PENCE, M. A., KREIKEMEYER, B., PODBIELSKI, A., HASTY, D. L. & DALE, J. B. 2009. Relationship between expression of the family of M proteins and lipoteichoic acid to hydrophobicity and biofilm formation in Streptococcus pyogenes. *PLoS One,* 4**,** e4166.

CUNNINGHAM, M. A., RONDEAU, E., CHEN, X., COUGHLIN, S. R., HOLDSWORTH, S. R. & TIPPING, P. G. 2000. Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. *J Exp Med,* 191**,** 455-62.

CVITKOVITCH, D. G. & HAMILTON, I. R. 1994. Biochemical change exhibited by oral streptococci resulting from laboratory subculturing. *Oral Microbiol Immunol,* 9**,** 209-17.

DOERN, C. D., ROBERTS, A. L., HONG, W., NELSON, J., LUKOMSKI, S., SWORDS, W. E. & REID, S. D. 2009. Biofilm formation by group A Streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). *Microbiology,* 155**,** 46-52.

DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev,* 15**,** 167-93.

ERIKSSON, B. K., ANDERSSON, J., HOLM, S. E. & NORGREN, M. 1998. Epidemiological and clinical aspects of invasive group A streptococcal infections and the streptococcal toxic shock syndrome. *Clin Infect Dis,* 27**,** 1428-36.

GOVAN, J. R. & DERETIC, V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiol Rev,* 60**,** 539-74.

HALL-STOODLEY, L., COSTERTON, J. W. & STOODLEY, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol,* 2**,** 95-108.

HILARIO, M. O. & TERRERI, M. T. 2002. Rheumatic fever and post-streptococcal arthritis. *Best Pract Res Clin Rheumatol,* 16**,** 481-94.

HUANG, Y. C., HSUEH, P. R., LIN, T. Y., YAN, D. C. & HSIA, S. H. 2001. A family cluster of streptococcal toxic shock syndrome in children: clinical implication and epidemiological investigation. *Pediatrics,* 107**,** 1181-3.

JESAITIS, A. J., FRANKLIN, M. J., BERGLUND, D., SASAKI, M., LORD, C. I., BLEAZARD, J. B., DUFFY, J. E., BEYENAL, H. & LEWANDOWSKI, Z. 2003. Compromised host defense on Pseudomonas aeruginosa biofilms: characterization of neutrophil and biofilm interactions. *J Immunol,* 171**,** 4329-39.

KAPUR, V., TOPOUZIS, S., MAJESKY, M. W., LI, L. L., HAMRICK, M. R., HAMILL, R. J., PATTI, J. M. & MUSSER, J. M. 1993. A conserved Streptococcus pyogenes extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb Pathog,* 15**,** 327-46.

KOCH, C. & HOIBY, N. 1993. Pathogenesis of cystic fibrosis. *Lancet,* 341**,** 1065-9.

KRETH, J., CHEN, Z., FERRETTI, J. & MALKE, H. 2011. Counteractive balancing of transcriptome expression involving CodY and CovRS in Streptococcus pyogenes. *J Bacteriol,* 193**,** 4153-65.

KRISTICH, C. J., LI, Y. H., CVITKOVITCH, D. G. & DUNNY, G. M. 2004. Esp-independent biofilm formation by Enterococcus faecalis. *J Bacteriol,* 186**,** 154-63.

LAPENTA, D., RUBENS, C., CHI, E. & CLEARY, P. P. 1994. Group A streptococci efficiently invade human respiratory epithelial cells. *Proc Natl Acad Sci U S A,* 91**,** 12115-9.

LAPPIN, E. & FERGUSON, A. J. 2009. Gram-positive toxic shock syndromes. *Lancet Infect Dis,* 9**,** 281-90.

LEID, J. G., WILLSON, C. J., SHIRTLIFF, M. E., HASSETT, D. J., PARSEK, M. R. & JEFFERS, A. K. 2005. The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing. *J Immunol,* 175**,** 7512-8.

LEMBKE, C., PODBIELSKI, A., HIDALGO-GRASS, C., JONAS, L., HANSKI, E. & KREIKEMEYER, B. 2006. Characterization of biofilm formation by clinically relevant serotypes of group A streptococci. *Appl Environ Microbiol,* 72**,** 2864-75.

LEONARD, B. A., WOISCHNIK, M. & PODBIELSKI, A. 1998. Production of stabilized virulence factor-negative variants by group A streptococci during stationary phase. *Infect Immun,* 66**,** 3841-7.

LEWIS, K. 2008. Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol,* 322**,** 107-31.

MARTIN, M. C., GOMEZ-JIMENEZ, J., ESTEBAN, F., SAURI, R., MOURELLE, M. I. & SALGADO, A. 1995. [Cytokines and nitric oxide in streptococcal toxic shock syndrome]. *Med Clin (Barc),* 104**,** 458-60.

MATHEE, K., CIOFU, O., STERNBERG, C., LINDUM, P. W., CAMPBELL, J. I., JENSEN, P., JOHNSEN, A. H., GIVSKOV, M., OHMAN, D. E., MOLIN, S., HOIBY, N. & KHARAZMI, A. 1999. Mucoid conversion of Pseudomonas aeruginosa by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology,* 145 (Pt 6)**,** 1349-57.

MATZ, C., MORENO, A. M., ALHEDE, M., MANEFIELD, M., HAUSER, A. R., GIVSKOV, M. & KJELLEBERG, S. 2008a. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. *ISME J,* 2**,** 843-52.

MATZ, C., WEBB, J. S., SCHUPP, P. J., PHANG, S. Y., PENESYAN, A., EGAN, S., STEINBERG, P. & KJELLEBERG, S. 2008b. Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. *PLoS One,* 3**,** e2744.

MAY, T. B., SHINABARGER, D., MAHARAJ, R., KATO, J., CHU, L., DEVAULT, J. D., ROYCHOUDHURY, S., ZIELINSKI, N. A., BERRY, A., ROTHMEL, R. K. & ET AL. 1991. Alginate synthesis by Pseudomonas aeruginosa: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin Microbiol Rev,* 4**,** 191-206.

MOLINARI, G. & CHHATWAL, G. S. 1999. Role played by the fibronectin-binding protein SfbI (Protein F1) of Streptococcus pyogenes in bacterial internalization by epithelial cells. *J Infect Dis,* 179**,** 1049-50.

OGAWA, T., TERAO, Y., OKUNI, H., NINOMIYA, K., SAKATA, H., IKEBE, K., MAEDA, Y. & KAWABATA, S. 2011a. Biofilm formation or internalization into epithelial cells enable Streptococcus pyogenes to evade antibiotic eradication in patients with pharyngitis. *Microb Pathog,* 51**,** 58-68.

OGAWA, T., TERAO, Y., SAKATA, H., OKUNI, H., NINOMIYA, K., IKEBE, K., MAEDA, Y. & KAWABATA, S. 2011b. Epidemiological characterization of Streptococcus pyogenes isolated from patients with multiple onsets of pharyngitis. *FEMS Microbiol Lett,* 318**,** 143-51.

OHARA-NEMOTO, Y., SASAKI, M., KANEKO, M., NEMOTO, T. & OTA, M. 1994. Cysteine protease activity of streptococcal pyrogenic exotoxin B. *Can J Microbiol,* 40**,** 930-6.

OLSEN, R. J. & MUSSER, J. M. 2010. Molecular pathogenesis of necrotizing fasciitis. *Annu Rev Pathol,* 5**,** 1-31.

OSTERLUND, A. & ENGSTRAND, L. 1995. Intracellular penetration and survival of Streptococcus pyogenes in respiratory epithelial cells in vitro. *Acta Otolaryngol,* 115**,** 685-8.

OSTERLUND, A. & ENGSTRAND, L. 1997. An intracellular sanctuary for Streptococcus pyogenes in human tonsillar epithelium--studies of asymptomatic carriers and in vitro cultured biopsies. *Acta Otolaryngol,* 117**,** 883-8.

PASSALI, D., LAURIELLO, M., PASSALI, G. C., PASSALI, F. M. & BELLUSSI, L. 2007. Group A streptococcus and its antibiotic resistance. *Acta Otorhinolaryngol Ital,* 27**,** 27-32.

PETERSSON, K., FORSBERG, G. & WALSE, B. 2004. Interplay between superantigens and immunoreceptors. *Scand J Immunol,* 59**,** 345-55.

PODBIELSKI, A., BECKERT, S., SCHATTKE, R., LEITHAUSER, F., LESTIN, F., GOSSLER, B. & KREIKEMEYER, B. 2003. Epidemiology and virulence gene expression of intracellular group A streptococci in tonsils of recurrently infected adults. *Int J Med Microbiol,* 293**,** 179-90.

PODBIELSKI, A. & KREIKEMEYER, B. 2001. Persistence of group A streptococci in eukaryotic cells--a safe place? *Lancet,* 358**,** 3-4.

REID, S. D., CHAUSSEE, M. S., DOERN, C. D., CHAUSSEE, M. A., MONTGOMERY, A. G., STURDEVANT, D. E. & MUSSER, J. M. 2006. Inactivation of the group A Streptococcus regulator srv results in chromosome wide reduction of transcript levels, and changes in extracellular levels of Sic and SpeB. *FEMS Immunol Med Microbiol,* 48**,** 283-92.

REID, S. D., MONTGOMERY, A. G. & MUSSER, J. M. 2004. Identification of srv, a PrfA-like regulator of group A streptococcus that influences virulence. *Infect Immun,* 72**,** 1799-803.

ROBERTS, A. L., CONNOLLY, K. L., DOERN, C. D., HOLDER, R. C. & REID, S. D. 2010. Loss of the group A Streptococcus regulator Srv decreases biofilm formation in vivo in an otitis media model of infection. *Infect Immun,* 78**,** 4800-8.

RODRIGUEZ-ITURBE, B. & BATSFORD, S. 2007. Pathogenesis of poststreptococcal glomerulonephritis a century after Clemens von Pirquet. *Kidney Int,* 71**,** 1094-104.

SCOTT, J. R., GUENTHNER, P. C., MALONE, L. M. & FISCHETTI, V. A. 1986. Conversion of an M- group A streptococcus to M+ by transfer of a plasmid containing an M6 gene. *J Exp Med,* 164**,** 1641-51.

SHAIKH, N., LEONARD, E. & MARTIN, J. M. 2010. Prevalence of streptococcal pharyngitis and streptococcal carriage in children: a meta-analysis. *Pediatrics,* 126**,** e557-64.

STOODLEY, P., SAUER, K., DAVIES, D. G. & COSTERTON, J. W. 2002. Biofilms as complex differentiated communities. *Annu Rev Microbiol,* 56**,** 187-209.

THENMOZHI, R., BALAJI, K., KUMAR, R., RAO, T. S. & PANDIAN, S. K. 2011. Characterization of biofilms in different clinical M serotypes of Streptococcus pyogenes. *J Basic Microbiol,* 51**,** 196-204.

TRENT, J. T. & KIRSNER, R. S. 2002. Diagnosing necrotizing fasciitis. *Adv Skin Wound Care,* 15**,** 135-8.

WESSELS, M. R. & BRONZE, M. S. 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. *Proc Natl Acad Sci U S A,* 91**,** 12238-42.

WOOD, D. N., CHAUSSEE, M. A., CHAUSSEE, M. S. & BUTTARO, B. A. 2005. Persistence of Streptococcus pyogenes in stationary-phase cultures. *J Bacteriol,* 187**,** 3319-28.

WOOD, D. N., WEINSTEIN, K. E., PODBIELSKI, A., KREIKEMEYER, B., GAUGHAN, J. P., VALENTINE, S. & BUTTARO, B. A. 2009. Generation of metabolically diverse strains of Streptococcus pyogenes during survival in stationary phase. *J Bacteriol,* 191**,** 6242-52.

APPENDIX

MEDIA AND SOLUTIONS

Phosphate Buffered Saline (0.1 M)

0.2 M K_2HPO_4	87.7 ml
0.2 M KH_2PO_4	12.3 ml
Water	to 200 ml

Autoclave and store at room temperature.

Todd Hewitt Broth

Todd Hewitt Powder (Oxoid)	36.4 g
Water	to 1000 ml

Autoclave and store at room temperature.

Agar: Add 1.5% Agar (Difco) before autoclave and store at 4°C