PHENOTYPIC AND GENOTYPIC DETERMINANTS OF COLONIZATION AND PATHOGENESIS IN GROUP B *STREPTOCOCCUS*

By

Robert Edward Parker

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Microbiology and Molecular Genetics—Doctor of Philosophy

2016

ABSTRACT

PHENOTYPIC AND GENOTYPIC DETERMINANTS
OF COLONIZATION AND PATHOGENESIS
IN GROUP B *STREPTOCOCCUS*

By

Robert Edward Parker

Group B *Streptococcus* (GBS) is a leading cause of sepsis and meningitis in neonates, an important factor in premature and still births worldwide, and a threat to immunocompromised and elderly adults. The prevention strategy for neonatal invasive disease targets maternal colonization, the primary risk factor, through late-gestational screening and intrapartum antibiotics. This approach has resulted in a significant decline in disease rates; however, this decrease has stagnated in the 2000s, and maternal re-colonization following treatment is common. For my doctoral work, I examined phenotypic and genotypic factors which facilitate colonization, antibiotic tolerance, and persistence of GBS including biofilm production, quorum sensing, and phenotypic heterogeneity. The assessment of biofilm formation across a diverse set of isolates, including colonizing and invasive clinical human strains, found that weak biofilm production correlated with genotype, pilus profile, and invasive disease. Furthermore, asymptomatic colonization was associated with strong biofilm production suggesting a colonization advantage for strong biofilm producers. The role of a putative quorum-sensing auto-inducing peptide, RgfD, was investigated through the creation of a deletion mutant through homologous recombination. In this work, *rgfD* was found to drive adherence to decidualized human endometrial cells through the upregulation of the regulator of fibrinogen-binding two-component system suggesting quorum-sensing in GBS is important for colonization. Lastly, the importance of phenotypic diversification in a single strain of GBS was assessed through the identification and characterization of a locked mutant small colony variant (SCV) derived from a clinical isolate. This is the first demonstration of SCV formation in GBS. The mutant SCV displayed increased penicillin tolerance and biofilm production, but reduced phagocytic uptake

by THP-1-derived macrophages. Furthermore, the SCV phenotype was inducible when treated with antibiotics or exposed to acidic pH, which, alongside whole transcriptome analysis, suggests variant-formation to be driven by stress response in GBS. The work contained herein furthers the understanding of GBS colonization and identifies key phenotypic and genotypic characteristics driving colonization and persistence that must be considered in the development of future therapeutics.

This thesis is dedicated to my sister, Kathryn, and brother, Phillip, upon whose love, strength, and intelligence I continually rely despite their physical absence.

ACKNOWLEDGMENTS

There are many people I should recognize for their guidance, friendship, and patience throughout this process. My mentor, Dr. Shannon Manning, has been incredible to me. I am certain I would not have been successful without her, so she deserves first mention. I am forever grateful that she gave me a chance to be part of her lab and realized not to listen to me when I tried to quit. It really means so much to have a mentor who cares. My committee members Dr. George Sundin, Dr. Ned Walker, and Dr. Chris Waters, have guided me to this point and deserve credit. We have been through tough times together, and I appreciate all the help and patience they have afforded me. I also would like to give special thanks to Dr. Terry Marsh for all of his assistance and reassurance to help me through the comprehensive exam.

Next, I must thank my partner and love of my life, Rita O'Brien. Rita has been a constant source of strength and love for me and has found a way to make me smile throughout this process. I still can't believe she has been able to deal with me this whole time, and I look forward to our future together.

My friends in the Lansing area have meant so much to me. Alex Bryan, Jared Talaga, Adam and Lacey Ingrao, Paul, Emily, and Franklin Nicholls, Gabriel and Nichole Biber, Alison Colby, and Jason Cox, are a few of the many people I would like to mention who have been an excellent adoptive family. I appreciate all the opportunities they have afforded me to expand my horizons beyond my work.

My friends from other parts of the country have also been an important source of strength for me. Jeremy Harris has gone out of his way to remain an active part of my life and I am eternally grateful, and John Pape has remained a steadfast friend upon whom I can rely. Jo Dery made the effort to visit and stay in touch. The re-entry of Ashrae Fax into my renewed my love for Michael Soter, Renee Haran, and Alex Chesney and provided a much-needed distraction to, strangely, keep me focused.

My family has been incredibly supportive throughout this pfrocess, and I thank and love them dearly. My brother, Richard, has been so good at keeping in touch although I drop the ball and providing love and support. My parents have been an ever-present source of love, comfort, support, and treats to help me stay motivated. Thank you for everything. Littlebear O'Brien has provided a wealth of inspiration and love, as have the rest of the Kantz, O'Brien, and Gould families. My immediate family, including Dingy the Dog, Soba Noodles, Mowgli MaParrot, and Charlie Bird, have been invaluable to me throughout this experience. I especially want to express gratitude to Dingy, who has never let me down. Without all of these crazy animals giving me perspective on a daily basis, this process would've been much more difficult.

I also want to thank all of the members of the Manning Lab, past and present. It has been a true pleasure to work with all of you, and I wish you all the best of success going forward. Thanks to my daily bicycle commute with Michelle Korir, I was able to maintain sanity in otherwise insane times. She has been a great friend to me. Pallavi Singh has been incredibly helpful to me and her humor and advice have proven salient. Wonhee Cha has also provided excellent counsel, and delicious M&Ms whenever necessary. Kathy Jernigan has been a great friend and benchmate. Clare Laut, Dave Knupp, and Jessica Plemmons all gave me more than I could have given them and made work more enjoyable. There is really too much to say about what I love and appreciate about the people I have met and worked with here, but here is a short list: Rim Al Safadi's training and humor, Rebekah Mosci's precise understanding, Sam Wengert's happy anger, Lindsey Ouellette's smile and patience, Beth Whittam's caring and laughter, Megan Shiroda's excitement and cupcakes, Scott Henderson's sweet but surly nature, Sanjana Mukherjee's large heart and singing, Jose Rodriguez's snorting spirit, Brian Nohomovich's sweet apathy, Jade Sha's questioning nature, Aaron Balogh's eagerness, Maria Tikhonenko's dancing, Matt Peter's quiet sarcasm, Moriah Moore's caring mockery, and many, many more people I know I must be leaving out. Thank you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GROUP B STREPTOCOCCAL DISEASE AND COLONIZATION

Introduction

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae*

Originally described in the 1880s as the causative agent in bovine mastitis infections, this non-motile, chain-forming, Gram-positive coccus was recognized in the 1960s as the pathogen responsible for the majority of neonatal mortality in industrialized nations. GBS is present as a commensal in up to 40% of healthy men and women in the gastrointestinal and/or urogenital tracts (1, 2). Phylogenetically-located within the Streptococcaceae family of the order Lactobacillales, GBS is so-named due to the β-hemolysis, or complete lysis of red blood cells, observed when grown on blood agar plates (3).

Invasive Disease

GBS is a leading cause of stillbirth, meningitis, and sepsis in neonates, and an emerging pathogen capable of causing invasive disease in susceptible adults. Increased efforts towards prevention of GBS disease in the US throughout the 1990s have resulted in a reduction of disease burden in neonates; however, this pathogen continues to plague both industrialized and developing nations, and there remain significant gaps in understanding mechanisms of pathogenesis (4, 5).

There are two types of GBS neonatal disease, early (EOD) and late (LOD) onset, which differ not only temporally but likely in mechanism of infection. EOD accounts for ~80% of neonatal infections and involves either breathing of infected amniotic fluid during birth or a transmission event across intact membranes (6). Heavy colonization of the mother is the major identified risk factor for the development of EOD (7).

LOD presents up to a few months after birth, occurs less frequently, and the risk factors and transmission are not well understood (6). LOD can result in meningitis leading to neurological defects and, possibly, death (8). Guidelines, established by the American College of Obstetricians and Gynecologists and CDC in 1996, recommend testing for colonization in the late 3rd trimester followed by intrapartum antibiotic prophylaxis during labor if GBS is present (5). These guidelines have resulted in a dramatic reduction in the cases of EOD; however, the prevalence of LOD cases has remained constant (9). Additionally, the initial decline of EOD rates has reached a plateau over the past decade due to a combination of suboptimal guideline implementation and a lack of other treatment options (10).

GBS disease progression includes sepsis, meningitis, pneumonia, cellulitis, osteomyelitis, and septic arthritis, and ~1200 infants in the United States are infected each year resulting in fatalities in 2-4% of cases and neurological defects in up to 50% of survivors (11, 12). Furthermore, colonization of the mother has been related to low birthweight, preterm birth, and stillbirth, suggesting detrimental effects even in the case of asymptomatic colonization (13). Economically, infection with GBS was found to be the most costly, and nearly 2 times higher over the first two years, of common neonatal infections in one United Kingdom study (14). GBS disease has also been increasingly recognized as a threat to non-pregnant adults over the past few decades. Risk factors such as age, race, and underlying medical condition have been linked to GBS-related skin infections, urinary tract infections, osteomyelitis, meningitis, and endocarditis (4, 15, 16).

Asymptomatic Colonization, Transmission, and Pathogenesis

Asymptomatic Colonization

Colonization and persistence are essential elements of GBS carriage and disease. Asymptomatic colonization is prevalent as studies have found up to 30% of healthy adults are infected in the gastrointestinal or genitourinary tracts, although variation in the sensitivity of detection methods likely underestimates carriage (1, 17, 18). There is no difference in colonization rates between men and women, regardless of pregnancy status (18–21). The majority of commensal infections are transitory with colonization status changing over time, but chronic carriers have been identified (17, 22). Furthermore, most chronic carriers have been found to be colonized by the same clone, even when tested at multiple body sites or over time, and recolonization occurs in approximately 40% of cases following successful antibiotic treatment indicating either an environmental, or, more likely, internal reservoir (18, 23, 24).

Transmission of Infection

The infection model for GBS pathogenesis suggests a vertical transmission event from mother to child during, or in some cases before, childbirth leading to infection of the neonate (25). Vertical transmission is known to occur in approximately 50% of births to colonized mothers (6). Nosocomial transmission has also been documented, although these cases are relatively rare (26, 27). Thus, maternal colonization is the key to the development of neonatal disease. Not surprisingly, adult invasive disease is also considered to have stemmed from previous asymptomatic infection (28). Sexual transmission can occur with risk of overall carriage, and especially vaginal, increasing with the number of partners (19, 29, 30).

Colonization is the primary risk factor for disease, and the identification of infection determinants driving chronic infection remains central to prevention efforts.

Pathogenesis

The pathogenesis of GBS can be broken down into three stages: host-cell attachment, colonization, and invasion. While the first two stages are important for a commensal relationship, invasion is essential to pathogenesis. Many novel proteins have been identified in GBS that assist the bacterium in one or more of these stages. *In vitro* experiments identified an adhesin, FbsA, which binds fibrinogen and aids in attachment and invasion of host cells (31). Additionally, presence or absence of *fbsB*, which encodes a protein shown to aid in invasion, is correlated with greater fibrinogen-binding ability (32–34). ScpB is a surface protein able to bind fibrinogen which facilitates attachment to host cells and also disables the innate immune system through cleavage of the complement factor, C5a (35). Two distinct pilus loci: PI-1 and PI-2 have been identified in the GBS genome. PI-1 is ubiquitous across CCs, and there are two variants of PI-2: PI-2a and PI-2b. These pili have been identified as playing a role in attachment to host cells and in biofilm formation (36–38). Hyaluronate lyase (*hylB)*, well-studied due to homologues in other species such as *Streptococcus pyogenes, S. pneumoniae*, *Staphylococcus aureus, and Clostridium perfringens,* has been shown to degrade the extracellular matrices of placental tissues (39, 40). GBS is also able to invade and persist, often through the induction of endocytic uptake (41), in a number of eukaryotic cell types, including both epithelial and endothelial (32, 42, 43). Furthermore, GBS is known to evade and persist within immune cells, offering one explanation as to why the immune system alone cannot clear an infection (44–46). The characteristic pore-forming β-hemolysin is important for invasion and persistence in macrophages(47), while the presence, chemical composition, and allelic variation of the protective capsule surrounding GBS cells is an important factor in immune evasion (48–50).

Interestingly, these virulence promoting factors vary in presence and sequence between isolates, resulting in variable phenotypes and offering insight into the differences in pathogenic potential of different GBS isolates.

Phenotypic and Genotypic Variability

Phenotypic Variability

While an understanding of the features important for colonization and invasiveness is incomplete, attempts to characterize both invasive and colonizing isolates have resulted in the discovery of phenotypic factors important to GBS pathogenesis. Some phenotypic determinants with a role in pathogenesis in GBS include serotype, growth lag in certain media, pigment production, and biofilm production (51–54). The question of what causes increased virulence of a particular isolate demands the continued discovery and search for variation between strains.

Capsule Type

Serotyping, or capsule typing, was one of the first techniques to classify isolates. This technique utilizes inherent antigenic variation of the polysaccharide capsule using mono- and polyclonal antibodies to determine the capsule type of a given strain (55). Multiple molecular-based capsule typing strategies have largely replaced serotyping, and provide equal or greater specificity and increased resolution to detect capsule variants that could otherwise be missed (56–59). To date, there are 10 reported capsule (CPS) types: Ia, Ib, and II through IX (60). Although each CPS type has been isolated from cases of invasive disease, CPS-III is disproportionately responsible for invasive disease, both in neonates and adults (4, 6, 61, 62).

Biofilm Production

It was recently discovered that GBS can form biofilms, or aggregates of cells surrounded by a protective self-produced matrix composed of polysaccharides, proteins, and/or DNA (54, 63–66). Biofilms are known to promote environmental persistence and chronic infections (67–70). The majority of bacterial infections are thought be biofilm related and GBS biofilm production may offer an explanation for persistent colonization (64, 71–73). Studies which assayed biofilm production in clinical isolates of GBS have shown significant variation in biofilm-forming ability between strains (54, 63). Kaur, et al, showed that high biofilm production is correlated with asymptomatically colonizing strains, whereas low biofilm production characterized invasive strains. Furthermore, it was demonstrated that optimal biofilm production occurred in the presence of glucose and at neutral pH (54). Acidic conditions were shown to enhance biofilm production of invasive strains when the biofilm assay was modified to include additional washing steps, growth in a shaking environment, and a different growth medium (63). However, many questions remain unanswered regarding biofilm production and GBS, and, given the importance of bacterial biofilms to human disease, the second chapter of this dissertation further explores the importance of biofilms to GBS colonization and pathogenesis.

Phase Variation

Natural selection demands phenotypic heterogeneity at the subspecies level. Generation of phenotypic diversification in bacteria can be accomplished in many ways, both heritable and transient including phage acquisition, horizontal gene transfer, transposable elements, replication errors, epigenetic modifications, and phase variation (74–81). Phase variation, in which individuals of a population exist in different, revertable phenotypes which may display increased fitness under certain environmental conditions, offers a non-fixed pathway and

includes the development of variants (82–85). Small colony variants (SCVs) have been identified for a number of bacterial species, including many pathogens (86). *S. aureus* is the best characterized Gram positive species that readily forms SCVs, although elucidation of the mechanism can be difficult due to frequent phenotypic reversion (87). SCVs isolated from *Staphylococcal* infections have been demonstrated as drivers of persistence and pathogenesis (88). Phase and non-phase variation have been documented within the streptococc*i*, including *S. pneumoniae* (89–91) , *S. pyogenes* (92)*, S. faecalis* (93)*, and S. tigurinus*(94)*;* however, Chapter 4 of this dissertation represents the first report of SCVs in GBS.

Genetic Variability

Phylogenetic Lineages

MLST, a molecular typing method used to identify sequence variation within seven conserved genes, has been used to identify distinct sequence types and, subsequently, link phylogenetic lineage and pathogenicity (95). This technique has also shown the vast majority of clinical isolates fall into four major clonal complexes (CCs): 1, 17, 19, and 23, named according to the predominant sequence type (ST) within each CC. Of these, CCs-17 and -19 have been associated with neonatal disease and CC-17, specifically, is more closely related to bovine-derived than human-derived strains (96, 97). CCs -1 and -23, however, have been associated with asymptomatic colonization in several prior studies (95, 98). Notably, although CPS III strains can belong to many lineages, the majority of CC-17 strains are CPS III indicating that increased pathogenicity is likely due to genetic variability across the whole genome, not just within the capsule operon , prompting further investigation into variable presence of sequence of other virulence factors (97).

Pilus Islands

Described as extracellular non-flagellar, adhesin-tipped, filamentous appendages constructed through the assembly of subunits, pili offer contact with the surrounding environment and are important for phage adsorption, conjugation, surface attachment, biofilm production, and DNA and protein translocation (99–103). Most examples come from Gram negative (G-) bacteria in which pili are categorized into distinct varieties according to mechanism of assembly, including type I, type IV, and curli types (36). However, there are a number of Gram positive pathogens for which pili have been identified and assigned important pathogenesis-promoting functions, including several *Streptococcus* species (36, 38, 104). Two different pilus islands were identified in the GBS genome encoding all the necessary genes for pilus assembly, including the structural backbone and adhesin LPXTG-motif containing proteins and assembly-oriented sortase enzymes which facilitate polymerization and cell wall attachment of structural proteins (37, 105, 106). Pilus Island 1 (PI-1) is a ~16 Kb genomic feature found in the majority of human isolates and containing 18 open reading frames (ORFs) (37, 107, 108). PI-1 is present in the same locus in the majority of human strains, but is absent in bovine-associated strains, suggesting the importance to human infection (37, 109). In accordance, PI-1 plays a role in immune evasion and is expressed in a pH-dependent manner, but, interestingly, has no proven role in surface attachment (110, 111). There are 2 mutually-exclusive variants of Pilus Island 2, PI-2a and PI-2b, both approximating 11kb in length (37). Despite overall similarity in sequence length between these two variants, sequence variation is significant as they contain unique backbones and ancillary proteins, as well as the addition of a signal peptidase in PI-2a and *rogB* regulator in PI-2b (37, 112). PI-2a is associated with adult invasive disease, increased biofilm production, and virulence in mice, while PI-2b is associated with neonatal invasive disease and found in both the hyperinvasive CC-17 and bovine lineages (72,

109, 113, 114). The importance of allelic variation in pilus genes on biofilm production across a large set of strains is addressed in Chapter 2 of this dissertation.

Virulence Regulation

Two Component Regulatory Systems

The regulation of virulence is critical for pathogenesis as the control of gene expression allows for adaptation and survival in diverse environments (115). The ability to recognize extracellular stimuli and respond is controlled by signal transduction systems (STS) and, in bacteria, one important STS is the two component system (TCS) In GBS, there are four experimentally verified TCSs with a role in virulence, the controller of virulence (CovR/S), the regulator of D-Alanyl-lipotechoic acid biosynthesis (DltR/S), the competence and β-lactam-resistance promoting system (CiaR/C), and the regulator of fibrinogen binding (RgfA/C). CovR/S is the best understood of the TCSs and studies have found it to have a global regulatory function in GBS. Through a deletion mutant, CovR/S was found to affect transcription of >7% of all genes in a CC-23 strain and phenotypic changes included greater adherence to epithelial cells and increased hemolytic activity (66). The DltR/S system was found to regulate genes responsible for the d-alanine (d-ala) content of lipotechoic acids. Disruption of this pathway resulted in a decreased resistance to colistin, a peptide antibiotic primarily effective against Gram-negatives, with susceptibility likely due to the increased electronegativity of a cell wall, as has been shown in *Bacillus subtilis* (66, 116). The importance of the CiaR/S system to GBS pathogenicity was discovered in an *in-vitro* screen of random transposon insertion mutants showing increased susceptibility to antimicrobial peptides (117). Further characterization showed a role for this TCS in intracellular survival and virulence in a mouse model, possibly through regulation of genes involved in proteolysis (117). The regulator

of fibrinogen binding (Rgf) system has an important role in host cell binding, and it has been shown to affect expression of membrane-bound proteins and affect fibrinogen binding through regulation of FbsB (33, 118).

Quorum Sensing in Gram Positives and the *Streptococceae*

The role of quorum sensing in pathogenesis is well documented (119). In gram positive bacteria, this phenomenon is largely driven by small peptides that are secreted into the extracellular milieu where they react with cell wall receptors and change the transcriptome through a STS. *Streptococcus* species are known to use quorum sensing to control competence, biofilm formation, and invasion in a host (120–122). The competence stimulating peptide in *S. pneumoniae*, for example, is encoded within the *com* operon along with the histidine kinase and response regulator, *comD* and *comE*, respectively (123). This same peptide in *Streptococcus mutans* has been demonstrated to function in the formation of a biofilm (68). Another quorum sensing pathway in the streptococci is mediated through autoinducer-2. This secreted peptide activates the LuxPQ STS resulting in phenotypic changes, such as biofilm formation in *S. gordonii* (124). The only verified cell to cell communication system in GBS involves a small hairpin protein (shp) which activates the regulator *rovS* driving virulence in a mouse model. Interestingly, expression and activation of the shp was specific to media and environment (125). As disease progression involves differential expression in response to changing host environments, examination of any additional putative auto-inducing peptides capable of environmentally-mediated regulation in GBS is merited.

rgfD and Quorum Sensing

The *rgf* two-component system in GBS has been demonstrated to affect *scpB*, *fbsA*, and *fbsB* transcription, and deletion of the response regulator and histidine kinase resulted in an altered fibrinogen-binding phenotype, as mentioned above (33). The genes have been suggested to be polycistronically transcribed as part of a putative four gene operon, *rgfBDAC*, with *rgfA* and *rgfC* coding for the response regulator and sensor kinase, respectively. Along with the TCS, the transcript codes for a putative quorum sensing peptide, RgfD, and its putative processor, RgfB (33, 118). Consequently, it is hypothesized but untested that the *rgfA/C* system facilitates GBS colonization via quorum sensing. *agr*, the *rgf* homologue present in *Staphylococcus* species, is important for virulence in an animal model through the regulation of secreted virulence factors and surface proteins in response to extracellular stimuli and density (126, 127). The importance of *rgfD*, the gene coding for the putative autoinducing peptide in the regulation of the rgf operon is addressed in Chapter 3 of this dissertation.

Conclusions

Successful treatment and cure of GBS disease in both neonatal and adult populations requires a holistic approach targeting multiple pathogenic determinants, both host and microbe. The current recommended treatment regimen of intrapartum antibiotic prophylaxis has greatly reduced the disease burden, yet this decline has reached a plateau over the past decade (12) and has contributed to increasing frequencies of antibiotic resistance in GBS and other bacteria (e.g., E. coli)(128, 129). Biofilms are known to confer greater antibiotic tolerance to inhabitants and are important to persistent colonization for many microbial pathogens. The epidemiological associations with biofilm production explored in the second chapter of this dissertation suggest biofilms may offer a good target for the clearance of chronic GBS infections. Vertical

transmission of GBS is common (6)and adhesin-mediated attachment to host cells is vital to the establishment and spread of infections (8, 34) . In Chapter 3, a putative quorum sensing AIP in GBS that modulates expression of a regulatory circuit important for fibrinogen binding is examined. This work offers the potential of an extracellular target to prevent initial attachment events and the spread of GBS to new infection sites. Lastly, phenotypic heterogeneity and the development of SCVs is examined in Chapter 4. The work contained within identifies a role for stress in the generation of SCVs and offers an explanation for persistent colonization. Furthermore, this chapter strengthens the argument for alternative treatments to antibiotic use while also identifying a complication to therapeutic development which was not previously known. Altogether, the work in this dissertation utilizes the study of bacterial physiology to provide guidance towards the development of new detection methods and therapeutics, which must account for biofilm production, temporal expression of potential targets, and a persister cell population, for the further reduction of disease burden in both adult and neonatal populations.

REFERENCES

REFERENCES

1. **Hickman ME**, **Rench M a**, **Ferrieri P**, **Baker CJ**. 1999. Changing epidemiology of Group B Streptococcal colonization. Pediatrics **104**:203–9.

2. **Manning SD**, **Neighbors K**, **Tallman P a**, **Gillespie B**, **Marrs CF**, **Borchardt SM**, **Baker CJ**, **Pearlman MD**, **Foxman B**. 2004. Prevalence of Group B *Streptococcus* colonization and potential for transmission by casual contact in healthy young men and women. Clin Infect Dis **39**:380–8.

3. **Makarova K**, **Slesarev A**, **Wolf Y**, **Sorokin A**, **Mirkin B**, **Koonin E**, **Pavlov A**, **Pavlova N**, **Karamychev V**, **Polouchine N**, **Shakhova V**, **Grigoriev I**, **Lou Y**, **Rohksar D**, **Lucas S**, **Huang K**, **Goodstein DM**, **Hawkins T**, **Plengvidhya V**, **Welker D**, **Hughes J**, **Goh Y**, **Benson A**, **Baldwin K**, **Lee J-H**, **Díaz-Muñiz I**, **Dosti B**, **Smeianov V**, **Wechter W**, **Barabote R**, **Lorca G**, **Altermann E**, **Barrangou R**, **Ganesan B**, **Xie Y**, **Rawsthorne H**, **Tamir D**, **Parker C**, **Breidt F**, **Broadbent J**, **Hutkins R**, **O'Sullivan D**, **Steele J**, **Unlu G**, **Saier M**, **Klaenhammer T**, **Richardson P**, **Kozyavkin S**, **Weimer B**, **Mills D**. 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci **103** :15611–15616.

4. **Phares CR**, **Lynfield R**, **Farley MM**, **Mohle-boetani J**, **Harrison LH**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Stefonek KR**, **Albanese BA**, **Zell ER**, **Schuchat A**, **Schrag SJ**. 2008. Epidemiology of invasive Group B Streptococcal disease in the United States , 1999-2005. J Am **299**:1999–2005.

5. **Verani JR**, **McGee L**, **Schrag SJ**. 2010. Prevention of perinatal group B streptococcal disease. 2002; 51. MMWR **59**: 1-22

6. **Schuchat A**. 1998. Epidemiology of Group B Streptococcal Disease in the United States : Shifting Paradigms. Clin Microbiol Rev **11**.

7. **Baker CJ**. 2000. Streptococcal Infections : Clinical aspects, microbiology, and molecular pathogenesis. Oxford University Press.

8. **Maisey HC**, **Doran KS**, **Nizet V**. 2008. Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. Expert Rev Mol Med **10**:e27.

9. **Dermer P**, **Lee C**, **Eggert J**, **Few B**. 2004. A history of neonatal Group B *Streptococcus* with its related morbidity and mortality rates in the United States. J Pediatr Nurs **19**:357–63.

10. **Verani JR**, **Spina NL**, **Lynfield R**, **Schaffner W**, **Harrison LH**, **Holst A**, **Thomas S**, **Garcia JM**, **Scherzinger K**, **Aragon D**. 2014. Early-onset group B streptococcal disease in the United States: potential for further reduction. Obstet Gynecol **123**:828–837.

11. **Nizet V**, **Kim KS**, **Stins M**, **Jonas M**, **Chi EY**, **Nguyen D**, **Rubens CE**. 1997. Invasion of brain microvascular endothelial cells by Group B Streptococci. Infect Immun **65**:5074–5081.

12. **Verani JR**, **McGee L**, **Schrag SJ**. 2010. Prevention of perinatal group B streptococcal

disease: Revised guidelines from CDC, 2010. Department of Health and Human Services, Centers for Disease Control and Prevention.

13. **Valkenburg-van den Berg AW**, **Sprij AJ**, **Dekker FW**, **Dörr PJ**, **Kanhai HHH**. 2009. Association between colonization with Group B *Streptococcus* and preterm delivery: a systematic review. Acta Obstet Gynecol Scand **88**:958–67.

14. **Schroeder E-A**, **Petrou S**, **Balfour G**, **Edamma O**, **Heath P**. 2009. The economic costs of Group B *Streptococcus* (GBS) disease: prospective cohort study of infants with GBS disease in England. Eur J Heal Econ **10**:275–285.

15. **Ulett KB**, **Benjamin WH**, **Zhuo F**, **Xiao M**, **Kong F**, **Gilbert GL**, **Schembri M a**, **Ulett GC**. 2009. Diversity of Group B *Streptococcus* serotypes causing urinary tract infection in adults. J Clin Microbiol **47**:2055–60.

16. **Skoff TH**, **Farley MM**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Harrison LH**, **Lynfield R**, **Mohle-Boetani J**, **Zansky S**, **Albanese B a**, **Stefonek K**, **Zell ER**, **Jackson D**, **Thompson T**, **Schrag SJ**. 2009. Increasing burden of invasive Group B Streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis **49**:85–92.

17. **Hansen SM**, **Uldbjerg N**, **Kilian M**, **Sørensen UBS**. 2004. Dynamics of *Streptococcus agalactiae* Colonization in Women during and after Pregnancy and in Their Infants. J Clin Microbiol **42**:83–89.

18. **Bliss SJ**, **Manning SD**, **Tallman P**, **Baker CJ**, **Pearlman MD**, **Marrs CF**, **Foxman B**. 2002. Group B *Streptococcus* Colonization in Male and Nonpregnant Female University Students: A Cross-Sectional Prevalence Study. Clin Infect Dis **34**:184–190.

19. **Regan JA**, **Klebanoff MA**, **Nugent RP**. 1991. The epidemiology of group B streptococcal colonization in pregnancy. Obstet Gynecol **77**:604–610.

20. **van der Mee-Marquet N**, **Fourny L**, **Arnault L**, **Domelier A-S**, **Salloum M**, **Lartigue M-F**, **Quentin R**. 2008. Molecular Characterization of Human-Colonizing *Streptococcus agalactiae* Strains Isolated from Throat, Skin, Anal Margin, and Genital Body Sites. J Clin Microbiol **46**:2906–2911.

21. **Brimil N**, **Barthell E**, **Heindrichs U**, **Kuhn M**, **Lütticken R**, **Spellerberg B**. 2006. Epidemiology of *Streptococcus agalactiae* colonization in Germany. Int J Med Microbiol **296**:39–44.

22. **Lewin EB**, **Amstey MS**. 1981. Natural history of group B *Streptococcus* colonization and its therapy during pregnancy. Am J Obstet Gynecol **139**:512–515.

23. **Tam T**, **Bilinski E**, **Lombard E**. 2012. Recolonization of group B *Streptococcus* (GBS) in women with prior GBS genital colonization in pregnancy. J Matern Fetal Neo M **25**:1987–1989.

24. **Manning SD**, **Lewis M**, **Springman AC**, **Lehotzky E**, **Whittam TS**, **Davies HD**. 2008. Genotypic diversity and serotype distribution of Group B *Streptococcus* isolated from women before and after delivery. Clin Infect Dis **46**:1829–37.

25. **Doran KS**, **Nizet V**. 2004. Molecular pathogenesis of neonatal Group B Streptococcal infection: no longer in its infancy. Mol Microbiol **54**:23–31.

26. **Morinis J**, **Shah J**, **Murthy P**, **Fulford M**. 2011. Horizontal transmission of group B *Streptococcus* in a neonatal intensive care unit. Pediatr Child Heath **16**:48–50.

27. **Noya FJD**, **Rench MA**, **Metzger TG**, **Colman G**, **Naidoo J**, **Baker CJ**. 1987. Unusual Occurrence of an Epidemic of Type Ib/c Group B Streptococcal Sepsis in a Neonatal Intensive Care Unit. J Infect Dis **155** :1135–1144.

28. **Tyrrell GJ**, **Senzilet LD**, **Spika JS**, **Kertesz DA**, **Alagaratnam M**, **Lovgren M**, **Talbot JA**, **Sy SHUS**. 2000. Invasive disease due to Group B Streptococcal infection in adults: Results from a Canadian, population-based, active laboratory surveillance study - 1996. J Infect Dis **182**:168–173.

29. **Manning SD**, **Tallman P**, **Baker CJ**, **Gillespie B**, **Marrs CF**, **Foxman B**. 2002. Determinants of co-colonization with Group B *Streptococcus* among heterosexual college couples. Epidemiology **13**.

30. **Meyn LA**, **Moore DM**, **Hillier SL**, **Krohn MA**. 2002. Association of Sexual Activity with Colonization and Vaginal Acquisition of Group B *Streptococcus* in Nonpregnant Women. Am J Epidemiol **155**:949–957.

31. **Schubert A**, **Zakikhany K**, **Pietrocola G**, **Meinke A**, **Speziale P**, **Eikmanns BJ**, **Reinscheid DJ**. 2004. The fibrinogen receptor FbsA promotes adherence of *Streptococcus agalactiae* to human epithelial cells. Infect Immun **72**:6197–6205.

32. **Gutekunst H**, **Eikmanns BJ**, **Reinscheid DJ**. 2004. The novel fibrinogen-binding protein FbsB promotes *Streptococcus agalactiae* invasion into epithelial cells. Infect Immun **72**:3495–3504.

33. **Al Safadi R**, **Mereghetti L**, **Salloum M**, **Lartigue M-F**, **Virlogeux-Payant I**, **Quentin R**, **Rosenau A**. 2011. Two-component system RgfA/C activates the *fbsB* gene encoding major fibrinogen-binding protein in highly virulent CC17 clone group B *Streptococcus*. PLoS One **6**:e14658.

34. **Rosenau A**, **Martins K**, **Amor S**, **Gannier F**, **Lanotte P**, **van der Mee-Marquet N**, **Mereghetti L**, **Quentin R**. 2007. Evaluation of the ability of *Streptococcus agalactiae* strains isolated from genital and neonatal specimens to bind to human fibrinogen and correlation with characteristics of the *fbsA* and *fbsB* genes. Infect Immun **75**:1310–1317.

35. **Bohnsack JF**, **Takahashi S**, **Hammitt L**, **Miller D V**, **Aly AA**, **Adderson EE**. 2000. Genetic Polymorphisms of Group B *Streptococcus scpB* Alter Functional Activity of a Cell-Associated Peptidase That Inactivates C5a. Infect Immun **68**:5018–5025.

36. **Telford JL**, **Barocchi MA**, **Margarit I**, **Rappuoli R**, **Grandi G**. 2006. Pili in gram-positive pathogens. Nat Rev Microbiol **4**:509–519.

37. **Rosini R**, **Rinaudo CD**, **Soriani M**, **Lauer P**, **Mora M**, **Maione D**, **Taddei A**, **Santi I**, **Ghezzo C**, **Brettoni C**, **Buccato S**, **Margarit I**, **Grandi G**, **Telford JL**. 2006. Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus*

agalactiae. Mol Microbiol **61**:126–141.

38. **Lauer P**, **Rinaudo CD**, **Soriani M**, **Margarit I**, **Maione D**, **Rosini R**, **Taddei AR**, **Mora M**, **Rappuoli R**, **Grandi G**, **Telford JL**. 2005. Genome analysis reveals pili in Group B Streptococcus. Science **309**:105.

39. **Girish KS**, **Kemparaju K**. 2007. The magic glue hyaluronan and its eraser hyaluronidase : A biological overview. Life Sci **80**:1921–1943.

40. **Gase K**, **Malke H**. 1998. The *hylB* gene encoding hyaluronate lyase : completion of the sequence and expression analysis. Biochim Biophys Acta 86–98.

41. **Tyrrell GJ**, **Kennedy A**, **Shokoples SE**, **Sherburne RK**. 2002. Binding and invasion of HeLa and MRC-5 cells by *Streptococcus agalactiae*. Microbiology **148**:3921–3931.

42. **van Sorge NM**, **Quach D**, **Gurney MA**, **Sullam PM**, **Nizet V**, **Doran KS**. 2009. The Group B Streptococcal Serine-Rich Repeat 1 Glycoprotein Mediates Penetration of the Blood-Brain Barrier. J Infect Dis **199** :1479–1487.

43. **Winram SB**, **Jonas M**, **Chi E**, **Rubens CE**. 1998. Characterization of group B streptococcal invasion of human chorion and amnion epithelial cells in vitro. Infect Immun **66**:4932–4941.

44. **Hunolstein V**. 1998. Group B Streptococci persist inside macrophages. Immunology **93**:86–95.

45. **Valenti-Weigand P**, **Benkel P**, **Rohde M**, **Chhatwal GS**. 1996. Entry and intracellular survival of group B streptococci in J774 macrophages. Infect Immun **64**:2467–2473.

46. **Kubica M**, **Guzik K**, **Koziel J**, **Zarebski M**, **Richter W**, **Gajkowska B**, **Golda A**, **Maciag-Gudowska A**, **Brix K**, **Shaw L**, **Foster T**, **Potempa J**. 2008. A Potential New Pathway for *Staphylococcus aureus* Dissemination: The Silent Survival of *S. aureus* Phagocytosed by Human Monocyte-Derived Macrophages. PLoS One **3**:e1409.

47. **Nizet V**, **Gibson RL**, **Chi EY**, **Framson PE**, **Hulse M**, **Rubens CE**. 1996. Group B streptococcal beta-hemolysin expression is associated with injury of lung epithelial cells. Infect Immun **64**:3818–3826.

48. **Rubens CE**, **Wessels MR**, **Heggen LM**, **Kasper DL**. 1987. Transposon mutagenesis of type III Group B *Streptococcus*: correlation of capsule expression with virulence. Proc Natl Acad Sci U S A **84**:7208–12.

49. **Wessels MR**, **Paoletti LC**, **Kasper DL**, **DiFabio JL**, **Michon F**, **Holme K**, **Jennings HJ**. 1990. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III Group B *Streptococcus*. J Clin Invest **86**:1428–33.

50. **Tettelin H**, **Masignani V**, **Cieslewicz MJ**, **Donati C**, **Medini D**, **Ward NL**, **Angiuoli S V**, **Crabtree J**, **Jones AL**, **Durkin a S**, **Deboy RT**, **Davidsen TM**, **Mora M**, **Scarselli M**, **Margarit y Ros I**, **Peterson JD**, **Hauser CR**, **Sundaram JP**, **Nelson WC**, **Madupu R**, **Brinkac LM**, **Dodson RJ**, **Rosovitz MJ**, **Sullivan S a**, **Daugherty SC**, **Haft DH**, **Selengut J**, **Gwinn ML**, **Zhou L**, **Zafar N**, **Khouri H**, **Radune D**, **Dimitrov G**, **Watkins K**,

O'Connor KJB, **Smith S**, **Utterback TR**, **White O**, **Rubens CE**, **Grandi G**, **Madoff LC**, **Kasper DL**, **Telford JL**, **Wessels MR**, **Rappuoli R**, **Fraser CM**. 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A **102**:13950–5.

51. **Musser JM**, **Mattingly SJ**, **Quentin R**, **Goudeau A**, **Selander RK**. 1989. Identification of a high-virulence clone of type III *Streptococcus agalactiae* (group B *Streptococcus*) causing invasive neonatal disease. Proc Natl Acad Sci U S A **86**:4731–5.

52. **Tapsall JW**. 1986. Pigment production by Lancefield-group-B streptococci (*Streptococcus agalactiae*). J Med Microbiol **21**:75–81.

53. **Whidbey C**, **Harrell MI**, **Burnside K**, **Ngo L**, **Becraft AK**, **Iyer LM**, **Aravind L**, **Hitti J**, **Adams Waldorf KM**, **Rajagopal L**. 2013. A hemolytic pigment of Group B *Streptococcus* allows bacterial penetration of human placenta. J Exp Med **210**:1265–1281.

54. **Kaur H**, **Kumar P**, **Ray P**, **Kaur J**, **Chakraborti A**. 2009. Biofilm formation in clinical isolates of Group B Streptococci from North India. Microb Pathog **46**:321–7.

55. **Lancefield RC**, **McCARTY M**, **Everly WN**. 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142**:165–179.

56. **Kong F**, **Gowan S**, **Martin D**, **James G**, **Gilbert GL**. 2002. Serotype identification of Group B Streptococci by PCR and sequencing. J Clin Microbiol **40**:216–226.

57. **Manning SD**, **Lacher DW**, **Davies HD**, **Foxman B**, **Whittam TS**. 2005. DNA polymorphism and molecular subtyping of the capsular gene cluster of Group B *Streptococcus*. J Clin Microbiol **43**:6113–6116.

58. **Imperi M**, **Pataracchia M**, **Alfarone G**, **Baldassarri L**, **Orefici G**, **Creti R**. 2010. A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. J Microbiol Methods **80**:212–4.

59. **Poyart C**, **Tazi A**, **Réglier-Poupet H**, **Billoët A**, **Tavares N**, **Raymond J**, **Trieu-Cuot P**. 2007. Multiplex PCR assay for rapid and accurate capsular typing of Group B Streptococci. J Clin Microbiol **45**:1985–1988.

60. **Slotved H**, **Kong F**, **Lambertsen L**, **Sauer S**, **Gilbert GL**. 2007. Serotype IX, a Proposed New *Streptococcus agalactiae* Serotype. J Clin Microbiol **45**:2929–36.

61. **Persson E**, **Berg S**, **Trollfors B**, **Larsson P**, **Ek E**, **Backhaus E**, **Claesson BEB**, **Jonsson L**, **Rådberg G**, **Ripa T**, **Johansson S**. 2004. Serotypes and clinical manifestations of invasive Group B Streptococcal infections in Western Sweden 1998-2001. Clin Microbiol Infect **10**:791–6.

62. **Baker CJ**, **Barrett FF**. 1974. Group B streptococcal infections in infants: the importance of the various serotypes. JAMA-J Am Med Assoc **230**:1158–1160.

63. **D'Urzo N**, **Martinelli M**, **Pezzicoli A**, **De Cesare V**, **Pinto V**, **Margarit I**, **Telford JL**, **Maione D**. 2014. Acidic pH strongly enhances *in vitro* biofilm formation by a subset of

hypervirulent ST-17 *Streptococcus agalactiae* strains. Appl Environ Microbiol **80**:2176–2185.

64. **Costerton JW**, **Stewart PS**, **Greenberg EP**. 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science **284**:1318–1322.

65. **Whitchurch CB**, **Tolker-Nielsen T**, **Ragas PC**, **Mattick JS**. 2002. Extracellular DNA required for bacterial biofilm formation. Science **295**:1487.

66. **Poyart C**, **Lamy MC**, **Boumaila C**, **Fiedler F**, **Trieu-Cuot P**. 2001. Regulation of D-alanyl-lipoteichoic acid biosynthesis in *Streptococcus agalactiae* involves a novel two-component regulatory system. J Bacteriol **183**:6324–6334.

67. **Vuong C**, **Voyich JM**, **Fischer ER**, **Braughton KR**, **Whitney AR**, **DeLeo FR**, **Otto M**. 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol **6**:269–275.

68. **Li Y**, **Tang N**, **Aspiras MB**, **Lau PCY**, **Lee JH**, **Ellen RP**, **Cvitkovitch DG**. 2002. A Quorum-Sensing Signaling System Essential for Genetic Competence in *Streptococcus mutans* Is Involved in Biofilm Formation. J Bacteriol **184**:2699–2708.

69. **Zhu J**, **Mekalanos JJ**. 2003. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Dev Cell **5**:647–656.

70. **Stewart PS**, **William Costerton J**. 2001. Antibiotic resistance of bacteria in biofilms. Lancet **358**:135–138.

71. **Lewis K**. 2007. Persister cells, dormancy and infectious disease. Nat Rev Micro **5**:48–56.

72. **Konto-Ghiorghi Y**, **Mairey E**, **Mallet A**, **Duménil G**, **Caliot E**, **Trieu-Cuot P**, **Dramsi S**. 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. PLoS Pathog **5**:e1000422.

73. **Benitz WE**, **Gould JB**, **Druzin ML**. 1999. Risk factors for early-onset Group B Streptococcal sepsis: estimation of odds ratios by critical literature review. Pediatrics **103**:e77–e77.

74. **Schloter M**, **Lebuhn M**, **Heulin T**, **Hartmann A**. 2000. Ecology and evolution of bacterial microdiversity. FEMS Microbiol Rev **24**:647–660.

75. **Woese CR**. 1987. Bacterial evolution. Microbiol Rev **51**:221–271.

76. **Cheetham BF**, **Katz ME**. 1995. A role for bacteriophages in the evolution and transfer of bacterial virulence determinants. Mol Microbiol **18**:201–208.

77. **Amábile-Cuevas CF**, **Chicurel ME**. 1993. Horizontal Gene Transfer. Am Sci **81**:332–341

78. **Levinson G**, **Gutman GA**. 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol **4** :203–221.

79. **Cox EC**. 1976. Bacterial mutator genes and the control of spontaneous mutation. Annu Rev Genet **10**:135–156.

80. **Rosenberg SM**. 2001. Evolving responsively: adaptive mutation. Nat Rev Genet **2**:504–515.

81. **Adam M**, **Murali B**, **Glenn NO**, **Potter SS**. 2008. Epigenetic inheritance based evolution of antibiotic resistance in bacteria. BMC Evol Biol **8**:52.

82. **Hallet B**. 2001. Playing Dr Jekyll and Mr Hyde: combined mechanisms of phase variation in bacteria. Curr Opin Microbiol **4**:570–581.

83. **Simon M**, **Zieg J**, **Silverman M**, **Mandel G**, **Doolittle R**. 1980. Phase variation: evolution of a controlling element. Science **209**:1370–1374.

84. **Van Der Woude MW**, **Bäumler AJ**. 2004. Phase and antigenic variation in bacteria. Clin Microbiol Rev **17**:581–611.

85. **Henderson IR**, **Owen P**, **Nataro JP**. 1999. Molecular switches—the ON and OFF of bacterial phase variation. Mol Microbiol **33**:919–932.

86. **Proctor RA**, **Von Eiff C**, **Kahl BC**, **Becker K**, **McNamara P**, **Herrmann M**, **Peters G**. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol **4**:295–305.

87. **Moisan H**, **Brouillette E**, **Jacob CL**, **Langlois-Bégin P**, **Michaud S**, **Malouin F**. 2006. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J Bacteriol **188**:64–76.

88. **Proctor RA**, **van Langevelde P**, **Kristjansson M**, **Maslow JN**, **Arbeit RD**. 1995. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin Infect Dis **20**:95–102.

89. **Allegrucci M**, **Sauer K**. 2007. Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. J Bacteriol **189**:2030–8.

90. **Allegrucci M**, **Sauer K**. 2008. Formation of *Streptococcus pneumoniae* non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. J Bacteriol **190**:6330–6339.

91. **Kim JO**, **Weiser JN**. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J Infect Dis **177**:368–377.

92. **Ravins M**, **Jaffe J**, **Hanski E**, **Shetzigovski I**, **Natanson-Yaron S**, **Moses AE**. 2000. Characterization of a mouse-passaged, highly encapsulated variant of Group A *Streptococcus* in *in vitro* and *in vivo* studies. J Infect Dis **182**:1702–1711.

93. **Green MT**, **Heidger PM**, **Domingue G**. 1974. Proposed reproductive cycle for a relatively stable L-phase variant of *Streptococcus faecalis*. Infect Immun **10**:915–927.

94. **Zbinden A**, **Quiblier C**, **Hernandez D**, **Herzog K**, **Bodler P**, **Senn MM**, **Gizard Y**, **Schrenzel J**, **François P**. 2014. Characterization of *Streptococcus tigurinus* small-colony variants causing prosthetic joint infection by comparative whole-genome analyses. J Clin Microbiol **52**:467–474.

95. **Jones N**, **Bohnsack JF**, **Takahashi S**, **Oliver KA**, **Chan M-S**, **Kunst F**, **Glaser P**, **Rusniok C**, **Crook DWM**, **Harding RM**. 2003. Multilocus sequence typing system for group B *Streptococcus*. J Clin Microbiol **41**:2530–2536.

96. **Lin F-YC**, **Whiting A**, **Adderson E**, **Takahashi S**, **Dunn DM**, **Weiss R**, **Azimi PH**, **Philips JB**, **Weisman LE**, **Regan J**. 2006. Phylogenetic lineages of invasive and colonizing strains of serotype III Group B Streptococci from neonates: a multicenter prospective study. J Clin Microbiol **44**:1257–1261.

97. **Bohnsack JF**, **Whiting A**, **Gottschalk M**, **Dunn DM**, **Weiss R**, **Azimi PH**, **Philips JB**, **Weisman LE**, **Rhoads GG**, **Lin F-YC**. 2008. Population structure of invasive and colonizing strains of *Streptococcus agalactiae* from neonates of six U.S. academic centers from 1995 to 1999. J Clin Microbiol **46**:1285–1291.

98. **Bisharat N**, **Crook DW**, **Leigh J**, **Harding RM**, **Ward PN**, **Coffey TJ**, **Maiden MC**, **Peto T**, **Jones N**. 2004. Hyperinvasive neonatal Group B *Streptococcus* has arisen from a bovine ancestor. J Clin Microbiol **42**:2161–2167.

99. **Anthony KG**, **Sherburne C**, **Sherburne R**, **Frost LS**. 1994. The role of the pilus in recipient cell recognition during bacterial conjugation mediated by F-like plasmids. Mol Microbiol **13**:939–953.

100. **Koebnik R**. 2001. The role of bacterial pili in protein and DNA translocation. Trends Microbiol **9**:586–590.

101. **Crawford EM**, **Gesteland RF**. 1964. The adsorption of bacteriophage R-17. Virology **22**:165–167.

102. **Pizarro-Cerda J**, **Cossart P**. 2006. Bacterial adhesion and entry into host cells. Cell **124**:715–727.

103. **Mandlik A**, **Swierczynski A**, **Das A**, **Ton-That H**. 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol **16**:33–40.

104. **Kreikemeyer B**, **Gámez G**, **Margarit I**, **Giard J-C**, **Hammerschmidt S**, **Hartke A**, **Podbielski A**. 2011. Genomic organization, structure, regulation and pathogenic role of pilus constituents in major pathogenic Streptococci and Enterococci. Int J Med Microbiol **301**:240–251.

105. **Margarit I**, **Rinaudo CD**, **Galeotti CL**, **Maione D**, **Ghezzo C**, **Buttazzoni E**, **Rosini R**, **Runci Y**, **Mora M**, **Buccato S**, **Pagani M**, **Tresoldi E**, **Berardi A**, **Creti R**, **Baker CJ**, **Telford JL**, **Grandi G**. 2009. Preventing bacterial infections with pilus-based vaccines: The Group B *Streptococcus* paradigm. J Infect Dis **199**:108–115.

106. **Nobbs AH**, **Rosini R**, **Rinaudo CD**, **Maione D**, **Grandi G**, **Telford JL**. 2008. Sortase A

utilizes an ancillary protein anchor for efficient cell wall anchoring of pili in *Streptococcus agalactiae*. Infect Immun **76**:3550–3560.

107. **Lalioui L**, **Pellegrini E**, **Dramsi S**, **Baptista M**, **Bourgeois N**, **Doucet-Populaire F**, **Rusniok C**, **Zouine M**, **Glaser P**, **Kunst F**. 2005. The SrtA Sortase of *Streptococcus agalactiae* is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. Infect Immun **73**:3342–3350.

108. **Dramsi S**, **Caliot E**, **Bonne I**, **Guadagnini S**, **Prévost M**, **Kojadinovic M**, **Lalioui L**, **Poyart C**, **Trieu-Cuot P**. 2006. Assembly and role of pili in Group B Streptococci. Mol Microbiol **60**:1401–1413.

109. **Springman AC**, **Lacher DW**, **Waymire EA**, **Wengert SL**, **Singh P**, **Zadoks RN**, **Davies HD**, **Manning SD**. 2014. Pilus distribution among lineages of Group B *Streptococcus*: an evolutionary and clinical perspective. BMC Microbiol **14**:159.

110. **Rinaudo CD**, **Rosini R**, **Galeotti CL**, **Berti F**, **Necchi F**, **Reguzzi V**, **Ghezzo C**, **Telford JL**, **Grandi G**, **Maione D**. 2010. Specific involvement of pilus type 2a in biofilm formation in Group B *Streptococcus*. PLoS One **5**:e9216.

111. **Jiang S**, **Park SE**, **Yadav P**, **Paoletti LC**, **Wessels MR**. 2012. Regulation and function of pilus island 1 in Group B *Streptococcus*. J Bacteriol **194**:2479–2490.

112. **Dramsi S**, **Dubrac S**, **Konto-Ghiorghi Y**, **Da Cunha V**, **Couvé E**, **Glaser P**, **Caliot E**, **Débarbouillé M**, **Bellais S**, **Trieu-Cuot P**. 2012. Rga, a RofA-like regulator, is the major transcriptional activator of the PI-2a pilus in *Streptococcus agalactiae*. Microb Drug Resist **18**:286–297.

113. **Martins ER**, **Andreu A**, **Melo-Cristino J**, **Ramirez M**. 2013. Distribution of pilus islands in *Streptococcus agalactiae* that cause human infections: Insights into evolution and implication for vaccine development. Clin Vaccine Immunol **20**:313–316.

114. **Papasergi S**, **Brega S**, **Mistou M-Y**, **Firon A**, **Oxaran V**, **Dover R**, **Teti G**, **Shai Y**, **Trieu-Cuot P**, **Dramsi S**. 2011. The GBS PI-2a pilus is required for virulence in mice neonates. PLoS One **6**:e18747.

115. **Winzer K**, **Williams P**. 2001. Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int J Med Microbiol **291**:131–43.

116. **Wecke J**, **Madela K**, **Fischer W**. 1997. The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*. Microbiology **143**:2953–2960.

117. **Quach D**, **van Sorge NM**, **Kristian S a**, **Bryan JD**, **Shelver DW**, **Doran KS**. 2009. The CiaR response regulator in Group B *Streptococcus* promotes intracellular survival and resistance to innate immune defenses. J Bacteriol **191**:2023–32.

118. **Spellerberg B**, **Rozdzinski E**, **Martin S**, **Weber-Heynemann J**, **Lütticken R**. 2002. *rgf* encodes a novel two-component signal transduction system of *Streptococcus agalactiae*. Infect Immun **70**:2434–2440.

119. **Kievit TR De**, **Iglewski BH**. 2000. Bacterial quorum sensing in pathogenic relationships. Infect Immun **68**.

120. **Havarstein LS**, **Coomaraswamyt G**, **Morrisont DA**. 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc Natl Acad Sci **92**:11140–11144.

121. **Vidal JE**, **Ludewick HP**, **Kunkel RM**, **Zähner D**, **Klugman KP**. 2011. The LuxS-dependent quorum-sensing system regulates early biofilm formation by *Streptococcus pneumoniae* strain D39. Infect Immun **79**:4050–60.

122. **Stroeher UH**, **Paton AW**, **Ogunniyi AD**, **Paton JC**. 2003. Mutation of *luxS* of *Streptococcus pneumoniae* affects virulence in a mouse model. Infect Immun **71**:3206–3212.

123. **Pestova E V**, **Håvarstein LS**, **Morrison D a**. 1996. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol Microbiol **21**:853–62.

124. **Blehert DS**, **Jr RJP**, **Xavier JB**, **Almeida JS**, **Kolenbrander PE**, **Palmer RJ**. 2003. Autoinducer 2 production by *Streptococcus gordonii* DL1 and the biofilm phenotype of a *luxS* mutant Are influenced by nutritional conditions. J Bacteriol **185**:4851–4860.

125. **Pérez-Pascual D**, **Gaudu P**, **Fleuchot B**, **Besset C**, **Rosinski-Chupin I**, **Guillot A**, **Monnet V**, **Gardan R**. 2015. RovS and Its Associated Signaling Peptide Form a Cell-To-Cell Communication System Required for *Streptococcus agalactiae* pathogenesis. MBio **6**.

126. **Traber KE**, **Lee E**, **Benson S**, **Corrigan R**, **Cantera M**, **Shopsin B**, **Novick RP**. 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology **154**:2265–74.

127. **Kong K-F**, **Vuong C**, **Otto M**. 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. Int J Med Microbiol **296**:133–9.

128. **Morales WJ**, **Dickey SS**, **Bornick P**, **Lim D V**. 1999. Change in antibiotic resistance of group B *Streptococcus*: impact on intrapartum management. Am J Obstet Gynecol **181**:310–314.

129. **Stoll BJ**, **Hansen N**, **Fanaroff AA**, **Wright LL**, **Carlo WA**, **Ehrenkranz RA**, **Lemons JA**, **Donovan EF**, **Stark AR**, **Tyson JE**. 2002. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med **347**:240–247.

CHAPTER 2

ASSOCIATION BETWEEN GENOTYPIC DIVERSITY AND BIOFILM PRODUCTION IN GROUP B *STREPTOCOCCUS*

ABSTRACT

Background: Group B *Streptococcus* (GBS) is a leading cause of sepsis and meningitis and an important factor in premature and still births. Biofilm production has been suggested to be important for GBS pathogenesis alongside many other elements, including phylogenetic lineage and virulence factors, such as pili and capsule type. A complete understanding of the confluence of these components, however, is lacking. To identify associations between biofilm phenotype, pilus profile and lineage, 293 strains from asymptomatic carriers, invasive disease cases, and bovine mastitis cases, were assessed for biofilm production using an *in-vitro* assay.

Results: Multilocus sequence type (ST) profile, pilus island profile, and isolate source were associated with biofilm production. Strains from invasive disease cases and/or belonging to the ST-17 and ST-19 lineages were significantly more likely to form weak biofilms, whereas strains producing strong biofilms were recovered more frequently from individuals with asymptomatic colonization.

Conclusions: These data suggest that biofilm production is a lineage-specific trait in GBS and may promote colonization of strains representing lineages other than STs 17 and 19. The findings herein also demonstrate that biofilms must be considered in the treatment of pregnant women, particularly for women with heavy GBS colonization.

Introduction

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae*, is a leading cause of neonatal sepsis and meningitis worldwide (1, 2). Originally identified as the etiological agent in bovine mastitis, GBS is present as a commensal in the gastrointestinal and urogenital tracts in 15-30% of healthy adults (3). Human cases of invasive GBS infections were reported in the early 1900's, and GBS was identified as the primary cause of neonatal infections, with up to a 50% mortality rate and devastating long term effects for survivors (4, 5). GBS is also responsible for soft tissue infections in elderly and immunocompromised individuals (6). In most neonatal infections, the pathogen is vertically transmitted before or during birth, however, in adults there are implications of transmission due to exposure to either infected humans or other animals (7-9). Understanding the interplay of factors driving transmission of and persistent infection with GBS is essential to the development of treatments leading to the prevention of disease.

Colonization and persistence are critical for the development of GBS-mediated disease in humans. Guidelines established in 1996 in the United States recommended screening for maternal colonization during the late third trimester followed by intrapartum antibiotic prophylaxis during labor (4). The institution of these guidelines has resulted in a dramatic decrease in the incidence of neonatal early onset disease (EOD), or cases within the first week of life. Studies have shown, however, that re-colonization of the mother occurs in up to 65% of cases following antibiotic treatment, which may explain why having a previous baby with invasive GBS disease is a risk factor for neonatal disease, and no changes have been reported in the incidence of late-onset disease (LOD), or infections occurring after the first week of life (10-12). Application of multilocus sequence typing (MLST) targeting seven reference genes, for instance, has identified specific sequence types (STs) and clonal complexes (CCs) to be associated with maternal colonization as well as neonatal disease. CCs 1 and 23 have previously been linked to asymptomatic colonization, while CCs-17 and -19 were found to

predominate among neonates, though differences in CC distributions have been noted across populations (13-16). Moreover, heavy colonization of the mother was suggested to be a risk factor for neonatal infections and preterm birth (17), though few studies have linked the density of maternal GBS colonization to specific bacterial factors.

Several factors have been found to be important for GBS colonization, the first step in pathogenesis. Proteins shown to facilitate binding to host cell surface components include the laminin-binding protein (Lmb), fibrinogen binding proteins (FbsA, FbsB, and ScpB), serine-repeat rich proteins (Srr-1 and Srr-2), and pili (18-24). Similarly, the chemical composition and antigenic variation of the polysaccharide capsule has been linked to virulence while survivability in different environments and biofilm formation were also suggested to be important, particularly in the case of persistent colonization (25-29). A biofilm is defined as an aggregation of cells in a distinctly sessile state surrounded by a self-produced matrix composed of polysaccharide as well as protein and DNA (30). For some bacterial pathogens, biofilm production is an important virulence determinant that has been linked to colonization and disease progression (30, 31). Biofilms offer protection in harsh environments that can include antimicrobials, extreme pH, and immune cells, thereby promoting the maintenance of a bacterial population that can contribute to chronic infection and heavy colonization (32-35). The specific environmental conditions found within biofilms can also exert a selective pressure that can enhance pathogenicity via the rise of phenotypic and genotypic variants (35, 36). Additional information on GBS biofilms was published in a recent review by Rosini and Margarit (37). Previous studies have shown that pili play an important role in biofilm formation in GBS, and each GBS genome encodes one or two distinct pilus islands (PI), PI-1 and PI-2 (20, 38, 39). PI-1 has been observed in high frequency among human strains combined with one of two genetically distinct PI-2 variants, PI-2a and PI-2b (40, 41), yet the effect of PI sequence diversity on biofilm production has not been addressed. Because the role of biofilms in GBS-mediated disease is not known, and this phenotype has been observed to vary between strains, we sought to characterize biofilm

production across strains to identify biofilm determinants including isolation source, phylogenetic lineage, and variability in both presence and sequence of pilus loci (25, 26). Furthermore, we sought to determine whether allelic variation within genes encoding the PIs impacts biofilm formation, as well as attachment to host cells, particularly among genotypes associated with neonatal disease. Because GBS pili have been posited as potential vaccination targets due to their importance in biofilms and adherence to and invasion of host cells, further understanding of allelic diversity and pilus-associated phenotypes could guide the development of new prevention strategies (40).

Results

Source and genotype are predictive of biofilm phenotypes

Assaying biofilm production in 293 strains, including 242 human and 51 bovine strains resulted in a range of absorbance values from 0.1 to 12.3 (Figure 2.1). Using the median absorbance value of 1.8 to classify the biofilm phenotype, 148 (50.5%) strains were categorized as strong biofilm producers and 145 (49.5%) were designated weak biofilm producers. In all, there was considerable variation by source with human-derived strains having decreased levels of biofilm production relative to the bovine-derived strains. A total of 138 (57.0%) of the 242 human-derived strains formed a weak biofilm compared to only six (11.8%) of the 51 bovine-derived strains. Indeed, the bovine strains were significantly more likely to form strong biofilms relative to the human strains overall (Odds ratio (OR): 10.0; 95% confidence interval (CI): 4.09, 24.21; $p < 0.0001$). The average OD_{595} for human strains was 1.7 (range: 0.1 to 5.0), while the average OD_{595} for bovine strains was 6.3 (range: 0.6 to 12.3). Among human-derived strains, an association was also observed by source, as maternal colonizing strains were more likely to be strong biofilm producers compared to neonatal invasive strains (OR: 1.8; 95% CI: 1.02, 3.06; $p = 0.04$). A total of 45 of the 98 (45.9%) maternal colonizing strains had a strong biofilm phenotype relative to only 32.5% (n=39) of the 120 invasive strains from newborns with sepsis or meningitis.

To examine phenotypic variation by genotypes, biofilm production levels were compared between STs and CCs (Figure 2.2). Interestingly, the majority (87.3%) of the weak biofilm producers belonged to CC-17 and CC-19; 75.7% (n=53) and 79.6% (n=70) of the strains belonging to these two lineages, respectively, were classified as weak. Although both lineages were overrepresented in the analysis, it is notable that no other lineages had weak biofilm producers outnumbering the strong producers. Among all 158 CC-17 and CC-19 strains

combined, 77.8% (n=123) formed weak biofilms compared to only 15.6% of the 135 strains belonging to all other CCs (X^2 = 113.0, p < 0.0001). By contrast, the lineages that were exclusively comprised of bovine strains (e.g., CCs 61 and 67) were most frequently classified as strong biofilm producers. Only three strains among all 32 strains representing CC-61 and CC-67 had weak levels of biofilm production. It is important to note that some CCs such as CC-23 and CC-1, which mostly contained human-derived strains, also contained a subset of three and four bovine-derived strains, respectively. After excluding these bovine strains from the analysis, however, both CCs 23 and 1 were still overrepresented with strong biofilm producing strains. Similarly, although there was only one representative of each, the bovine-derived strains representing CC-17 and CC-19 formed strong biofilms unlike the remainder of the strains within these two lineages. Also, when the 51 bovine strains were compared to 75 human strains comprising the lineages previously associated with asymptomatic carriage (e.g., CCs 1, 7, 12, and 23), there was no significant difference in biofilm phenotype frequencies.

In addition, we examined associations between disease presentation and biofilm phenotype for 70 (62.5%) and 42 (37.5%) isolates recovered from neonates with EOD and LOD, respectively. These isolates represented CCs 1 (n=5), 12 (n=2), 17 (n=53), 19 (n=40), and 23 (n=8) as well as singletons (n=4). When all 112 isolates were examined together, there was no association between biofilm phenotype and disease onset; however, when stratified by CC, weak biofilm-formers belonging to CCs 17 and 19 were significantly more likely to cause EOD. Among the EOD cases, strong biofilm producers belonging to lineages other than CCs 17 and 19 were 28.7 times more likely to cause EOD (CI: 6.75, 121.69; p < 0.0001) than strains of CCs 17 and 19. A similar comparison could not be examined for LOD cases as all were caused by CC-17 strains in this study.

Biofilm production in GBS is influenced by PI occupancy and variation in PI genes

Biofilm production varied across strains with different PI profiles. Strains containing a PI-2 variant alone were significantly more likely to produce strong biofilms compared to strains with a PI-2 variant as well as PI-1 (OR: 10.4; 95% CI: 4.91, 22.00; $p < 0.0001$). Weak biofilm production was more common in strains with both PI-1 and either PI-2 variant as 54.9% of the 142 PI-1/PI-2a-positive strains and 70.3% of the 81 PI-1/2b-positive strains formed weak biofilms. Significantly more PI-1/2b strains, however, formed weak biofilms compared to the PI-1/2a strains (OR: 1.9; 95% CI: 1.09, 3.48; $p < 0.02$). Although an equal percentage of strains with exclusively PI-2a (n=22; 84.6%) or PI-2b (n=39; 88.6%) were capable of forming strong biofilms ($p = 0.63$), differences were noted in the absorbance values (Figure 2.3). Specifically, strains with PI-2a alone had a mean absorbance value of 2.6 ± 1.0 compared to strains with PI-2b alone (6.5 ± 3.2; Mann-Wilcox test, $p \leq 0.0001$). Strains with PI-2a alone also had a significantly higher mean absorbance value than strains with both PI-1 and PI-2a (1.9 ± 1.5; Mann-Wilcox test, $p \leq 0.0001$), which also was true for strains containing both PI-1 and PI-2b (1.5 ± 0.6) relative to those with PI-2b alone (Mann-Wilcox test, $p \leq 0.0001$). Because all strains with PI-2b alone were recovered from bovines, a comparison could not be made between PI-2b- and PI-2a-positive strains from humans.

To examine the association between genetic variation in PI genes and biofilm formation, we stratified biofilm strength by alleles detected in *gbs59*, the ancillary pilus protein in PI-2a, and *san1519*, the PI-2b adhesin. Variable biofilm production levels were observed among strains with different alleles of both genes. For *gbs59*, six alleles were identified and five of these six alleles (alleles 2-6) were significantly more common in strong biofilm producers (OR: 20.6; 95% CI: 9.27, 45.62; $p \leq 0.0001$). Only the *gbs59* allele 1, which predominated in 89 of the 168 PI-2a-positive strains, was more frequently detected in strains that formed weak biofilms (Table 1). The majority (n=87; 97.8%) of strains containing *gbs59* allele 1 belonged to CC-19, and more of

these strains were recovered from neonates (n=45; 52%) than pregnant women (n=39; 44.8%); one strain originated from a bovine. Indeed, the neonatal CC-19 strains containing the *gbs59* allele 1 were significantly less likely to form strong biofilms relative to the maternal CC-19 strains with other alleles ($p \leq 0.02$). Similar findings were observed for PI-2b, which were predominantly represented by CC-17. Strains with *san1519* allele 2 were significantly more likely to form weak biofilms relative to strains with *san1519* alleles 1 and 3 (OR: 17.3; 95% CI: 6.99, 42.81; $p \leq 0.0001$). No difference in frequency was observed for CC-17 strains with *san1519* allele 2 between neonates and pregnant women (Fisher's exact $p \leq 1.0$). Among the 32 bovine strains with *san1519* allele 3 from the bovine specific lineages, CCs 61 and 67, most (n=29; 90.6%) formed strong biofilms.

Multivariate analysis identifies source and genotype as important predictors of enhanced biofilm production

To further identify predictive features of strong biofilm production in GBS, we conducted a multivariate analysis including the following variables: pilus profile, source, and genotype. Among all GBS strains examined, only bovine source was moderately associated with strong biofilm production (OR: 3.6; 95% CI: 0.92, 13.80; $p = 0.07$). A positive association was observed for the presence of PI-2a, but it was also not statistically significant (OR: 2.7; 95% CI: 0.75, 9.90; $p = 0.13$) when adjusted for strain source and genotype. After excluding bovine strains from the analysis (Table 2), the presence of PI-2a remained associated with strong biofilm production; however, the association was still insignificant. Importantly, strains representing both CCs 17 and 19 were significantly less likely to form strong biofilms after controlling for source (invasive versus colonizing) and presence of PI-2a relative to other PI combinations. Unlike the univariate analysis, strains belonging to CCs 1 and 23 were not more likely to produce strong biofilms in the multivariate analysis.

The CC-17 lineage displays decreased association with Telomerase-immortalized Human Endothelial Cells (T-HESCs)

Variation in the ability to associate with decidualized T-HESC was observed among the 32 strains chosen to represent CCs 1, 17, and 23 with different biofilm phenotypes. The 16 CC-17 strains evaluated had T-HESC attachment levels between 0.003% and 0.199% with an average of 0.062 ± 0.048%. The eight CC-1 strains had slightly greater association levels to T-HESC (0.015% to 1.145%) than the CC-17 strains, whereas association levels for the eight CC-23 strains were highly variable (range: 0.004% to 21.68%). Interestingly, association with T-HESCs by CC-17 strains was significantly decreased when compared to both CCs 1 and 23 combined with averages of 0.057% and 0.245%, respectively (Mann-Wilcox test, p-value < 0.0005) (Figure 2.4a). After stratifying association levels by biofilm production, no difference was observed within or between lineages. When source was considered, however, CC-17 strains from invasive disease cases associated with T-HESCs at higher levels than CC-17 maternal colonizing strains, with averages of 0.077% and 0.037%, respectively. (Mann-Wilcox test, p-value <0.03) (Figure 2.4b).

Discussion

Because colonization of the host, or mother in the case of neonatal infection, is currently the primary predictor of GBS transmission and subsequent disease development (3, 42), we examined biofilm production in strains recovered from patients with invasive disease, individuals with asymptomatic colonization, and bovines with mastitis. Through this study we have demonstrated that biofilm production varies considerably across this diverse set of GBS strains. The phenotypic variation observed is in accordance with previous studies reporting absorbance values ranging from < 0.1 to >12 for GBS and other pathogens (25, 26, 43-45). The absorbance value of 1.8 was used to distinguish between weak and strong biofilm production. This value is more conservative and higher than the OD_{595} values of 0.5 and 0.65 used in two prior studies with a similar biofilm assay, but it is lower than the value of 3.0 used in another study with a modified assay (25, 26, 46). In our study, the distribution of absorbance values was not bimodal and hence, the 1.8 cut-off value was used to optimize sensitivity and specificity for the classification of weak versus strong biofilm producers. It is important to note, however, that any cut-off value carries a degree of subjectivity. In addition, differences between studies may be due to the media or type of plates used for the assays as both can alter the strength of a developing biofilm. In our study, we used THB plus 1% glucose and tissue-culture treated polystyrene plates compared to TSB or untreated polystyrene plates in other studies (26, 46). Because all strains were examined in similar conditions, our results are internally comparable, though external comparisons cannot be reliably made to data generated in other studies. It is important to note that we also assessed biofilm production in a subset of strains in other conditions including THB without glucose and in differing pHs as well as in the T-HESC infection media. The same trends were observed between isolates in these conditions, though the absorbance values were lower overall (data not shown).

Among all 293 strains examined, those strains originally recovered from bovines were capable of forming the strongest biofilms relative to the strains isolated from humans. Because there was no difference in biofilm phenotypes between strains representing the colonizing human lineages and the bovine lineages, these data suggest that strong biofilm production is more important for colonization in humans and bovines when compared to neonatal disease. It is therefore possible that unique genes or allelic variation within specific genes present in the bovine and human colonizing lineages, but lacking in invasive human strains, are important for biofilm phenotypes (47). For example, we found that the human-derived strains possessing both PI-1 and either PI-2 variant had reduced biofilm production compared to those strains without PI-1. These data suggest an inhibitory effect of PI-1, an operon encoding a specific pilus type that is absent in bovine-derived strains belonging to the bovine-specific lineages (41). Although a previous study found no effect on biofilm production in a PI-1 knockout (46), only one serotype Ia GBS strain was examined. Hence, it is possible that strains with varying genetic backgrounds, such as those belonging to CC-17 or CC-19 may behave differently when PI-1 is deleted. Similar to findings from a prior study (46), allelic variation within the genes comprising each PI may contribute to variation in the level of biofilm production observed. We found that specific alleles of *gbs59* and *san1519* were linked to strong biofilm formation while *san1519* allele 2, found exclusively in CC-17 strains, was associated with weak biofilm production. Since *san1519* allele 3 was restricted to the bovine strains (41), then this may also explain the association with strong biofilms. Despite these associations, future studies should focus on assessing the role of sequence variation on pili functionality and biofilms as other factors unique to specific lineages are also likely to be important. Nonetheless, given the proposed development of pilus-based vaccines for the treatment of GBS-mediated disease, these data highlight the importance of considering sequence variation in future vaccine development efforts similar to pilus-based vaccines targeting fimbriae in *Escherichia coli* and *Salmonella enterica*.

Another possible explanation for the increased biofilm production of the bovine-derived lineages is the presence of the lactose metabolism operon, Lac.2. This operon has been detected in the majority of bovine genomes, and upregulation of genes within the operon as well as genes important for glucose metabolism, have been documented following growth in milk (47, 48). A prior study of *Streptococcus mutans* demonstrated enhanced biofilm production in the presence of lactose, a key component of milk (49). Although it is possible that the ability to metabolize lactose and form strong biofilms is important for GBS survival in the bovine mammary gland to counteract the regular flow of milk, our *in vitro* biofilm assays were conducted without the addition of lactose. These data therefore suggest that other genes or gene combinations are more important for biofilm production *in vitro*. A prior study conducted by Ebrahimi *et al.* (50) also showed biofilm production to be a common feature of bovine strains *in vitro.* Consequently, it is clear that future studies should focus on mutagenesis of genes unique to bovine strains to determine their impact on biofilm formation and disease development *in vivo*.

In contrast to the consistently strong levels of biofilm production observed for the bovine strains, biofilm levels were highly variable between human-derived strains. Despite this variation, we found that strains of CC 17 and 19, the two CCs most commonly associated with invasive neonatal disease (14, 15), had significantly decreased levels of biofilm production compared to other CCs even after adjusting for PI profile and source. Likewise, we identified increased biofilm production in all but one of the bovine-derived strains representing lineages that were previously associated with asymptomatic carriage in humans (14). Although the latter associations were less clear in the multivariate model, these findings indicate a correlation between weak biofilm production and increased pathogenicity. Furthermore, strains from neonates with invasive disease were more likely to form weak biofilms compared to colonizing strains recovered from pregnant women, which is a similar trend as was reported previously (26). When disease onset was taken into account, weak biofilm production was associated with

EOD exclusively for strains belonging to CCs 17 and 19, while neonates with EOD caused by strains belonging to lineages other than CC-17 and CC-19 were significantly more likely to produce strong biofilms. These data suggest different roles for biofilm formation in colonization and disease among the lineages. Because maternal transmission is frequently implicated in cases of EOD, strong biofilm production in the less virulent lineages may result in the transmission of greater bacterial densities. More studies, however, are needed to assess the role that biofilms play in both EOD and transmission. The association between weak biofilm production and invasive disease across all isolates is in accordance with results in *Streptococcus pneumoniae*, which demonstrated decreased pathogenicity in biofilm-associated cells explained by an altered transcriptome favoring colonization over invasiveness (51). While our assay assessed optimal biofilm production *in vitro*, the plasticity of this trait due to environmental conditions encountered during colonization and pathogenesis was not explored. It remains possible that the biofilm production reported here may not reflect the ability of specific isolates to form biofilms in every environment. The elucidation of conditions that may trigger attachment and biofilm production is critical to determine the role of GBS biofilms in disease and colonization.

Decreased biofilm production within CC-17 and -19 strains is interesting given the protection conferred to biofilm-associated bacteria and the prior finding that these two lineages persisted better in women despite antibiotic treatment (11). It is therefore possible that weak biofilm producers belonging to CCs 17 and 19 utilize different environmental cues to induce biofilm formation, or have distinct persistence strategies that do not rely on biofilms. In support of the former, a prior study found that exposure to acidic pH was an important factor for biofilm production by a subset of isolates belonging to ST-17 (25). Because we observed no difference in T-HESC association levels between strong and weak biofilm producers overall, it is likely that biofilms are less important for host cell association. Biofilms take longer to form and cannot be reliably examined in these tissue culture assays, yet we expect that strong biofilm producers will

have higher attachment levels and bacterial densities over time and hence, have a colonization advantage. We also expect these densities to vary across environments, genotypes, and individual isolates as was shown in our prior study between two CC-17 strains (52). Indeed, it is possible that invasion of host cells is critical for strains that are not capable of forming strong biofilms, which may be more apparent *in vivo*. Although invasion frequencies were not calculated in this study, we expect them to be low given our prior findings (52) and importantly, differences would still be detectable using cell association frequencies that includes both the attached and invaded bacterial populations. These data are also in line with the epidemiological associations observed between CC-17 strains and invasive disease (14, 15) and our observation that invasive CC-17 strains had higher association levels compared to the colonizing CC-17 strains.

Together, these data highlight the phenotypic variability among GBS strains and support the hypothesis that strategies other than biofilm production are important for initial host cell attachment and persistence in some strains. Identifying alternative strategies requires further study, though variation in the ability to evade or survive within immune cells, invade the epithelia, and tolerate antibiotics, are all likely to be important. For those GBS strains that are capable of forming strong biofilms and also have enhanced association with host cells, it is possible that similar adherence mechanisms are used for each process. These mechanisms are likely to vary across genotypes and could be attributable to variation in different combinations of undefined or well-known surface proteins such as Lmb, Fbs, ScpB, Srr, and pili (18-24). Indeed, strains from colonizing lineages have been shown to contain greater genetic diversity than invasive lineages like CC-17, which is well represented in our data, as variability in both biofilm production and host cell attachment was higher in colonizing lineages (53-55). The observed differences in association levels between invasive and colonizing CC-17 strains, however, also suggest that variation between CC-17 strains exists. Additional studies are therefore needed to define the specific mechanisms of host cell attachment as well as biofilm

production in diverse GBS strain populations; such studies will facilitate the identification of unique therapeutic or vaccine targets. Furthermore, because biofilms confer protection from antibiotics and immune system effectors, and contribute to the development of chronic infections in multiple bacterial pathogens, these findings posit biofilm production in GBS as clinically important for colonization in lineages other than CCs 17 and 19. Despite the generally weak biofilm production observed in disease-associated lineages, GBS colonization is an important risk factor for neonatal infections as well as opportunistic infections in susceptible individuals regardless of bacterial genotype. Hence, eradicating or thwarting biofilm production should be considered in the development of novel treatment and prevention strategies for GBS-mediated diseases.

Quantifying biofilm production among 293 GBS strains from diverse sources demonstrated variation in the ability to form strong biofilms among strains belonging to different genotypes and from distinct sources. Those strains originating from bovines were capable of forming strong biofilms relative to strains from humans, though invasive versus colonizing human-derived strains belonging to CCs 17 and 19 were more likely to produce weak biofilms. Specific PI profiles and allelic variation within PI genes were also important for strong biofilm production, but no difference was observed in the ability of strong and weak biofilm producers to associate with T-HESCs. In all, these findings suggest that biofilm production is important for a subset of GBS strains and should be considered in the treatment of GBS-positive pregnant women to limit transmission to newborns.

Materials and Methods

Bacterial Strains

A total of 293 GBS strains representing 73 STs and eight CCs were characterized in this study. A complete list of the strains evaluated can be found in Supplemental Table 1. Most strains were recovered from the blood or cerebral spinal fluid of neonates (invasive strains; *n*=120) or vaginal/rectal swabs of pregnant women (colonizing strains; *n*=88). Approval to characterize the de-identified human strains was provided by the institutional review board of Michigan State University. For comparison, 51 strains from quarter milk samples previously recovered from bovines with clinical or subclinical mastitis were characterized (56) and a reference set of 35 human-derived strains of varying STs and serotypes was included to compare biofilm production across phylogenetically distinct lineages. Reference strains included genome and control strains (*n*=14) as well as ten strains each from adults with invasive disease and non-pregnant women. Except where otherwise indicated, GBS cultures were grown overnight in Todd-Hewitt (TH) broth at 37°C with 5% CO_2. Strains were previously characterized for the PI type and allelic variation within the PI-2a backbone protein gene (*gbs59*) and the PI-2b adhesin gene (*san1519*) (41).

Biofilm Assays

Overnight cultures inoculated from freezer stocks were grown in TH broth, and then diluted 1:20 in fresh TH supplemented with 1% glucose (THG). A total of 100 µl of the diluted culture was added to a 96-well plate with four technical replicates per strain. Cells were grown under static conditions at 37°C with 5% CO_2 for 20 hrs. Following incubation, unattached bacteria were removed by washing twice with PBS (200ul), and attached bacteria were stained with 100 µl crystal violet for 10 minutes. Unbound crystal violet was removed by washing three times with PBS, and bound crystal violet was solubilized with 200 ul of 95% ethanol. Biofilm

production was quantified through absorbance readings (OD_{595}) using a plate reader (Beckman Coulter, Inc.) and measurements were calculated as the sample value minus the media (blank) control. All assays were repeated at least three times with at least three technical replicates. All OD_{595} values above 1.8, the median value of all strains tested, represented strains that produce a strong biofilm. To determine this cutoff value, absorbance values were log-transformed and found to pass the D'Agostino and Pearson test of normality (57). Chi-square (χ^2) and the Fisher's Exact test for sample sizes less than five were used to examine associations with biofilm production (weak versus strong) using SAS (version 9.3); a P-value < 0.05 was considered significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to describe the univariate relationships. Multivariate analyses were conducted using logistic regression to identify predictors of strong biofilm production among the human-derived strains.

Association with Telomerase-Immortalized Human Endometrial Cells (T-HESC)

T-HESCs were decidualized as previously described (58) by growing the cells to ~50% confluence and treating with 0.5mM 8-bromo-cAMP (Sigma-Aldrich; St. Louis, MO) for three to six days. Decidualization was confirmed by examining the expression of prolactin and insulin-like growth factor (IGF) binding protein 1. Assays were not performed until the cells reached 100% confluency; no part of the bottom of the well was exposed to avoid attachment of bacteria to the plastic plates. GBS strains were selected for testing based on phylogenetic lineage (CC) and biofilm phenotype with equal representation of weak and strong biofilm producers for each CC tested**.** An equal number of strains from cases of invasive disease and asymptomatic colonization were also evaluated when possible.

Bacterial strains were grown overnight in TH broth, washed once with PBS and resuspended in T-HESC infection medium, as previously described, except infecting inoculums were taken directly from overnight growth (52). Host cells were washed three times with PBS

prior to adding GBS at a multiplicity of infection (MOI) of one bacterial cell per host cell. After a two-hour incubation at 37^{o}C with 5% CO_2, 100 µL of supernatant was removed and serial diluted to determine final bacterial growth. Wells were then washed three times with PBS to remove non-adherent bacteria. To determine the number of associated bacteria, host cells were lysed with 0.1% Triton X-100 (Sigma) for 30 min at 37^{o}C. Lysates were gently vortexed to further disrupt the host cells and liberate intracellular bacteria. After serial dilution, lysates were plated on THA, incubated overnight at 37^{o}C, and CFUs were counted. All data were expressed as a percentage (number of associated divided by the total number of bacteria) after the two hour infection period. Individual assays were run in triplicate and each strain was tested at least three times.

ACKNOWLEDGEMENTS

This study was supported in part by the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) in collaboration with the Bill and Melinda Gates Foundation (project N015615). This funding agency did not play a role in the design, analysis, and interpretation of data or in the preparation of the manuscript. Student support was provided in part by the Thomas S. Whittam and the Rudolph Hugh Graduate Fellowships at Michigan State University.

APPENDIX

APPENDIX

Table 2.1. Frequency of strong biofilm production among strains with distinct pilus island (PI) alleles and PI-1.

Pilus island allele (a)	Total number of strains	Strong biofilm (%), n	Clonal complexes represented (n)	PI-1 presence (%)
PI-2a, a1	89	21.4 (n=19)	19 (n=87), 1 (n=1) , 12 (n=1)	98.9
PI-2a, a2	16	87.5 (n=14)	23 (n=8), 1 (n=7), S (n=1)	93.8
PI-2a, a3	5	100.0 (n=5)	12 (n=3), 1 (n=1), 7 (n=1)	60.0
PI-2a, a4	6	83.3 (n=5)	S (n=5), 1 (n=1)	66.7
PI-2a, a5	21	90.5 (n=19)	23 (n=17), S (n=2), 19 (n=1), 7 (n=1)	14.3
PI-2a, a6	31	77.4 (n=24)	1 (n=17), 12 (n=9), 23 (n=4), S (n=1)	93.6
PI-2b, a1	11	72.7 (n=8)	1 (n=8), 7 (n=2), S (n=1)	100.0
PI-2b, a2	69	21.7 (n=16)	17 (n=69)	100.0
PI-2b, a3	45	86.7 (n=39)	61 (n=20), 67 (n=12), S (n=11), 1 (n=1), 17 (n=1)	2.2

S = Singleton

Table 2.2. Multivariate analysis of characteristics associated with strong biofilm production in human-derived strains.

Characteristics	Adjusted OR* (95% CI)	p-value†
Pilus Island (PI)		
Other PI combinations	1.0	--
PI-2a presence	4.0 (0.85,19.02)	0.08
Clonal complexes (CCs)		
Other CCs	1.0	--
CC-1	0.7 (0.18, 3.02)	0.66
CC-23	1.0 (0.19, 5.46)	0.99
CC-17	0.2 (0.03, 1.01)	0.05
CC-19	0.04 (0.01, 0.14)	<0.0001
Strain source		
Asymptomatic colonization	1.0	--
Invasive disease	1.2 (0.57, 2.33)	0.68

*OR, odds ratio; 95% CI, 95% confidence interval.

†Walds Chi-square test.

Figure 2.1. Source, frequency, and strength of biofilm production among 293 group B streptococcal strains by biofilm absorbance (OD_{595}). Bar height represents the percentage of isolates within each absorbance category listed on the x-axis. Strong biofilm producers were classified as having an OD_{595} of 1.8 or greater.

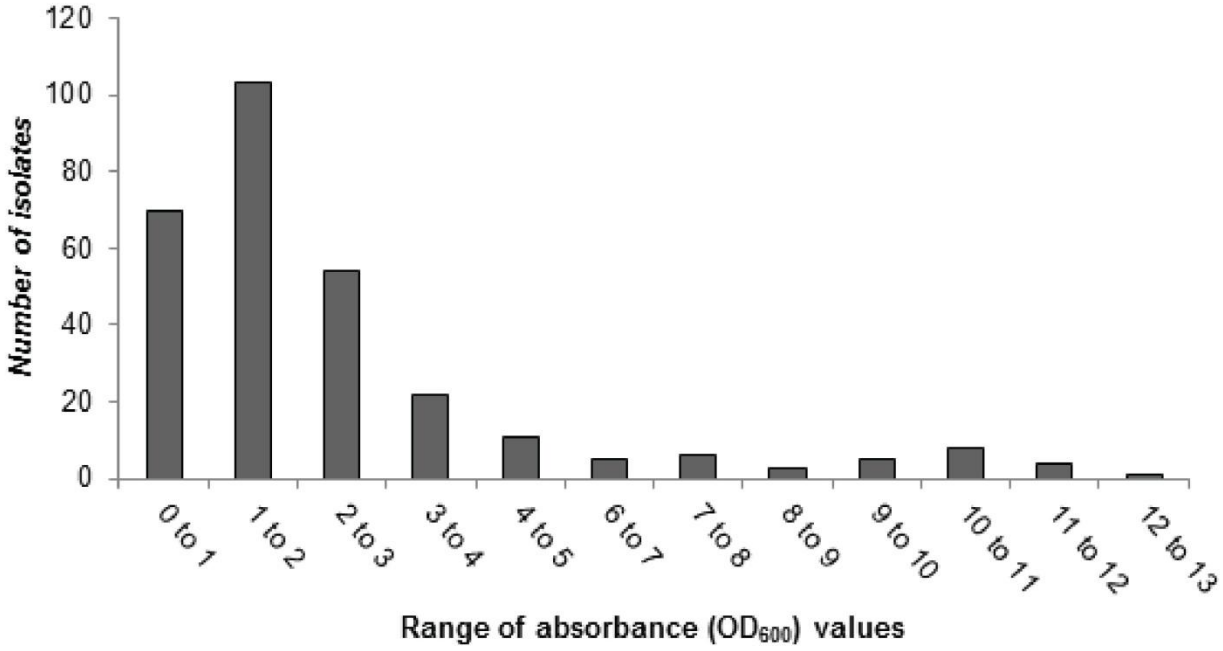


Figure 2.2. Variation in biofilm production among group B *Streptococcus* strains representing distinct phylogenetic lineages. The Neighbor-joining phylogeny grouped the 73 sequence types (STs) into eight clonal complexes (CCs), which are represented with different colors. Bovine strains are indicated in red and the frequency (%) of strains forming a strong (dark grey) versus weak (light grey) biofilm is shown within each CC as individual pie charts.

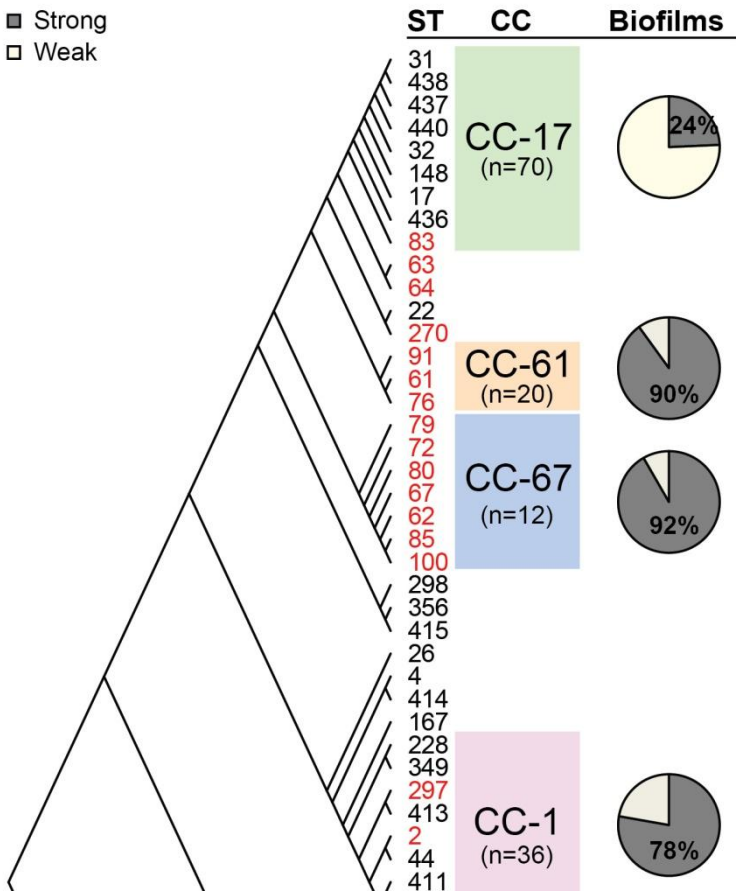


Figure 2.2. (cont'd)

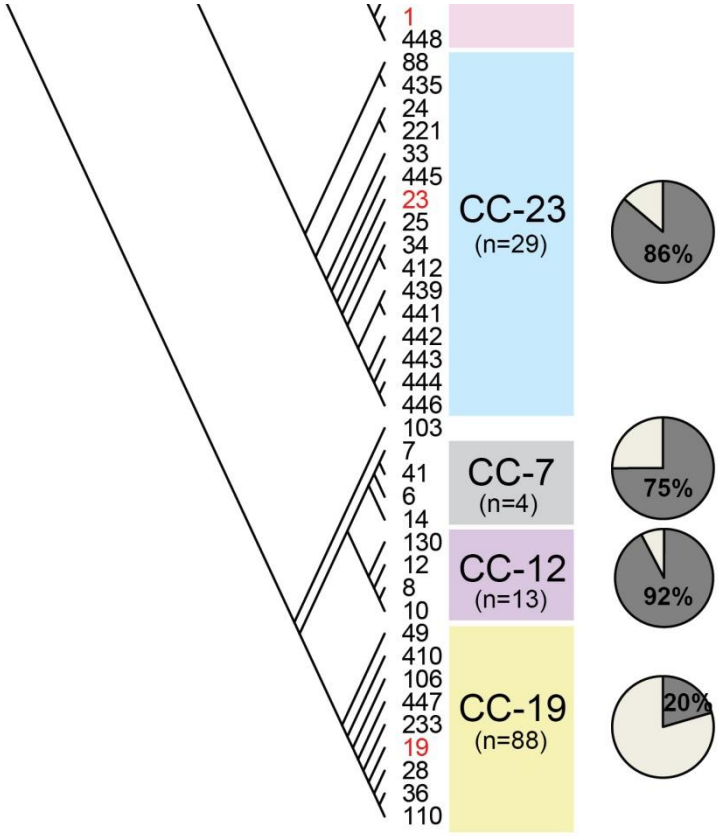


Figure 2.3. Biofilm absorbance among group B *Streptococcal* strains with different pilus island (PI) profiles. Center line of boxes is average absorbance, boxes represent the middle two quartiles, and whisker bars represent highest and lowest quartiles.

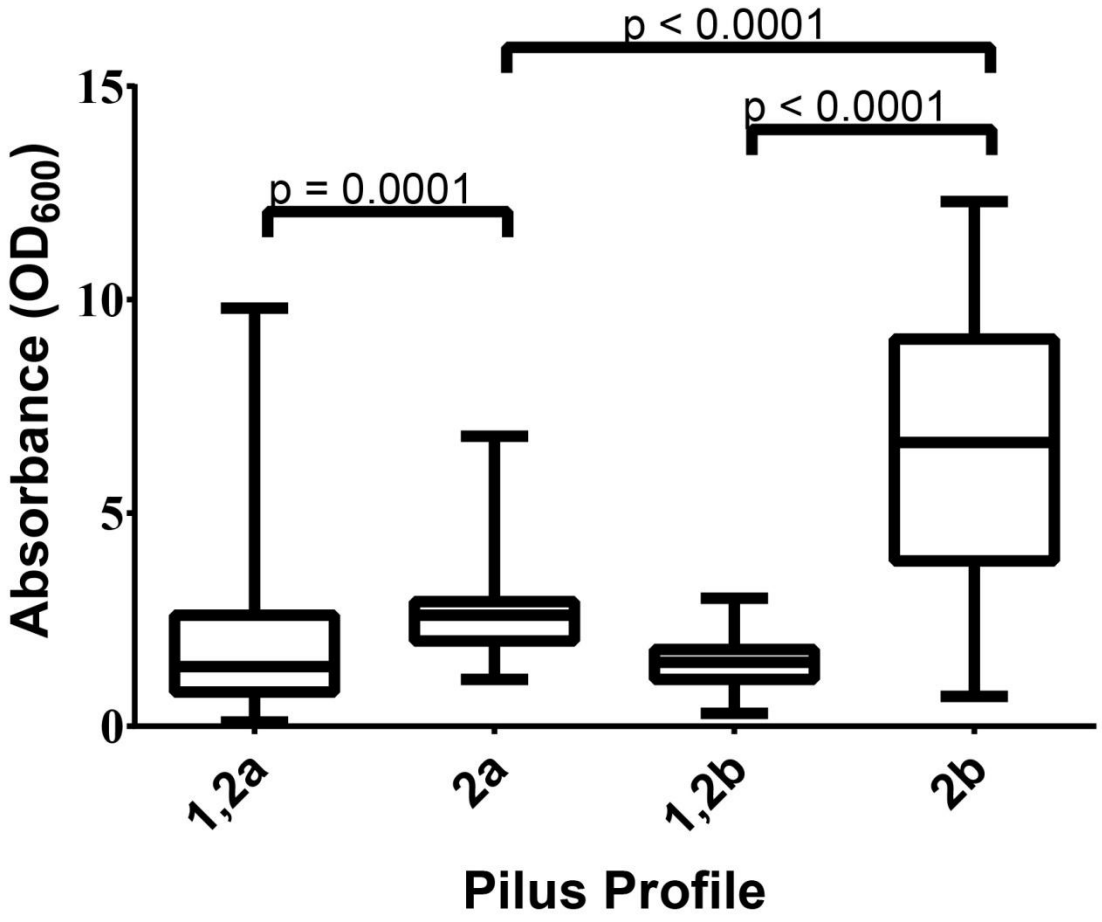
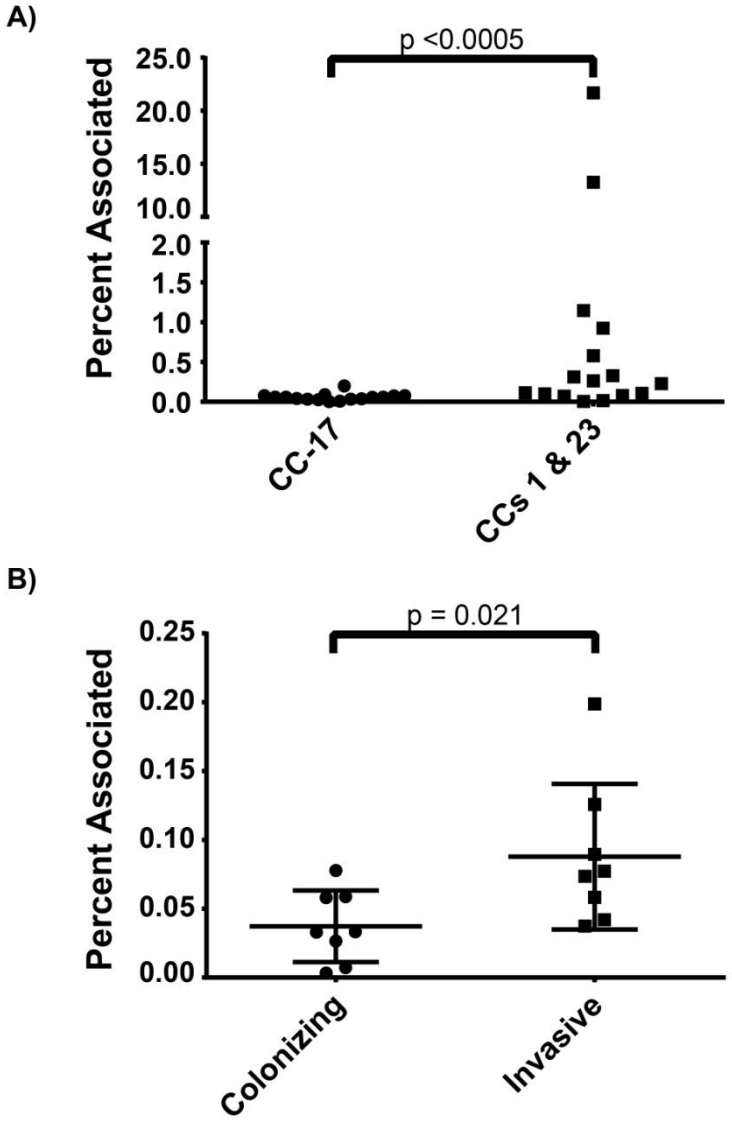


Figure 2.4. Variation in bacterial association with decidualized T-HESCs. Percent association among **A)** 32 strains representing distinct phylogenetic lineages or clonal complexes (CCs); and **B)** 16 strains belonging solely to CC-17 by source. Individual data points represent the average of three experiments with the largest horizontal lines and error bars representing the mean of data points and standard deviations, respectively.



REFERENCES

REFERENCES

1. **Furyk JS**, **Swann O**, **Molyneux E**. 2011. Systematic review: neonatal meningitis in the developing world. Trop Med Int Heal **16**:672–679.

2. **Thigpen MC**, **Whitney CG**, **Messonnier NE**, **Zell ER**, **Lynfield R**, **Hadler JL**, **Harrison LH**, **Farley MM**, **Reingold A**, **Bennett NM**. 2011. Bacterial meningitis in the United States, 1998–2007. N Engl J Med **364**:2016–2025.

3. **Hickman ME**, **Rench M a**, **Ferrieri P**, **Baker CJ**. 1999. Changing epidemiology of Group B Streptococcal colonization. Pediatrics **104**:203–9.

4. **Centers for Disease Control**, **Prevention**. 2002. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR.

5. **Phares CR**, **Lynfield R**, **Farley MM**, **Mohle-boetani J**, **Harrison LH**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Stefonek KR**, **Albanese BA**, **Zell ER**, **Schuchat A**, **Schrag SJ**. 2008. Epidemiology of invasive Group B Streptococcal disease in the United States , 1999-2005. J Am **299**:1999–2005.

6. **Tyrrell GJ**, **Senzilet LD**, **Spika JS**, **Kertesz DA**, **Alagaratnam M**, **Lovgren M**, **Talbot JA**, **Sy SHUS**. 2000. Invasive disease due to Group B Streptococcal infection in adults: Results from a Canadian, population-based, active laboratory surveillance study - 1996. J Infect Dis **182**:168–173.

7. **Manning SD**, **Neighbors K**, **Tallman P a**, **Gillespie B**, **Marrs CF**, **Borchardt SM**, **Baker CJ**, **Pearlman MD**, **Foxman B**. 2004. Prevalence of Group B *Streptococcus* colonization and potential for transmission by casual contact in healthy young men and women. Clin Infect Dis **39**:380–8.

8. **Spellerberg B**. 2000. Pathogenesis of neonatal *Streptococcus agalactiae* infections. Microbes Infect **2**:1733–1742.

9. **Manning SD**, **Springman AC**, **Million AD**, **Milton NR**, **McNamara SE**, **Somsel PA**, **Bartlett P**, **Davies HD**. 2010. Association of Group B *Streptococcus* colonization and bovine exposure: A prospective cross-sectional cohort study. PLoS One **5**:e8795.

10. **Hansen SM**, **Uldbjerg N**, **Kilian M**, **Sørensen UBS**. 2004. Dynamics of *Streptococcus agalactiae* Colonization in Women during and after Pregnancy and in Their Infants. J Clin Microbiol **42**:83–89.

11. **Manning SD**, **Lewis MA**, **Springman AC**, **Lehotzky E**, **Whittam TS**, **Davies HD**. 2008. Genotypic diversity and serotype distribution of group B *Streptococcus* isolated from women before and after delivery. Clin Infect Dis **46**:1829–37.

12. **Schrag SJ**, **Zywicki S**, **Farley MM**, **Reingold AL**, **Harrison LH**, **Lefkowitz LB**, **Hadler JL**, **Danila R**, **Cieslak PR**, **Schuchat A**. 2000. Group B Streptococcal disease in the era of intrapartum antibiotic prophylaxis. N Engl J Med **342**:15–20.

13. **Bisharat N**, **Crook DW**, **Leigh J**, **Harding RM**, **Ward PN**, **Coffey TJ**, **Maiden MC**, **Peto**

T, Jones N. 2004. Hyperinvasive neonatal Group B *Streptococcus* has arisen from a bovine ancestor. J Clin Microbiol **42**:2161–2167.

14. **Manning SD**, **Springman AC**, **Lehotzky E**, **Lewis MA**, **Whittam TS**, **Davies HD**. 2009. Multilocus sequence types associated with neonatal Group B Streptococcal sepsis and meningitis in Canada. J Clin Microbiol **47**:1143–1148.

15. **Jones N**, **Bohnsack JF**, **Takahashi S**, **Oliver KA**, **Chan M-S**, **Kunst F**, **Glaser P**, **Rusniok C**, **Crook DWM**, **Harding RM**. 2003. Multilocus sequence typing system for group B Streptococcus. J Clin Microbiol **41**:2530–2536.

16. **Bekker V**, **Bijlsma MW**, **van de Beek D**, **Kuijpers TW**, **van der Ende A**. 2016. Incidence of invasive group B streptococcal disease and pathogen genotype distribution in newborn babies in the Netherlands over 25 years: a nationwide surveillance study. Lancet Infect Dis **14**:1083–1089.

17. **Hillier SL**, **Nugent RP**, **Eschenbach DA**, **Krohn MA**, **Gibbs RS**, **Martin DH**, **Cotch MF**, **Edelman R**, **Pastorek JG**, **Rao AV**, **McNellis D**, **Regan JA**, **Carey JC**, **Klebanoff MA**. 1995. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. N Engl J Med **333**:1737–1742.

18. **Cheng Q**, **Stafslien D**, **Purushothaman SS**, **Cleary P**. 2002. The Group B Streptococcal C5a peptidase is both a specific protease and an invasin. Infect Immun **70**:2408–2413.

19. **Gutekunst H**, **Eikmanns BJ**, **Reinscheid DJ**. 2004. The novel fibrinogen-binding protein FbsB promotes *Streptococcus agalactiae* invasion into epithelial cells. Infect Immun **72**:3495–3504.

20. **Konto-Ghiorghi Y**, **Mairey E**, **Mallet A**, **Duménil G**, **Caliot E**, **Trieu-Cuot P**, **Dramsi S**. 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. PLoS Pathog **5**:e1000422.

21. **Sharma P**, **Lata H**, **Arya DK**, **Kashyap AK**, **Kumar H**, **Dua M**, **Ali A**, **Johri AK**. 2013. Role of pilus proteins in adherence and invasion of *Streptococcus agalactiae* to the lung and cervical epithelial cells. J Biol Chem **288**:4023–34.

22. **Sheen TR**, **Jimenez A**, **Wang N-Y**, **Banerjee A**, **van Sorge NM**, **Doran KS**. 2011. Serine-rich epeat proteins and pili promote *Streptococcus agalactiae* colonization of the vaginal tract. J Bacteriol **193**:6834–6842.

23. **Spellerberg B**, **Rozdzinski E**, **Martin S**, **Weber-Heynemann J**, **Schnitzler N**, **Lütticken R**, **Podbielski A**. 1999. Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. Infect Immun **67**:871–878.

24. **Tenenbaum T**, **Bloier C**, **Adam R**, **Reinscheid DJ**, **Schroten H**. 2005. Adherence to and invasion of human brain microvascular endothelial cells are promoted by fibrinogen-binding protein FbsA of *Streptococcus agalactiae*. Infect Immun **73**:4404–4409.

25. **D'Urzo N**, **Martinelli M**, **Pezzicoli A**, **De Cesare V**, **Pinto V**, **Margarit I**, **Telford JL**, **Maione D**. 2014. Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 *Streptococcus agalactiae* strains. Appl Environ Microbiol **80**:2176–2185.

26. **Kaur H**, **Kumar P**, **Ray P**, **Kaur J**, **Chakraborti A**. 2009. Biofilm formation in clinical isolates of Group B Streptococci from North India. Microb Pathog **46**:321–7.

27. **Rubens CE**, **Wessels MR**, **Heggen LM**, **Kasper DL**. 1987. Transposon mutagenesis of type III Group B *Streptococcus*: correlation of capsule expression with virulence. Proc Natl Acad Sci U S A **84**:7208–12.

28. **Tettelin H**, **Masignani V**, **Cieslewicz MJ**, **Donati C**, **Medini D**, **Ward NL**, **Angiuoli S V**, **Crabtree J**, **Jones AL**, **Durkin a S**, **Deboy RT**, **Davidsen TM**, **Mora M**, **Scarselli M**, **Margarit y Ros I**, **Peterson JD**, **Hauser CR**, **Sundaram JP**, **Nelson WC**, **Madupu R**, **Brinkac LM**, **Dodson RJ**, **Rosovitz MJ**, **Sullivan S a**, **Daugherty SC**, **Haft DH**, **Selengut J**, **Gwinn ML**, **Zhou L**, **Zafar N**, **Khouri H**, **Radune D**, **Dimitrov G**, **Watkins K**, **O'Connor KJB**, **Smith S**, **Utterback TR**, **White O**, **Rubens CE**, **Grandi G**, **Madoff LC**, **Kasper DL**, **Telford JL**, **Wessels MR**, **Rappuoli R**, **Fraser CM**. 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A **102**:13950–5.

29. **Wessels MR**, **Paoletti LC**, **Kasper DL**, **DiFabio JL**, **Michon F**, **Holme K**, **Jennings HJ**. 1990. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III Group B Streptococcus. J Clin Invest **86**:1428–33.

30. **Costerton JW**, **Stewart PS**, **Greenberg EP**. 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science (80-) **284**:1318–1322.

31. **Lewis K**. 2005. Persister cells and the riddle of biofilm survival. Biochem **70**:267–274.

32. **Li Y-H**, **Tang N**, **Aspiras MB**, **Lau PCY**, **Lee JH**, **Ellen RP**, **Cvitkovitch DG**. 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. J Bacteriol **184**:2699–2708.

33. **Vuong C**, **Voyich JM**, **Fischer ER**, **Braughton KR**, **Whitney AR**, **DeLeo FR**, **Otto M**. 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol **6**:269–275.

34. **Zhu J**, **Mekalanos JJ**. 2003. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Dev Cell **5**:647–656.

35. **Stewart PS**, **William Costerton J**. 2001. Antibiotic resistance of bacteria in biofilms. Lancet **358**:135–138.

36. **Hall-Stoodley L**, **Stoodley P**. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol **13**:7–10.

37. **Rosini R**, **Margarit I**. 2015. Biofilm formation by *Streptococcus agalactiae*: influence of environmental conditions and implicated virulence factors. Front Cell Infect Microbiol **5**:6.

38. **Lauer P**, **Rinaudo CD**, **Soriani M**, **Margarit I**, **Maione D**, **Rosini R**, **Taddei AR**, **Mora M**, **Rappuoli R**, **Grandi G**, **Telford JL**. 2005. Genome analysis reveals pili in Group B *Streptococcus*. Science **309**:105.

39. **Rosini R**, **Rinaudo CD**, **Soriani M**, **Lauer P**, **Mora M**, **Maione D**, **Taddei A**, **Santi I**,

Ghezzo C, **Brettoni C**, **Buccato S**, **Margarit I**, **Grandi G**, **Telford JL**. 2006. Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. Mol Microbiol **61**:126–141.

40. **Margarit I**, **Rinaudo CD**, **Galeotti CL**, **Maione D**, **Ghezzo C**, **Buttazzoni E**, **Rosini R**, **Runci Y**, **Mora M**, **Buccato S**, **Pagani M**, **Tresoldi E**, **Berardi A**, **Creti R**, **Baker CJ**, **Telford JL**, **Grandi G**. 2009. Preventing bacterial infections with pilus-based vaccines: The Group B *Streptococcus* paradigm. J Infect Dis **199**:108–115.
41. **Springman AC**, **Lacher DW**, **Waymire EA**, **Wengert SL**, **Singh P**, **Zadoks RN**, **Davies HD**, **Manning SD**. 2014. Pilus distribution among lineages of Group B *Streptococcus*: an evolutionary and clinical perspective. BMC Microbiol **14**:159.

42. **Ferrieri P**, **Cleary PP**, **Seeds AE**. 1977. Epidemiology of Group-B Streptococcal carriage in pregnant women and newborn infants. J Med Microbiol **10**:103–114.

43. **Lee H-W**, **Koh YM**, **Kim J**, **Lee J-C**, **Lee Y-C**, **Seol S-Y**, **Cho D-T**. 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin Microbiol Infect **14**:49–54.

44. **Mathur T**, **Singhal S**, **Khan S**, **Upadhyay D**, **Fatma T**, **Rattan A**. 2006. Detection of biofilm formation among the clinical isolates of staphylococci: An evaluation of three different screening methods. Indian J Med Microbiol **24**:25–29.

45. **Shin JH**, **Kee SJ**, **Shin MG**, **Kim SH**, **Shin DH**, **Lee SK**, **Suh SP**, **Ryang DW**. 2002. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol **40**:1244–1248.

46. **Rinaudo CD**, **Rosini R**, **Galeotti CL**, **Berti F**, **Necchi F**, **Reguzzi V**, **Ghezzo C**, **Telford JL**, **Grandi G**, **Maione D**. 2010. Specific involvement of pilus type 2a in biofilm formation in Group B *Streptococcus*. PLoS One **5**:e9216.

47. **Richards VP**, **Lang P**, **Bitar PDP**, **Lefébure T**, **Schukken YH**, **Zadoks RN**, **Stanhope MJ**. 2011. Comparative genomics and the role of lateral gene transfer in the evolution of bovine adapted *Streptococcus agalactiae*. Infect Genet Evol **11**:1263–1275.

48. **Richards VP**, **Choi SC**, **Bitar PDP**, **Gurjar AA**, **Stanhope MJ**. 2013. Transcriptomic and genomic evidence for *Streptococcus agalactiae* adaptation to the bovine environment. BMC Genomics **14**:920.

49. **Assaf D**, **Steinberg D**, **Shemesh M**. 2015. Lactose triggers biofilm formation by *Streptococcus mutans*. Int Dairy J **42**:51–57.

50. **Ebrahimi A**, **Moatamedi A**, **Lotfalian S**, **Mirshokraei P**. 2013. Biofilm formation, hemolysin production and antimicrobial susceptibilities of *Streptococcus agalactiae* isolated from the mastitis milk of dairy cows in Shahrekord district, Iran. Vet Res Forum an Int Q J **4**:269–272.

51. **Sanchez CJ**, **Kumar N**, **Lizcano A**, **Shivshankar P**, **Dunning Hotopp JC**, **Jorgensen JH**, **Tettelin H**, **Orihuela CJ**. 2011. *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. PLoS One **6**:e28738.

52. **Korir ML**, **Knupp D**, **LeMerise K**, **Boldenow E**, **Loch-Caruso R**, **Aronoff DM**, **Manning SD**. 2014. Association and virulence gene expression vary among serotype III Group B *Streptococcus* isolates following exposure to decidual and lung epithelial cells. Infect Immun **82**:4587–4595.

53. **Brochet M**, **Couvé E**, **Zouine M**, **Vallaeys T**, **Rusniok C**, **Lamy M-C**, **Buchrieser C**, **Trieu-Cuot P**, **Kunst F**, **Poyart C**. 2006. Genomic diversity and evolution within the species *Streptococcus agalactiae*. Microbes Infect **8**:1227–1243.

54. **Fluegge K**, **Wons J**, **Spellerberg B**, **Swoboda S**, **Siedler A**, **Hufnagel M**, **Berner R**. 2011. Genetic differences between invasive and noninvasive neonatal Group B Streptococcal isolates. Pediatr Infect Dis J **30**.

55. **Springman AC**, **Lacher DW**, **Wu G**, **Milton N**, **Whittam TS**, **Davies HD**, **Manning SD**. 2009. Selection, recombination, and virulence gene diversity among Group B Streptococcal genotypes. J Bacteriol **191**:5419–5427.

56. **Sukhnanand S**, **Dogan B**, **Ayodele MO**, **Zadoks RN**, **Craver MPJ**, **Dumas NB**, **Schukken YH**, **Boor KJ**, **Wiedmann M**. 2005. Molecular subtyping and characterization of bovine and human *Streptococcus agalactiae* isolates. J Clin Microbiol **43**:1177–1186.

57. **D'Agostino, R, Pearson E**. 1973. Tests for departure from normality. Empirical results for the distributions of b2 and√ b1. Biometrika **60**:613–622.

58. **Brosens J**, **Takada S**, **Acevedo CH**, **Lewis MP**, **Kirby PL**, **Symes EK**, **Krausz T**, **Purohit A**, **Gellersen B**, **White JO**. 1996. Human endometrial fibroblasts immortalized by simian virus 40 large T antigen differentiate in response to a decidualization stimulus. Endocrinology **137**:2225–2231.

CHAPTER 3

THE CONTRIBUTION OF *RGFD* TO THE REGULATION OF THE *RGF* OPERON AND HOST CELL ASSOCIATION IN GROUP B *STREPTOCOCCUS*

ABSTRACT

Background: *Streptococcus agalactiae* (GBS) is a normal inhabitant in the microbiome, present in up to 40% of healthy adults; however, this opportunistic pathogen is able to breach restrictive host barriers and persist in harsh and changing conditions during pathogenesis. This study sought to identify a role for quorum sensing, a form of cell to cell communication, in the regulation of the fibrinogen-binding (*rgf*) two-component system and the ability to bind to immobilized fibrinogen and host epithelial cells, *in vitro,* through the creation of a deletion mutant lacking the ability to produce the putative autoinducing peptide, RgfD.

Results: Sequence variation in the *rgf* operon was detected between 40 clinically-isolated strains and within the *rgfD* gene of 21 strains; A non-synonymous SNP resulting in a truncated translation was detected in the seven strains in the ST-17 lineage**.** Expression of genes within the *rgf* operon, assessed using qPCR, were significantly decreased in the mutant throughout exponential growth with the biggest difference (-3.3-fold) occurring at higher cell densities. In contrast to previous findings, expression differences between the *rgfB* and *rgfC* genes suggest non-polycistronic transcription. Attachment to fibrinogen was 1.6-fold higher and association with decidualized endometrial cells was decreased 1.3-fold. *rgfC* expression, when exposed to endometrial cells, was reduced 22-fold in the mutant providing additional evidence that this putative quorum sensing molecule is important for GBS colonization in the human host.

Conclusions: These data demonstrate a role for the putative quorum-sensing peptide, RgfD, in GBS pathogenesis through regulation of *rgfA/C*, and offer a novel target for the development of future therapeutics aimed at preventing or treating GBS colonization, vertical transmission to neonates, and disease.

Introduction

Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is a medically relevant pathogen and leading cause of meningitis and sepsis in neonates. GBS is present as a commensal in up to 40% of healthy men and women in the gastrointestinal and/or urogenital tracts but is an opportunistic pathogen presenting a danger to newborns, pregnant women, the chronically ill, and the elderly (1–5). Although there has been a reduction in neonatal disease cases in the past 30 years, GBS remains a health problem in both industrialized and developing nations, and there remain significant gaps in understanding the molecular mechanisms of pathogenesis (4).

The identification of features that drive one strain of GBS to become invasive while another remains benign is incomplete. Several studies utilizing multilocus sequence typing (MLST), a method targeting seven reference genes, have shown that most isolates belong to one of four clonal complexes (CCs): 1, 17, 19, and 23, with CC-17 identified associated with increased virulence (6, 7). This suggests the CC-17 lineage likely contains unique features which impact disease development and progression (8, 9).

While GBS is well adapted to survival in the host, crossing restrictive barriers still presents a challenge to this opportunist, and progression to disease for GBS requires complex regulation of an array of virulence factors, and, in the development of invasive disease, the crossing of restrictive tissues such as the placental and blood-brain barrier (10, 11). The ability to respond to environment cues through transcriptome remodeling allows for adaptation and survival (12). This ability to recognize extracellular stimuli and respond is controlled by signal transduction systems (STS), and the most common STS found in bacteria are two-component systems (TCS) (13). A typical TCS is composed of a membrane-bound sensor kinase, which reacts to an extracellular stimulus by phosphorylating, and thus activating, a specific response regulator which acts as a transcription factor driving downstream behavioral changes (14).

The number of TCS coded in a bacterial chromosome has been shown to positively correlate to genome size at a rate of ~2.3 TCSs per 1 MB for genomes up to 5MB (15), and GBS has a disproportionately high number of TCSs with 17-20 predicted for a 2.2MB genome (16–18). Several of these TCSs have been further studied and have demonstrated roles in pathogenesis, including the controller of virulence (CovR/S)(19), the regulator of D-Alanyl-lipotechoic acid biosynthesis (DltR/S)(20, 21), the competence and β-lactam-resistance promoting system (CiaR/C)(22), and the regulator of fibrinogen binding (RgfA/C)(23–25).

The *rgf* system promotes binding of host cell components through regulation of cell surface proteins, including fibronectin binding protein, *scpB*, and fibrinogen binding proteins, *fbsA*, and *fbsB* (23). This TCS is present in the genome of every CC-17 isolate, but is variably present in other CCs (23). Furthermore, CC-17 is the only lineage in which the combination of *rgf* and *fbsB* are found (23). Thus, *rgf* has a role in host cell attachment, but the regulation of this operon is currently unknown. Interestingly, the histidine kinase and response regulator are part of a four gene operon, *rgfBDAC*, which was identified as polycistronically transcribed (24). Along with the TCS, the transcript codes for a putative quorum sensing peptide, RgfD, and its putative processor, RgfB, but the function of these proteins has yet to be proven (23, 24). We hypothesized that the *rgfA/C* system may facilitate GBS colonization and/or pathogenesis via regulation by quorum sensing. The *rgf* operon is homologous to the accessory gene regulatory system, (*agr*) which is found in many *Staphylococci* species and is important for virulence through the regulation of secreted virulence factors and surface proteins (26). The *agr* operon is autocatalytic and transcription and AgrD signaling is sufficient for sensor kinase activationg (27). Upon phosphorylation of the response regulator, AgrA, by the sensor kinase, AgrC, an alternative promoter is activated; This promoter drives expression of the intracellular effector, a regulatory RNA called RNAIII (27). RNAIII, encoded in the reverse orientation directly upstream of the *agr* operon, is responsible for downstream transcriptome alterations (28). However, regulation of this operon is complex and includes many other identified factors, of which AgrD is

only one (26, 27, 29, 30). Regardless, it is one of the best characterized quorum-sensing circuits in Gram-positive pathogens (26, 31). The genes encoding *agrA*, and *agrC*, show 55% and 45% homology to *rgfA* and *rgfC*, respectively (24). The *agr* operon is a mutable locus, *in vivo,* and strains have been isolated from patients with mutations conferring a non-hemolytic, non-invasive phenotype (26, 32). Interestingly, analysis of the genome of NEM316, a serotype III GBS strain isolated from a fatal case of neonatal septicemia, uncovered a large deletion encompassing *rgfD* and part of *rgfC* (16). The effect of this deletion in this strain is not currently known. A further study found that deletion of *rgfA* resulted in increased virulence in a murine model, possibly through increased sialic acid production (25). However, the importance of *rgfD*, the putative auto-inducing peptide, on the regulation of the *rgf* operon has not been assessed. The work described below investigated the role of *rgfD* in biofilm production, host cell adherence, expression of the *rgf* operon across growth stages and when exposed to decidualized endothelial cells, and the whole transcriptome during log phase growth.

Results

rgf operon and *rgfD* sequence variation between strains of GBS

As a truncated variation of the *rgf* operon, in which complete *rgfD* and *rgfA* genes are absent, has been previously identified (23) , we perfomed a multiple sequence alignment of the *rgf* operon between the genomes of 40 clinical strains previously characterized for biofilm production in Chapter 2 and used these data to build a topological phylogenetic tree (Figure 3.1). The strains were representative of five STs and two singletons, including STs: 1 (n=6), 12 (n=2), 17 (n=7), 19 (n=10), and 23 (n=13), and S (n=2). There were 19 strains found to contain a complete rgf operon and 21 with the truncated allele. While ST-17 strains lacked variation in the operon and clustered together, other STs displayed high variability. Particularly, ST-19 and -23 strains were found to harbor either the complete or truncated *rgf* operon. Because allelic variation in the *agr* system has been related to biofilm production in *S. aureus* (33), we assessed the importance of *rgf* allelic variation on biofilm phenotypes. Of the 40 strains, 16 (40%) were strong biofilm producers and 24 (60%) were weak. No relationship was observed using a Chi-Squared test between biofilm phenotype and complete or truncated *rgf* operon: Among those with a complete rgf operon, 42.1% were strong and 57.9% were weak while the percentage of strong and weak biofilm producers was 38.1% and 61.9% for those with a truncated *rgf* operon, respectively.

In the *agr* system of *S. aureus*, for instance, allelic variation in the autoinducing peptide (AIP) has been shown to be important for competitive inhibition of the operon both within and between *Staphylococccus* species (34). Our comparison of *rgfD* sequences and the phylogenetic analysis of 21 strains with a complete *rgf* operon (Figure 3.2) found the ST-17s to group together, but not with other STs as a result of a non-synonymous T54A single nucleotide polymorphism (SNP). This SNP results in a truncated coding sequence due to a stop codon

after 17 amino acids. Sequence similarity in ST-17 was in agreement with earlier findings indicating less genomic variability within the ST-17 lineage relative to others (35). However, a single ST-17 isolate, GB97, did harbor a second synonymous SNP (G40T), indicating sequence variation within this ST. There was also a distinct branch including two strains representing ST-12 and ST-19 containing two unique SNPs, G21A and G102A. The G21A SNP is non-synonymous and results in an amino acid change of methionine to isoleucine, while the G102A SNP is synonymous.

rgf operon is upregulated in a density-dependent manner and is not polycistronically transcribed

Because quorum-sensing controlled systems are characterized by density-dependent expression as the concentration of extracellular inducer increases, the expression of *rgfB* was assessed over time in three separate CC-17 strains. All three strains contained complete *rgf* operons, with a similar level of upregulation at increasing cell densities for each (Figure 3.3). At OD_{595} = 0.68 ± 0.07, *rgfB* expression was upregulated by 6.1 ± 1.7 fold and as density increased to OD_{595}= 0.87 ± 0.05, *rgfB* transcription increased by 12.5 ± 7.6 fold. Each of these points were calculated relative to OD_{595} = 0.2. While transcription levels of *rgfB* and *rgfA* were similar at early- and mid-log phase, significant expression differences in early stationary phase were identified between *rgfB* and *rgfA* with relative transcription levels of 2.5 ± 1.3 and 0.5 ± 0.3, respectively, in contradiction to previous findings of polycistronic transcription (24).

As the *rgf* operon was expressed in a density-dependent manner, expression was then compared between the *ΔrgfD* mutant and WT through the expression of the sensor kinase (*rgfC*), the putative transporter *(rgfB),* and a gene (*fbsB*) known to be activated by the *rgf* operon (23). At early-log and mid-log phase growth, expression of *rgfC* was significantly decreased in the mutant; however there was no difference in *rgfB* or *fbsB* (Figure 3.4). At OD_{595}=0.4, the WT

and mutant had significantly different relative transcript quantities of 0.12 ± 0.03 and 0.05 ± 0.04, respectively (T-Test P-value <0.05). Similarly, expression at OD_{595}=0.6 in the WT was significantly higher than in the mutant with relative transcript quantities of 0.10 ± 0.03 compared to 0.03 ± 0.01 (T-test P-value <0.01). Upon entry into stationary phase at OD = ~0.8, expression again became variable, although it was consistent within biological replicates of the same experiment.

rgfD decreases fibrinogen binding and increases host cell association

Because the *rgf* operon was previously shown to affect binding of immobilized human fibrinogen in-vitro(23), the Δ*rgfD* mutant was evaluated for its ability to bind fibrinogen. Notably, *ΔrgfD* was found to bind fibrinogen 1.7 ± 0.2 fold better than the WT (0.54% ± 0.13% compared to 0.33% ± 0.14%) (Figure 3.5). Next, the mutant and wild-type were tested to ascertain whether increased attachment to fibrinogen in the mutant resulted in increased association with decidualized T-HESCs. Interestingly, the mutant was decreased 1.3-fold in association with host cells compared to the wild type with 0.23% ± 0.14% and 0.29% ± 0.21% (ratio T-test P-value <0.03) (Figure 3.6). As the association assay was performed in different conditions than the earlier expression analysis, we tested *rgfC* expression in the mutant and wild-type to confirm whether the differential regulation was the same under these conditions. Following 2 hours of exposure to decidualized T-HESCs, there was a 22.8-fold reduction in *rgfC* expression in the mutant compared to the WT with relative transcription values of 0.0019 ± 0014 and 0.043 ± 0.019 (Figure 3.7).

Effect of *rgfD* deletion on the transcriptome

To further assess the role of *rgfD* in GBS, we performed a whole transcriptome comparison between the WT and *ΔrgfD* mutant at mid-log phase using RNA sequencing. We found nine significantly upregulated and 28 significantly downregulated genes (Tables 3.1 and 3.2). As expected, *rgfA* and *rgfC* were both downregulated by 4.6-fold; however, the biggest decrease was observed in genes located directly upstream of the *rgf* operon. Gene 1751, encoding a phage transcriptional repressor was downregulated by 29.8-fold, which was the largest difference observed. Remaining genes had predicted functions of transport, signal transduction, carbohydrate and amino acid metabolism, and nucleic acid binding, as well as several which were hypothetical proteins. As with the qPCR data, no difference was observed in *rgfB* transcription levels. A conjugal transfer protein was the most highly upregulated with 2.6-fold increased expression while the remaining upregulated genes were involved in metabolism, transport, membrane proteins, or transcription/ regulation.

Discussion

Based on sequence, operon structure, and functional similarity, we hypothesized that the *rgf* system was highly similar to the *agr* system of *S. aureus* through the confirmation of *rgfD* as coding for an AIP which stimulates expression and phosphorylation of the two-component system encoded by *rgfA* and *rgfC*. Towards this end, a non-polar deletion mutant lacking *rgfD*, Δ*rgfD,* was constructed and used to identify a role in *rgf*-mediated phenotypes.

Variation has been previously identified in the *agr* loci both within the AIP and across the whole operon (36, 37), and sequence alterations within the AIP, *agrD*, alter the activation of the sensor kinase, *agrC*, offering strain-specificity to the quorum sensing message (37). We identified sequence variation in the putative AIP of ST-17 strains in, *rgfD*, in the form of 2 SNPS. Interestingly, one of these SNPs is non-synonymous and results in a stop codon and, thus, truncation of the protein. While we did not further assess the importance of this truncation, it is notable that the AgrD peptide is post-translationally modified and reduced to a single 8 amino acid peptide (38). Thus, the truncated *rgfD* coding transcript may be sufficient produce a functional AIP, though further work is required to assess this. Interestingly, given the posited function of *rgfB* in peptide processing and transport, post-translational modification is common for secreted peptides, such as bacteriocins, in lactic acid bacteria (14, 39). Indeed, the *agr* system has been implicated in the production of epidermin (40), thus, due to the number of conserved hypothetical proteins in close genomic proximity which were downregulated in our transcriptome data, there may yet be a role for the *rgf* system in the production and secretion of yet unknown bacteriocins in GBS. Additionally, it is notable that those genes located directly upstream of the *rgf* operon may code for intracellular effectors such as is activated in S. aureus by *agr* expression (27). Future efforts should focus on the characterization of these genes and the identification of promoter regions.

In accordance with a comparative genomics study in which they found significant variation in the *rgf* operon across 251 genomes (41), we discovered many strains with the deletion of *rgfD* and truncation of *rgfA* that has been reported previously in the hyper-invasive NEM 316 (16). Although this allele was present in many other CCs, there were no examples in CC-17 strains offering further confirming the truncation event to have occurred outside of this hyperinvasive genotype, or to be horizontally acquired. Although we did not evaluate the effect of this truncation on the pathogenic-potential of a specific GBS strain, future work should focus on generating a mutant with the truncated *rgf* operon in a CC-17 strain.

In our *ΔrgfD* mutant, we discovered an increase in fibrinogen-binding ability. Given the proven role for *rgfA* and *C* in fibrinogen-binding (23), this finding offers support to the notion that *rgfD* has a role in activation of this two-component system and fits with the finding in *S. aureus* that *agr* inhibits expression of fibronectin-binding proteins (42). Furthermore, reduced biofilm has been related to deletion of the *agr* operon in *S. aureus,* and we observed no difference in biofilm production in our mutant (43). We did, however, discovered a decrease in association with decidualized T-HESCs that correlated with decreased transcription of *rgfC* in the mutant. This is particularly interesting because the ability to attach to host cells has been shown to be variable in GBS (44), and it is plausible, given the large variation between strains in expression of *rgf* in high density cultures, that this operon may be at least partially responsible. It is notable that non-replicating stationary cells were used to assess fibrinogen-binding while growing log phase cells were used in association assays as the contradictory results between these assays serve to highlight the importance of metabolic state on *rgf* expression and function.

In contrast with previous findings, we discovered there is non-polycistronic transcription within the *rgf* operon with both qPCR and RNA seq with difference seen between *rgfB* and *rgfC* expression levels (24). This finding suggests that regulation is more complicated and cannot be solely attributed to *rgfD.* This is in agreement with the complicated *agr* regulation in *S. aureus*

for which there are several factors besides AgrD affecting *agr* expression (29, 30). Furthermore, the existence of multiple regulators may explain our inability to complement our mutation using an extra-chromosomal expression plasmid under control of the constitutive *rofA* promoter. As our complementation attempts focused solely on *rgfD*, future efforts may be successful using an expression plasmid with a native *rgf* promoter or expressing the whole *rgf* operon. Regardless, we were able to confirm Northern Blot results indicating density-dependent *rgf* expression(24) with qPCR of three separate CC-17 strains, although there was high variability in fold change observed at the final time point. This variabiity is likely due to entry into stationary phase, at which point the transcriptome is widely altered. Furthermore, quorum quenching is known to occur following entry into stationary phase in several bacterial species (45, 46), presumably to conserve energy, and this is consistent with the differences noted in high density rgf expression in GBS.

As disease progression involves differential expression in response to changing host environments, quorum sensing offers a potential explanation for variable pathogenic potential observed between GBS lineages. This work identified a role for the putative quorum-sensing AIP, *rgfD,* as a regulator of the sensor kinase, *rgfC,* as well as in host-cell association and fibrinogen-binding. Thus, *rgfD* remains a good candidate as coding for an AIP in GBS. Future studies should focus on the complementation of our mutation as well as assessing the importance of sequence variation, including the truncation of *rgfD* in ST-17 strains and the effect of the *rgfD* deletion and *rgfA* truncation observed in many non-CC-17 strains.

Materials and Methods

Bacterial Strain and Growth Conditions

GB00451, a serotype III, ST-17 strain isolated from the blood of a case of neonatal invasive disease was used in this study. GBS was cultured in Todd-Hewitt broth (THB) or agar (THA) or Trypticase soy agar plus 5% sheep's blood (Becton Dickinson) at 37°C with 5% CO_2. Growth curves were done in THB under the above conditions with samples taken for determination of OD_{595} at indicated times.

Sequence Analysis

41 *rgf* operon sequences were extracted from the NCBI database and analyzed by DNAStar software (DNA Star). Multiple alignments were performed using the ClustalW algorithm in the MEGAlign program (DNA Star). Phylogenetic trees were generated using MEGA6 (DNAStar).

RNA Extraction, cDNA synthesis, and Quantitative Real-Time PCR

RNA was extracted and cDNA was synthesized, and transcripts quantified as previously described (44). Briefly, for collection, samples were added to 2 volumes RNA Protect (Qiagen), pelleted and stored at -80C until extraction. RNA was then extracted using an RNEasy Kit following the “Enzymatic Lysis and Proteinase K Digestion” protocol. DNA was removed with the Turbo DNase kit (Ambion) according to manufacturer’s directions. Resulting purified RNA was quantified and 1µg was used for reverse-transcription with the iScript Reverse Transcription Kit (BioRad). iQ SYBR Supermix (Biorad) was used for Quantitative RT-PCR (qRT-PCR) in 15µL reactions with 10 µM (each) gene-specific primers. Specific products were amplified and quantified using a CFX384 Touch Real-Time PCR detection system (Bio-Rad) under the

following conditions: 1 cycle of 3 min at 95°C and 39 cycles of 95°C for 10 s and 60°C for 30 s. Relative transcript quantities were calculated using the comparative threshold cycle (C_T) method ($2^{-\Delta CT}$) (47) with *gyrA* as the internal control gene.

rgfD Mutagenesis and Complementation

Mutagenesis was performed as previously described using a double-homologous recombination strategy with the pG+host5 thermosensitive plasmid(48) for the deletion of *rgfD* as previously described (49). Flanking regions were amplified by PCR both upstream, using primers rgfD_del1 (ccgcggatccccactttactcatgggtgactt) and rgfd_del2 (*cccatccactaaacttaaaca*gcattccaaactttgtaaggagtc), and downstream, using primers rgfD_del3 (*tgtttaagtttagtggatggg*ttttattcaacaggcacgtttag) and rgfD_del4 (ggggggtaccaaaacttcttcaatccttctgct). Complementary DNA sequences in the primers are in italics and *Bam*HI and *Hind*III restrictions sites are underlined. An assembly PCR resulting in a single product was performed using equal amounts of the flanking products with the primers rgfD_del1 and rgfD_del4. Restriction digests using the resulting product and the plasmid pG+Host5 were performed followed by ligation and electroporation into Max Efficiency DH5α E. coli electrocompetent cells (ThermoFisher) with a Micropulser (Biorad). The resulting plasmid, confirmed by PCR amplification with primers PGhost 4630 and PGhost 5117 and sequencing of the resulting product, was then electroporated into GBS strain GB00451 and grown at 28°C with erythromycin (2 µg/ml) as the selection agent. Chromosomal integration of pG+host:ΔrgfD was selected for by growth on agar at 40°C in the presence of antibiotic. Excision and loss of the plasmid was stimulated by growth at 28°C without antibiotic pressure in broth for 6 generations at which point cultures were diluted and plated. Single colonies were tested for erythromycin susceptibility to ensure plasmid loss and PCR using primers rgfD_del5 (tcatactcgtcgtgctctgg) and rgfD_del6 (caactctatgtgaccttaatgacg) to identify those with successful gene deletion. The resulting mutant was identified as GB_00451ΔrgfD.

Complementation of *rgfD* was attempted with the pLZ12 plasmid with the *rofA* promoter sequence (50). *rgfD* was amplified with Plz:rgfD_F: (CGC**GGATCCA**GGAGGACAGCT*ATG*CGAAGTTTGGAATGCATGAG) and Plz:rgfD_R: (AAAA**CTGCAG**TTCTCTCTAAACGTGCCTGTTG), digested with PSTI and BamHI enzymes, and ligated into the plz12 plasmid. Restriction sites are shown in bold, ribosomal binding site is underlined, and start codon is in italics. The constructed plasmid was transformed into DH5α MAX Efficiency Chemically competent cells (Invitrogen) and transformants identified as chloramphenicol resistant. The plasmid was then electroporated as described above into GB_00451ΔrgfD competent cells, and transformants were selected for growth in Todd Hewitt agar plus chloramphenicol at 3µg/mL.

Association Assay

T-HESCs were decidualized as previously described (51). Briefly, cells were grown to approximately 50% confluence and treated with 0.5 mM 8-bromo-cyclic amp (cAMP) (Sigma) for 3 to 6 days. Decidualization was confirmed by examining the expression of prolactin and insulin-like growth factor (IGF)-binding protein 1, which are upregulated following decidualization. Assays were performed when cells reached 100% confluence. Bacterial strains were grown in THB overnight, washed once with phosphate-buffered saline (PBS), and resuspended in infection medium (HESC medium with 2% charcoal-treated FBS, no ITS+, and no antibiotics). Prior to infection, host cells were washed three times with PBS. They were then infected with GBS strains in the infection medium at a multiplicity of infection (MOI) of one bacterial cell per host cell. After 2 h of incubation at 37°C with 5% CO_2, wells were sampled, serially diluted, and plated to determine final growth. Then wells were washed three times with PBS to remove non-adherent bacteria, and host cells were lysed with 0.1% Triton X-100 (Sigma) for 30 min at 37°C. Lysates were subjected to gentle vortex mixing to further disrupt the host cells and liberate

intracellular bacteria. After serial dilution, lysates were then plated on THA and incubated overnight at 37°C, and CFU were counted. All data were expressed as percentages of the total number of bacteria per well after the 2-h infection. Assays were run in triplicate at least three times.

Fibrinogen Binding Assay

Binding to fibrinogen was assessed as previously described (52). Briefly, wells of a flat bottomed 96 well plate were incubated for 18h at 4°C with 29.4nM human fibrinogen (Sigma). Overnight cultures were washed and diluted in phosphate buffered saline. 100uL, containing $5x10^4$ to $5x10^5$ CFUs, was added to each well. Plates were incubated for 90 minutes at 37°C and 5% CO_2. Wells were then washed with PBS 3x to remove unbound cells and the serine protease mixture (Sigma) was added and plates were incubated an additional 15 minutes before samples were taken, diluted, and plated on THB agar. Percent was calculated as the number of bound CFUs divided by the inoculum CFUs. Statistical significance was determined using a two-tailed Student's T-test assuming unequal variance between averages of 3 experiments.

RNA Preparation for RNAseq

Bacterial RNA was isolated from cultures grown as described above. Samples were taken during log phase growth (OD595 = 0.4) and following 1 hour of macrophage exposure. Samples were extracted and DNA was removed as previously described (44), using RNAprotect Bacteria Reagent (Qiagen) and an RNeasy minikit (Qiagen). Briefly, samples were subjected to enzymatic and mechanical lysis and extracted following the RNeasy protocol. Following extraction, samples were treated with a Turbo DNA-free kit (Ambion) and checked for DNA contamination by PCR without prior reverse transcription (RT). For macrophage exposed samples, RNA was precipitated following Turbo DNase treatment and bacterial RNA was separated from mammalian following the standard protocol of a MICROBEnrich Kit (Ambion).

Following microbial RNA enrichment, samples were quantitated with a Qubit fluorometer (Qubit) uising standard manufacturer protocols and subjected to rRNA removal with a Ribo-Zero rRNA removal for Gram-Positive bacteria (Epicentre). Samples were again quantitated with a fluorometer and analyzed using the Agilent RNA Pico 6000 kit and Agilent bioananalyzer (Agilent) to ensure high quality and purity. Following this, 100ng was added to a 1.5mL Eppendorf tube, dried, and resuspended into 5µL RNase-free H_2O for library preparation, sequencing, and analysis. RNA library was prepared and sequenced by the MSU Research Technology Support Facility using a modified standard protocol which skipped the oligo-dT bead step with a TruSeq Stranded mRNA library sample preparation kit (Illumina). Paired-end reads were performed by an Illumina HiSeq 2500 Rapid Run flow cell (Illumina) with Rapid SBS reagents and bases were called using Illumina Real Time Analysis v1.18.64 and converted to FastQ files by Illumina Bcl2fastq v.1.8.4.

Whole Transcriptome Analysis

Trimmomatic (version 0.3) was used to remove Illumina adapters from the paired end raw sequence reads and for quality control: reads with average quality score less than 28 were dropped, and reads were clipped if average quality score in a window of 20 falls below 30. Tophat2 (version 2.0.11) was used to map the filtered reads to the GBSCOH1 bacterial reference genome as outlined in the gene feature files (.gff) from NCBI. Cufflinks (version 2.2) was used to identify differentially expressed genes from the reads mapped to reference transcriptomes, which gives the log_2 fold change in gene-expression. We then computed the p-values and the q-values (false discovery rates) for the log_2 fold change data and identified genes with FDR < 0.5 as the differentially expressed genes. P-values are calculated as the cumulative distribution function of the normally distributed z-scores for the log_2 fold expression change for each gene. Scripts for data analyses are available upon request.

ACKNOWLEDGEMENTS

This study was supported in part by the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) in collaboration with the Bill and Melinda Gates Foundation (project N015615). This funding agency did not play a role in the design, analysis, and interpretation of data or in the preparation of the manuscript. Student support was provided in part by the Thomas S. Whittam and the Rudolph Hugh Graduate Fellowships at Michigan State University. We wish to thank Dr. Rim Al Safadi for her help in mutant construction.

APPENDIX

APPENDIX

Table 3.1. Upregulated genes in the mutant relative to the WT identified in whole transcriptome comparisons. Gene ID is the gene identifier number in the COH1 reference genome and product is from genome information on Genbank (Accession# GCA_000689235.1).

Gene ID	Product	Fold Change	qval
1784	Conjugal Transfer Protein	2.60	0.01
1801	Cell Wall Surface Anchor	2.52	0.02
575	Amidase	2.22	0.10
1841	Hypothetical	2.06	0.21
34	Sugar ABC transporter	2.06	0.21
603	Endopeptidase	2.03	0.25
1955	Hypothetical - BRO family (DNA transcription or replication)	2.00	0.29
447	BioY family protein (biotin transport)	1.98	0.29
740	Transcription factor	1.90	0.40

Table 3.2. Downregulated genes in the mutant relative to the WT identified in whole transcriptome comparisons. Gene ID is the gene identifier number in the COH1 reference genome and product is from genome information on Genbank (Accession# GCA_000689235.1).

Gene ID	Product	Fold Change	q value
1751	Phage Transcriptional Repressor	-29.8	<0.01
1397	Glyoxalase Family Protein	-10.6	<0.01
1752	Transport ATP-binding Protein	-10.1	<0.01
1749	Putative Membrane Protein	-10.0	<0.01
1750	ABC transporter, ATP-binding	-8.3	<0.01
1754	Sensor Histidine Kinase (RgfA)	-4.6	<0.01
1753	Response Regulator (RgfC)	-4.6	<0.01
1694	PTS System, Nacgalactosamine-specific IIB component	-4.2	<0.01
1747	Putative Membrane Protein	-3.6	<0.01
1942	Hypothetical	-3.6	<0.01
1940	Phage Encoded Transcriptional Regulator (ArpU family)	-3.3	<0.01
1154	Hypothetical	-3.3	0.01
1656	Putative Transaldolase (Carbohydrate Metabolism)	-3.2	0.01
1726	LacB (Carbohydrate Metabolism)	-3.2	0.01
186	Putative Holin-like Protein (LrgA Family)	-3.2	0.01
1889	Hypothetical	-3.2	0.01
254	Nucleic Acid Binding	-2.7	0.07
577	Hypothetical	-2.7	0.08
564	Hypothetical	-2.6	0.08
1956	DNA Binding	-2.5	0.12
1547	Hypothetical	-2.5	0.15
87	Hypothetical	-2.5	0.15
35	Transport	-2.3	0.29
1844	Transferase	-2.3	0.29
1926	Amino Acid Metabolsim	-2.3	0.30
1944	Hypothetical	-2.3	0.33
1044	Hypothetical	-2.2	0.37
1179	Membrane Component	-2.2	0.39

Figure 3.1. Phylogenetic tree of *rgf* operon alleles and CC relationships. A multiple sequence alignment using the ClustalW algorithm found high similarity between all strains with complete operons and between strains with the truncation. The CC-17 contained identical sequences and formed their own clade. Tree shows topology only, and numbers denoting branches are strain identifiers. Bars represent CC frequency of each node and yellow color on tree identifies those strains with a complete *rgf* operon while purple highlights those with a truncation.

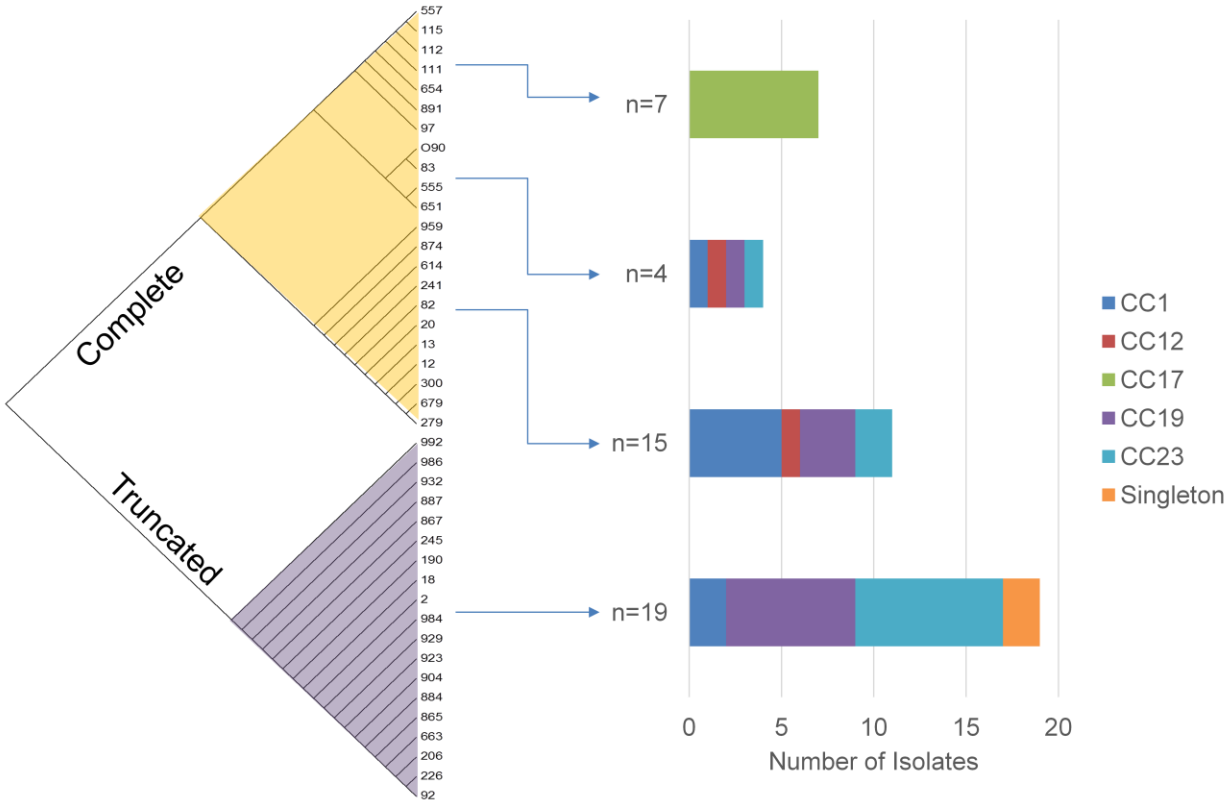


Figure 3.2. r*gfD* sequence varies between strains. An alignment of *rgfD* sequences from the 21 sequenced strains shows the ST-17s to group together, and no correlation between biofilm phenotype and *rgfD* sequence. Red and blue colors in the table represent weak and strong biofilm phenotype while numbers represent strain IDs and ST.

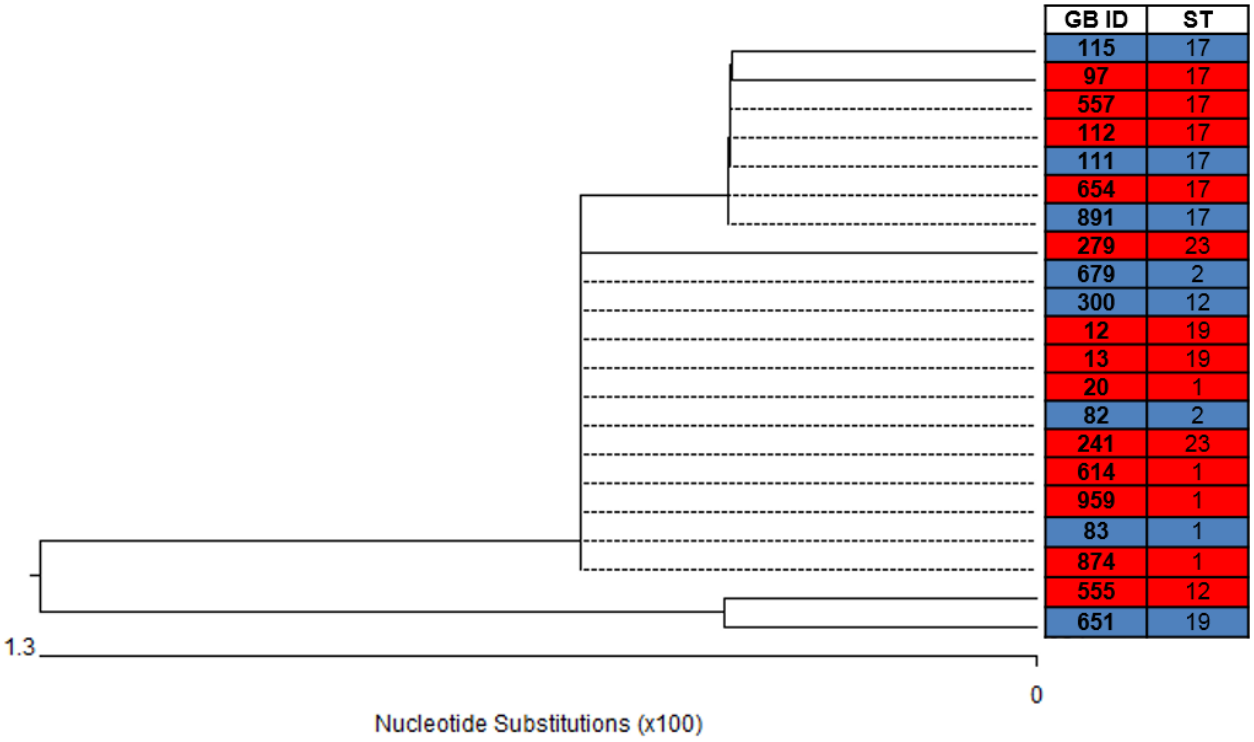


Figure 3.3. *rgf* operon is upregulated in a density-dependent manner in CC-17 strains. *rgf* expression of 3 CC-17 strains was assessed using qPCR at different culture densities. Error bars represent standard deviation between the strains at a given time point. Y-Axis is fold change as determined by ΔΔCT method relative to the earliest data point, and X-axis represents OD_{595} of the culture.

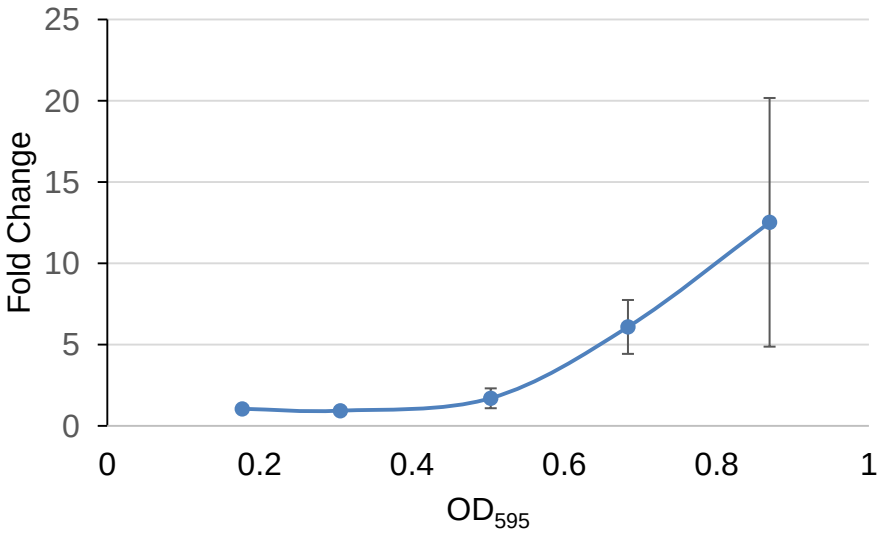


Figure 3.4. *rgfD* is necessary for full *rgfC* expression. Relative transcript quantity of *rgfB, rgfC*, and *fbsB* was compared between WT and mutant at early mid-log (OD_{595} = 0.4) and late mid-log (OD_{595} = 0.6). Blue bars represent WT and red bars represent *ΔrgfD*. Error bars are standard deviation of 4 biological replicates and single and double star represent p-value < 0.5 and < 0.1, respectively.

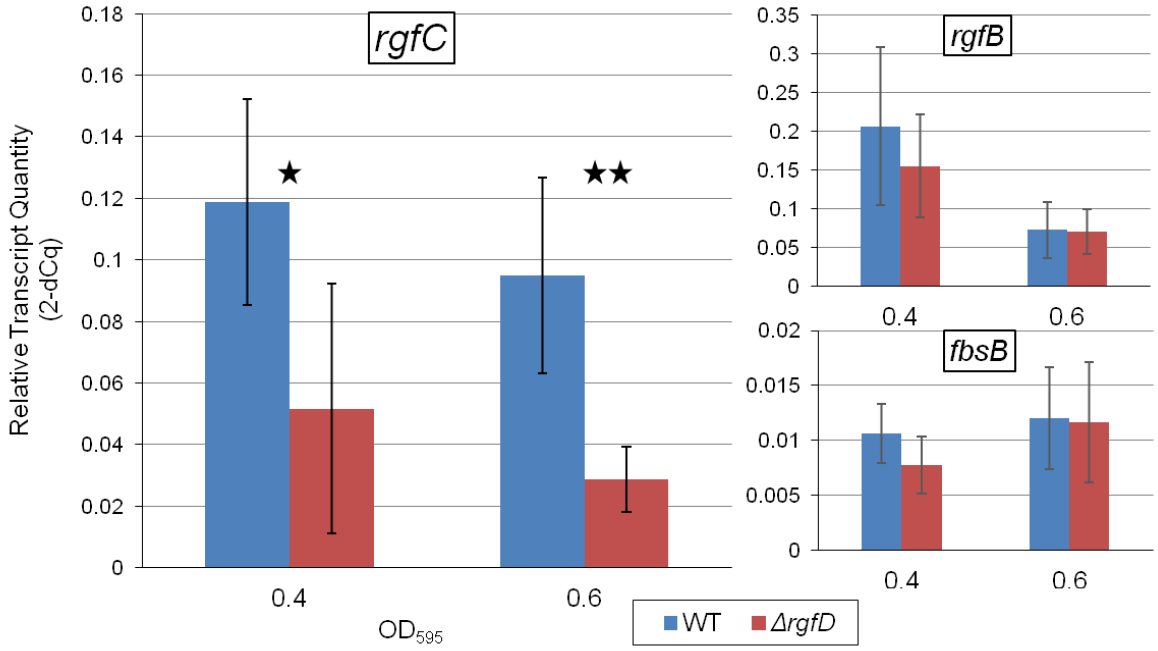


Figure 3.5. *rgfD* deletion increases fibrinogen-binding ability. Fibrinogen attachment was compared between the WT and *ΔrgfD* strains and found significantly higher attachment by the mutant. Blue and red bars represent WT and *ΔrgfD*, respectively, and error bars represent standard deviation between averages of 4 experiments. Y-axis is percent attachment as determined by CFU bound divided by the initial inoculum. Star signifies student t-test p-value <0.03.

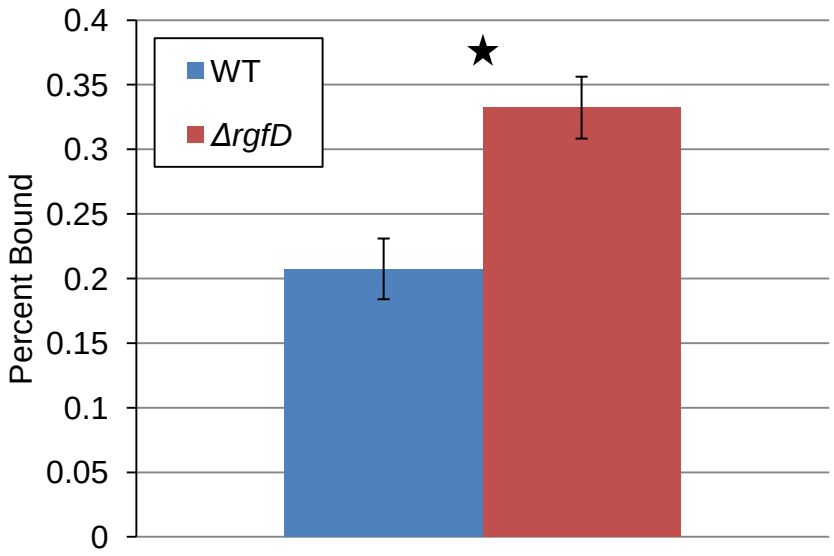


Figure 3.6. *rgfD* is necessary for optimal association with decidualized T-HESCs. The percentage of T-HESC-associated WT and *ΔrgfD* CFUs was determined with an association assay and WT showed significantly increased attachment. Bars represent average of 4 experiments, error bars represent standard deviation between 4 experiments, and star signifies significant difference (P-value < 0.03).

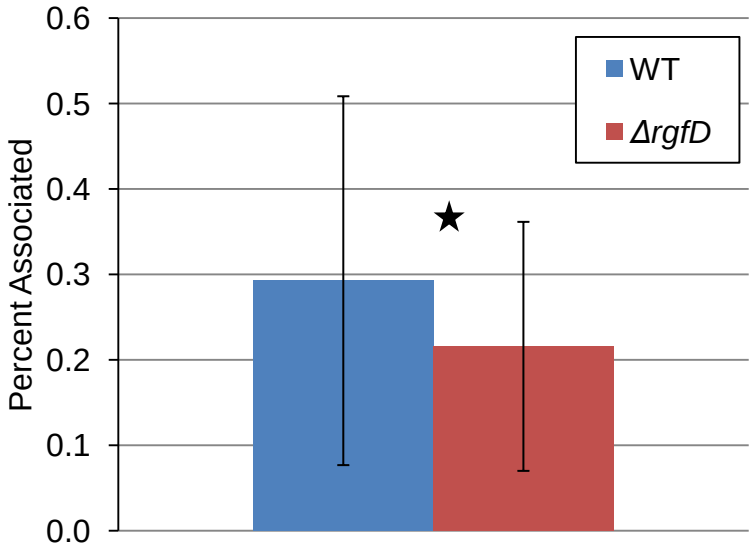
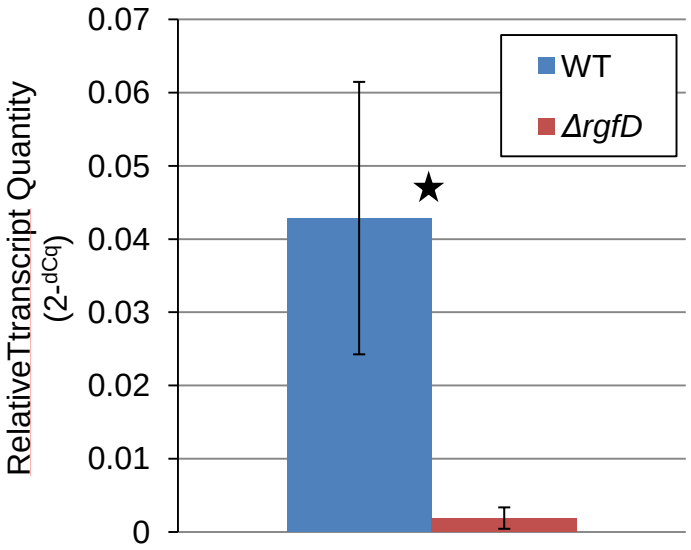


Figure 3.7. *rgfC* is upregulated by *rgfD* following exposure to decidualized T-HESCs. Expression of *rgfC* was measured by qPCR from RNA extracted at the end of the infection period of an association assay and found a 22.8-fold higher relative transcript quantity in the WT compared to *ΔrgfD*. Error bars represent standard deviation between 3 experiments and star represents significant difference with p-value < 0.02.



REFERENCES

REFERENCES

1. **Manning SD**, **Neighbors K**, **Tallman P a**, **Gillespie B**, **Marrs CF**, **Borchardt SM**, **Baker CJ**, **Pearlman MD**, **Foxman B**. 2004. Prevalence of Group B *Streptococcus* colonization and potential for transmission by casual contact in healthy young men and women. Clin Infect Dis **39**:380–8.

2. **Hickman ME**, **Rench M a**, **Ferrieri P**, **Baker CJ**. 1999. Changing epidemiology of Group B Streptococcal colonization. Pediatrics **104**:203–9.

3. **Ferrieri P**, **Cleary PP**, **Seeds AE**. 1977. Epidemiology of Group-B Streptococcal carriage in pregnant women and newborn infants. J Med Microbiol **10**:103–114.

4. **Phares CR**, **Lynfield R**, **Farley MM**, **Mohle-boetani J**, **Harrison LH**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Stefonek KR**, **Albanese BA**, **Zell ER**, **Schuchat A**, **Schrag SJ**. 2008. Epidemiology of invasive Group B Streptococcal disease in the United States , 1999-2005. J Am **299**:1999–2005.

5. **Skoff TH**, **Farley MM**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Harrison LH**, **Lynfield R**, **Mohle-Boetani J**, **Zansky S**, **Albanese B a**, **Stefonek K**, **Zell ER**, **Jackson D**, **Thompson T**, **Schrag SJ**. 2009. Increasing burden of invasive Group B Streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis **49**:85–92.

6. **Jones N**, **Bohnsack JF**, **Takahashi S**, **Oliver KA**, **Chan M-S**, **Kunst F**, **Glaser P**, **Rusniok C**, **Crook DWM**, **Harding RM**. 2003. Multilocus sequence typing system for group B *Streptococcus*. J Clin Microbiol **41**:2530–2536.

7. **Bisharat N**, **Crook DW**, **Leigh J**, **Harding RM**, **Ward PN**, **Coffey TJ**, **Maiden MC**, **Peto T**, **Jones N**. 2004. Hyperinvasive neonatal Group B *Streptococcus* has arisen from a bovine ancestor. J Clin Microbiol **42**:2161–2167.

8. **Maisey HC**, **Doran KS**, **Nizet V**. 2008. Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. Expert Rev Mol Med **10**:e27.

9. **Rajogopal L**. 2009. Understanding the regulation of Group B Streptococcal virulence factors. Future Microbiol **4**:201–221.

10. **Doran KS**, **Nizet V**. 2004. Molecular pathogenesis of neonatal Group B Streptococcal infection: no longer in its infancy. Mol Microbiol **54**:23–31.

11. **Huang S-H**, **Stins MF**, **Kim KS**. 2000. Bacterial penetration across the blood-brain barrier during the development of neonatal meningitis. Microbes Infect **2**:1237–1244.

12. **Winzer K**, **Williams P**. 2001. Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int J Med Microbiol **291**:131–43.

13. **Hoch J a**. 2000. Two-component and phosphorelay signal transduction. Curr Opin Microbiol **3**:165–70.

14. **Kleerebezem M**, **Quadri LEN**, **Kuipers OP**, **de Vos WM**. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol Microbiol **24**:895–904.

15. **Ulrich LE**, **Koonin E V**, **Zhulin IB**. 2005. One-component systems dominate signal transduction in prokaryotes. Trends Microbiol **13**:52–6.

16. **Glaser P**, **Rusniok C**, **Buchrieser C**, **Chevalier F**, **Frangeul L**, **Msadek T**, **Zouine M**, **Couvé E**, **Lalioui L**, **Poyart C**, **Trieu-Cuot P**, **Kunst F**. 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Mol Microbiol **45**:1499–513.

17. **Tettelin H**, **Masignani V**, **Cieslewicz MJ**, **Eisen J a**, **Peterson S**, **Wessels MR**, **Paulsen IT**, **Nelson KE**, **Margarit I**, **Read TD**, **Madoff LC**, **Wolf AM**, **Beanan MJ**, **Brinkac LM**, **Daugherty SC**, **DeBoy RT**, **Durkin a S**, **Kolonay JF**, **Madupu R**, **Lewis MR**, **Radune D**, **Fedorova NB**, **Scanlan D**, **Khouri H**, **Mulligan S**, **Carty H a**, **Cline RT**, **Van Aken SE**, **Gill J**, **Scarselli M**, **Mora M**, **Iacobini ET**, **Brettoni C**, **Galli G**, **Mariani M**, **Vegni F**, **Maione D**, **Rinaudo D**, **Rappuoli R**, **Telford JL**, **Kasper DL**, **Grandi G**, **Fraser CM**. 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc Natl Acad Sci U S A **99**:12391–6.

18. **Tettelin H**, **Masignani V**, **Cieslewicz MJ**, **Donati C**, **Medini D**, **Ward NL**, **Angiuoli S V**, **Crabtree J**, **Jones AL**, **Durkin a S**, **Deboy RT**, **Davidsen TM**, **Mora M**, **Scarselli M**, **Margarit y Ros I**, **Peterson JD**, **Hauser CR**, **Sundaram JP**, **Nelson WC**, **Madupu R**, **Brinkac LM**, **Dodson RJ**, **Rosovitz MJ**, **Sullivan S a**, **Daugherty SC**, **Haft DH**, **Selengut J**, **Gwinn ML**, **Zhou L**, **Zafar N**, **Khouri H**, **Radune D**, **Dimitrov G**, **Watkins K**, **O'Connor KJB**, **Smith S**, **Utterback TR**, **White O**, **Rubens CE**, **Grandi G**, **Madoff LC**, **Kasper DL**, **Telford JL**, **Wessels MR**, **Rappuoli R**, **Fraser CM**. 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A **102**:13950–5.

19. **Lamy M-C**, **Zouine M**, **Fert J**, **Vergassola M**, **Couve E**, **Pellegrini E**, **Glaser P**, **Kunst F**, **Msadek T**, **Trieu-Cuot P**, **Poyart C**. 2004. CovS/CovR of Group B *Streptococcus*: a two-component global regulatory system involved in virulence. Mol Microbiol **54**:1250–68.

20. **Wecke J**, **Madela K**, **Fischer W**. 1997. The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*. Microbiology **143**:2953–2960.

21. **Poyart C**, **Pellegrini E**, **Marceau M**, **Baptista M**, **Jaubert F**, **Lamy M-C**, **Trieu-Cuot P**. 2003. Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. Mol Microbiol **49**:1615–1625.

22. **Quach D**, **van Sorge NM**, **Kristian S a**, **Bryan JD**, **Shelver DW**, **Doran KS**. 2009. The CiaR response regulator in Group B *Streptococcus* promotes intracellular survival and resistance to innate immune defenses. J Bacteriol **191**:2023–32.

23. **Al Safadi R**, **Mereghetti L**, **Salloum M**, **Lartigue M-F**, **Virlogeux-Payant I**, **Quentin R**, **Rosenau A**. 2011. Two-component system RgfA/C activates the *fbsB* gene encoding

major fibrinogen-binding protein in highly virulent CC17 clone group B *Streptococcus*. PLoS One **6**:e14658.

24. **Spellerberg B**, **Rozdzinski E**, **Martin S**, **Weber-Heynemann J**, **Lütticken R**. 2002. *rgf* encodes a novel two-component signal transduction system of *Streptococcus agalactiae*. Infect Immun **70**:2434–2440.

25. **Gendrin C**, **Lembo A**, **Whidbey C**, **Burnside K**, **Berry J**, **Ngo L**, **Banerjee A**, **Xue L**, **Arrington J**, **Doran KS**, **Tao WA**, **Rajagopal L**. 2015. The Sensor Histidine Kinase RgfC Affects Group B Streptococcal Virulence Factor Expression Independent of Its Response Regulator RgfA. Infect Immun **83** :1078–1088.

26. **Traber KE**, **Lee E**, **Benson S**, **Corrigan R**, **Cantera M**, **Shopsin B**, **Novick RP**. 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology **154**:2265–74.

27. **Novick RP**, **Projan SJ**, **Kornblum J**, **Ross HF**, **Ji G**, **Kreiswirth B**, **Vandenesch F**, **Moghazeh S**. 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Gen Genet **248**:446–58.

28. **Morfeldt E**, **Tegmark K**, **Arvidson S**. 1996. Transcriptional control of the *agr* dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Mol Microbiol **21**:1227–1237.

29. **Roux A**, **Todd DA**, **Velázquez J V**, **Cech NB**, **Sonenshein AL**. 2014. CodY-mediated regulation of the *Staphylococcus aureus* Agr system integrates nutritional and population density signals. J Bacteriol **196**:1184–1196.

30. **Chien Y**, **Cheung AL**. 1998. Molecular Interactions between Two Global Regulators,*sar* and *agr*, in *Staphylococcus aureus*. J Biol Chem **273**:2645–2652.

31. **Kong K-F**, **Vuong C**, **Otto M**. 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. Int J Med Microbiol **296**:133–9.

32. **Wright JS**, **Traber KE**, **Corrigan R**, **Benson SA**, **Musser JM**, **Novick RP**. 2005. The *agr* radiation: An early event in the evolution of staphylococci. J Bacteriol **187**:5585–5594.

33. **Cafiso V**, **Bertuccio T**, **Santagati M**, **Demelio V**, **Spina D**, **Nicoletti G**, **Stefani S**. 2007. *agr*-Genotyping and transcriptional analysis of biofilm-producing *Staphylococcus aureus*. FEMS Immunol Med Microbiol **51**:220–7.

34. **Waters CM**, **Bassler BL**. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol **21**:319–46.

35. **Springman AC**, **Lacher DW**, **Wu G**, **Milton N**, **Whittam TS**, **Davies HD**, **Manning SD**. 2009. Selection, recombination, and virulence gene diversity among Group B Streptococcal genotypes. J Bacteriol **191**:5419–5427.

36. **Takeuchi S**, **Maeda T**, **Hashimoto N**, **Imaizumi K**, **Kaidoh T**, **Hayakawa Y**. 2001. Variation of the agr locus in *Staphylococcus aureus* isolates from cows with mastitis. Vet Microbiol **79**:267–274.

37. **Ji G**, **Beavis R**, **Novick RP**. 1997. Bacterial Interference Caused by Autoinducing

Peptide Variants. Science (80-) **276**:2027–2030.

38. **Ji G**, **Beavis RC**, **Novick RP**. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci **92**:12055–12059.

39. **Klaenhammer TR**. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev **12**:39–85.

40. **Kies S**, **Vuong C**, **Hille M**, **Peschel A**, **Meyer C**, **Götz F**, **Otto M**. 2003. Control of antimicrobial peptide synthesis by the *agr* quorum sensing system in *Staphylococcus epidermidis*: activity of the lantibiotic epidermin is regulated at the level of precursor peptide processing. Peptides **24**:329–338.

41. **Faralla C**, **Metruccio MM**, **De Chiara M**, **Mu R**, **Patras KA**, **Muzzi A**, **Grandi G**, **Margarit I**, **Doran KS**, **Janulczyk R**. 2014. Analysis of Two-Component Systems in Group B *Streptococcus* Shows That RgfAC and the Novel FspSR Modulate Virulence and Bacterial Fitness. MBio **5**.

42. **Saravia-Otten P**, **Müller HP**, **Arvidson S**. 1997. Transcription of *Staphylococcus aureus* fibronectin binding protein genes is negatively regulated by *agr* and an *agr*-independent mechanism. J Bacteriol **179**:5259–5263.

43. **Vuong C**, **Saenz HL**, **Götz F**, **Otto M**. 2000. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J Infect Dis **182**:1688–93.

44. **Korir ML**, **Knupp D**, **LeMerise K**, **Boldenow E**, **Loch-Caruso R**, **Aronoff DM**, **Manning SD**. 2014. Association and virulence gene expression vary among serotype III Group B *Streptococcus* isolates following exposure to decidual and lung epithelial cells. Infect Immun **82**:4587–4595.

45. **Surette MG**, **Bassler BL**. 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc Natl Acad Sci **95**:7046–7050.

46. **Barber CE**, **Tang JL**, **Feng JX**, **Pan MQ**, **Wilson TJG**, **Slater H**, **Dow JM**, **Williams P**, **Daniels MJ**. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. Mol Microbiol **24**:555–566.

47. **Schmittgen TD**, **Livak KJ**. 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc **3**:1101–1108.

48. **Biswas I**, **Gruss A**, **Ehrlich SD**, **Maguin E**. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol **175**:3628–3635.

49. **Schubert A**, **Zakikhany K**, **Schreiner M**, **Frank R**, **Spellerberg B**, **Eikmanns BJ**, **Reinscheid DJ**. 2002. A fibrinogen receptor from Group B *Streptococcus* interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol Microbiol **46**:557–569.

50. **Hanson BR**, **Lowe BA**, **Neely MN**. 2011. Membrane Topology and DNA-Binding Ability of the Streptococcal CpsA Protein. J Bacteriol **193** :411–420.

51. **Brosens J**, **Takada S**, **Acevedo CH**, **Lewis MP**, **Kirby PL**, **Symes EK**, **Krausz T**,

Purohit A, **Gellersen B**, **White JO**. 1996. Human endometrial fibroblasts immortalized by simian virus 40 large T antigen differentiate in response to a decidualization stimulus. Endocrinology **137**:2225–2231.

52. **Rosenau A**, **Martins K**, **Amor S**, **Gannier F**, **Lanotte P**, **van der Mee-Marquet N**, **Mereghetti L**, **Quentin R**. 2007. Evaluation of the ability of *Streptococcus agalactiae* strains isolated from genital and neonatal specimens to bind to human fibrinogen and correlation with characteristics of the *fbsA* and *fbsB* genes. Infect Immun **75**:1310–1317.

CHAPTER 4.

IDENTIFICATION OF PHENOTYPIC DIVERSIFICATION AND CHARACTERIZATION OF A STABLE IMMUNOEVASIVE SMALL COLONY VARIANT IN THE HYPERVIRULENT CC-17 LINEAGE OF GROUP B *STREPTOCOCCUS*

ABSTRACT

Background: Group B *Streptococcus* (GBS) is an important cause of neonatal sepsis and meningitis largely caused by vertical transmission at or during birth. The implementation of CDC-recommended guidelines for the prevention of neonatal disease successfully reduced rates for early-onset (EOD) but not late-onset (LOD) disease cases. Microdiversity, and the production of persister cells, offers one possible explanation for persistent colonization and transmission despite the administration of intrapartum antibiotics. Small colony variants (SCVs) have been implicated in persistence and pathogenesis for several bacterial pathogens, but the importance of phenotypic heterogeneity and SCVs has not been studied in GBS. In this study, we identified SCV formation and isolated a mutant SCV that was unable to revert to WT morphology in a GBS isolate belonging to the hyperinvasive, ST-17 lineage. We compared the ability of the mutant SCV to tolerate antibiotics, form biofilms, attach to fibrinogen and endometrial cells, and be phagocystosed by macrophages.

Results: Although revertable SCV induction was observed in both stressors, SCV frequency was significantly higher when exposed to penicillin compared to acidic pH. A locked mutant SCV was isolated and displayed ~2.5-fold increased biofilm production, two-fold increased penicillin tolerance. A seven-fold decrease in phagocytosis by macrophages and a four-fold decrease in in-vitro fibrinogen-binding relative to the wild type were also detected.

Conclusions: These data represent the first identification and characterization of SCVs in GBS and suggest microdiversity is an important contributor to pathogenesis. Furthermore, the presence of SCVs may partially explain why LOD rates have remained unchanged since disease prevention guidelines were implemented.

Introduction

Streptococcus agalactiae (GBS) is the primary etiological agent of neonatal invasive disease in the industrialized world and an emerging pathogen in adults with compromised immune systems (1–3). There are two types of GBS neonatal disease, early (EOD) and late (LOD) onset. EOD occurs during the first week of life, accounts for ~80% of neonatal infections, and is the result of vertical transmission at or directly preceding birth. LOD occurs after 1 week of life up to 3 months of age and, though vertical and maternal transmission has been suggested to be important, the delay in the development of disease is not understood (4, 5). The implementation of guidelines for the prevention of neonatal infections have resulted in a significant decline in EOD incidence, but not LOD incidence, over the past two decades in the USA (2, 6). Furthermore, the decrease in EOD levels has stabilized from 2000 to the present (7, 8). Asymptomatic colonization is common amongst healthy adults, but is also the primary risk factor for the development of both types of disease, thus reduced density or elimination of GBS colonization is the target of pre-birth intervention (9–11). Successful clearance of the infection, however, is often transient and up to 60% of women who are treated with antibiotics are subsequently re-colonized, suggesting a reservoir, either internal or environmental, is contributing to persistent colonization (12, 13). Further understanding and identification of bacterial factors influencing persistence is required to guide the development of diagnostic tests and therapeutics towards the elimination of GBS neonatal disease.

More virulent strains of GBS have been found to represent specific phylogenetic lineages. Multilocus sequence typing (MLST), targeting seven reference genes, found that two-thirds of clinical isolates fell into four major clonal complexes (CCs): 1, 17, 19, and 23 (14). Of these, CCs -17 and -19 isolates have been associated with neonatal disease (15–18). CC-17 strains have been linked to LOD as well as persistence in women following antibiotic treatment during childbirth, raising the possibility that some genotype-specific factors may contribute to both

pathogenesis and persistent colonization (12, 18). A previous study showed that the average duration of carriage of GBS to be 13.7 weeks in a healthy adult female in the absence of treatment (19). Long-term, persistent colonization through antibiotic tolerance, immune evasion, or other unknown mechanisms, may partially explain why LOD rates remain unchanged despite the implementation of preventative guidelines (6). Although the mechanism of persistence is not known, chronic carriage of GBS has been shown to increase the likelihood of transmission to neonates in both a murine model and clinical studies (20–22).

Attempts to uncover the cause of enhanced virulence of the CC-17 lineage have identified unique virulence factors and phenotypes that play a role in virulence and/or colonization including proteins, regulatory elements, and biofilm production (14, 23–27). For example, we identified an association between strong biofilm production and asymptomatic colonization in Chapter 2 of this dissertation. Environmental selection is important for variation, and harsh conditions have been demonstrated to affect phenotypic plasticity through the production of variants in clonal populations of bacteria (28–30). Phase variation, a bacterial survival strategy in which a proportion of the population exists in an altered state, has been noted for a number of pathogens (31–33), particularly for those containing highly immunogenic features such as flagella or pili. The mechanism of this variation is usually accomplished through alternative regulatory pathways that can alter the transcriptome in ways that can affect substrate attachment, growth rate, size, and immunogenicity (34). Small colony variants (SCVs) have also been described to be important for persistence and pathogenesis in a number of bacterial pathogens, including *Staphylococcus aureus* (35), *Vibrio cholera* (36)*, Pseudomonas aeruginosa* (37)*, Burkholderia cepacia* (38)*, and Neisseria gonorrhoeae* (39). SCVs in *S. aureus* were documented in the early 1900s, and this pathogen remains one of the best-studied cases of variant formation (35). In this bacterium, SCVs are present as a slow growing sub population that have been isolated from disease cases and have been implicated as essential to persistent and chronic infections (34). SCVs form characteristic small colonies, and many are

auxotrophes with a mutation often in the hemin biosynthetic, *hemB,* or the menadione biosynthetic, *menD,* genes (40, 41). *hemB* and m*enD* are essential to the formation of hemin and menaquinone synthesis, respectively, and mutations result in electron transport deficiencies (35). Interestingly, these mutants form stronger biofilms (42), display increased attachment to eukaryotic cell components(43), and persist intracellularly (44). Although variant formation has been documented within the streptococci, including *S. pneumonia* (45, 46), *S. pyogenes* (47)*, S. faecalis* (48) ,*and S tigurinus*(49), no studies have been conducted in GBS. Hence, the goal of the current study was to assess the ability of GBS to form SCVs in the presence of stressors and to identify a role for SCVs in colonization and pathogenesis.

Results

Stress-related SCV formation

Initially, SCVs were observed growing on Todd Hewitt agar (THA) plates following growth assays in which GBS was subjected to stressors that mimicked those environments typically encountered during pathogenesis. Following exposure to antibiotics and acidic pH, for instance, a greater frequency of SCVs were observed in multiple GBS strains relative to the same strains without these exposures. As SCVs have not been previously investigated in GBS, a modified Luria-Delbruck fluctuation test (50) was used to assess the role of specific stressors, namely penicillin and low pH (3.9) in the frequency of SCV development. In this assay, a single culture of GB00654, which was previously isolated from a pregnant woman, was grown to log phase before subculture to four distinct tubes. At this point, a sample was diluted and plated to quantify the abundance of SCVs in the initial inoculum before adding stressors. Following 24 hours of exposure to 1µg penicillin, the percentage of SCVs was similar between repeated measurements of the same culture (flask) and from the three separate tubes. The single repeated frequency averaged 15.0% ± 3.4% of SCV formation in the flask, while the three tube measurements averaged at 17.2% ± 6.2% (Figure 4.1). Similarly, a 24 hour exposure to acidic media revealed the same trend except with lower values of 3.7% ± 0.2% and 2.8% ± 2.1% SCVs for the flask and tubes, respectively. These data revealed no significant variation between individually cultured tubes and the larger flask; however, a difference was noted between antibiotic and acid stress. Specifically, a significantly higher frequency of SCVs was seen following exposure to peniciilin compared to low pH with averages of 0.16% ± 0.05% and 0.05% ± 0.03%, respectively (P-value = 0.0006)

Characterization of SCVs and isolation of a mutant SCV

Following exposure to penicillin, single SCV colony picks were resuspended in phosphate buffered saline and spread on new THA agar plates to assess reversion. Although most showed only WT morphology upon subculture, all subsequent colonies from one particular colony resulted in a homogenous plate of SCVs. Passage of this SCV for 10 generations did not result in reversion to WT colony morphology, thereby allowing further comparisons to be made between the stable or "locked" mutant SCV and the isogenic WT. Following overnight growth in broth, the SCV culture was dispersed throughout the tube with a smaller pellet on the bottom while WT showed limited dispersal, aggregates, and a large pellet on the bottom (Figure 4.2, panels A & B). SEM examination of cells from single colonies grown on agar revealed mutant SCV cells to be lacking extacellular debris seen on the surface of WT cells (Figure 4.2, panels C & D). Growth of the mutant SCV on a THA plate results in a small colony morphology, seen in comparison with WT colonies on a single plate in Figure 4.2, panel E.

The mutant SCV also displayed a decreased growth rate compared to WT culture (Figure 4.3), ultimately reaching stationary phase at a lower final OD_{595}, suggesting either a growth defect or a morphological difference affecting absorbance readings. When grown on TSA + 5% sheep's blood, the SCV was still capable of lysing the red blood cells via the β-hemolysin and grew to normal size, indicating some form of auxotrophy. Supplementation of THB media with hemin or thymidine, however, was insufficient to restore the WT phenotype in broth. Growth on Granada plates showed pigment production to be unaffected in SCVs. Notably, differences in the minimum inhibitory concentration (MIC) was observed as the SCVs had a MIC of 94 ng/µL for penicillin compared to 47 ng/µL for the WT. No difference in the MIC was observed between the mutant SCV and the WT for ampicillin as both displayed an MIC of 32ng/µL. Although the mutant SCV had an increased MIC for penicillin, according to CLSI

guidelines, both the mutant SCV and WT remained classified as susceptible to both penicillin and ampicillin.

Variation in biofilm production and attachment to distinct host matrices

Since biofilm formation has been associated with the generation of SCVs in other pathogens (34), we compared biofilm production *in-vitro* between the WT and SCV mutant. Interestingly, the SCV mutant had enhanced biofilm production compared to the WT with absorbance (A_{595}) values of 0.44 ± 0.05 and 0.17 ± 0.11, respectively (Figure 4.4). SEM on fully developed biofilms was also examined and found a less-developed biofilm by the SCV relative to the WT with a distinct lack of fully developed channels (Figure 4.5).

Because SCVs are often characterized as dormant persister cells (34), we hypothesized that the presence of membrane-bound adhesins may be decreased. To test this, we used an *in-vitro* fibrinogen binding assay to compare the ability of the WT and mutant SCV to bind fibrinogen. At 1.3% $\pm$ 0.6% attachment, the SCV displayed a significant 4-fold reduction in fibrinogen-binding ability compared to 5.1% $\pm$ 1.2% by the WT (Figure 4.6) (two-tailed student's t-test p-value =0.018). As both fibrinogen-binding and biofilm production have been shown to affect host-cell binding in *S. aureus* (51), we assessed the ability of the SCV to associate with decidualized T-HESCs, a pathogenically-relevant cell that makes up the uterine lining. Given the difference observed in fibrinogen-binding, we expected the SCV to show reduced ability to bind to T-HESCs. No difference in assocation, however, was observed between the WT and SCV mutant with 0.61% ± 0.20% and 0.51% ± 0.25%, respectively (Figure 4.7).

Differences in the transcriptional profiles of the mutant SCV and WT by RNA sequencing

Transcriptomic analysis of the SCV mutant and WT grown to log-phase detected differential expression in 35 genes at a q-value cutoff of 0.5 (Table 2a and 2b). Of these genes, 11 were upregulated and 24 were downregulated. Among the upregulated genes, the largest fold change was observed for two adjacent genes: GBSCOH1_1581, a gene involved in transport across the membrane, was upregulated 24.3 ± 10 fold, and GBSCOH1_1580, coding for a universal stress protein, was upregulated 11.5 ±3.8 fold. The majority of the remaining differentially expressed genes were involved in metabolism and nutrient transport, suggesting significant metabolic changes are important to the SCV phenotype. Furthermore, three hypothetical genes were identified.

Phagocytic uptake and intracellular survival of the mutant SCV

Enhanced phagocytic uptake and survival has been described for SCVs in *S. aureus*, so, as GBS is known to survive in the phagocytic vacuole, we hypothesized that SCV uptake may be increased as well. To test this hypothesis, phagocytic uptake was assessed using THP-1 derived macrophages. After 1 hour, phagocytic uptake was significantly reduced in the WT (3.23% ± 1.51%) relative to the mutant SCV (0.46% ± 0.25%) (Figure 4.8) (P-value = 0.028). Survival after 24 hours, normalized to phagocytosed cells at 1 hour, was identical between the WT and mutant SCV.

Discussion

Phenotypic heterogeneity in a bacterial population is an important mechanism of survival and persistence, and, thus, pathogenesis (34). The identification of SCVs following exposure to specific stressors and the isolation of a stable SCV mutant derived from a clinical isolate of the hyperinvasive ST-17 lineage, is novel and has yet to be described for GBS. Isolation of a stable SCV allows the study of SCVs without the complication of phenotypic plasticity as the majority of SCVs revert to WT morphology upon subculture and passage. As has been noted in *S. aureus*, there exist multiple genetic pathways to obtain the SCV phenotype (35), thus, while the data reported here is useful in determining a role for variants in colonization and pathogenesis it may not be representative of all SCVs observed in GBS. Nonetheless, we have demonstrated that the SCV phenotype could be important for GBS evasion of immune responses and persistent colonization, and must be accounted for in the detection and treatment of GBS-mediated disease.

The similar frequency of SCVs following exposure to acidic pH and penicillin indicates there are stress-induced, heritable genetic alterations resulting in phenotypic diversification. While these results are in contrast to those observed in *S. aureus*, in which SCVs are present in the initial inoculum and selected for both *in vivo* and under stressful conditions (52), they are consistent with results observed in *Burkholderia psuedomallei,* in which SCVs were only isolated following antibiotic stress (38). Also similar to our finding that antibiotic and acid stress induce SCV formation, *S. pneumoniae* variants have been shown to be selected for following exposure to exogenous stresses such as peroxide and during biofilm-associated growth (46, 53, 54). These results suggest that specific environmental cues are important for the generation of SCVs, but the possibility remains that growth characteristics and the transient nature of GBS morphological variants following sub-culture may prevent detection of SCVs present in the original inoculum. In agreement with the latter notion is the reversion of SCVs to WT

morphology on blood agar, which also suggests that SCVs are auxotrophic. Several examples of auxotrophy in SCVs have been described in other species, and this has largely been associated with electron transport deficiencies and a metabolic shift towards fermentation (34). Stable SCVs have been generated in *S. aureus* through interruption and/or deletion of genes important to the synthesis of electron transport components (41, 55); the slowed growth of our SCV mutant relative to the WT suggests electron transport may be important for variant formation in GBS. This hypothesis is further supported by our transcriptomic data that identified downregulation of a putative oxidoreductase, an enzyme often involved in electron transport. Furthermore, in accordance with other findings of increased antibiotic tolerance in SCVs of other species (34), the mutant SCV identified in this study had a higher MIC for penicillin relative to the WT. Interestingly, ampicillin MIC was unchanged for the mutant SCV the same, implicating a difference in cell membrane as ampicillin is a modified-penicillin better able to cross membrane barriers. Furthermore, the inability of *S. aureus* SCVs to use oxygen as a terminal electron acceptor results in a reduced membrane potential, which has been shown to promote increased antibiotic tolerance and even resistance to gentamicin and other aminoglycosides (56). Although we did not assess the membrane potential of the SCV mutant, this is of interest given the finding that in gentamicin-resistant strains of GBS, the addition of penicillin promote increased clearance, *in vitro* (57, 58). Whether SCV presence contributes to gentamicin-resistance has yet to be determined; however, the increased penicillin tolerance of the SCV mutant may indicate its importance in persistent GBS infections and the recolonization of women that occurs following antibiotic treatment during childbirth (12).

In contrast to *S. aureus*, neither pigment production, nor hemolytic ability, was affected in the mutant SCV (59). This is of particular interest because it suggests SCVs may be equally virulent despite delayed growth and morphological alterations. Indeed, we observed no difference in association to T-HESCs between the SCV relative to the WT. Unlike the T-HESCs, decreased fibrinogen binding, an important measurement of pathogenicity in

Streptococcus and Staphylococcus spp. (60, 61) of the SCV was noted. This suggests that either association with T-HESCs is not related to the expression of membrane-anchored fibrinogen-binding proteins, or that differences in the assays are affecting expression of these factors. These findings further differentiated GBS SCVs from *S. aureus*, in which greater adhesion to host cells and increased fibrinogen-binding is observed (61). Phagocytic uptake was drastically reduced in the mutant SCV, while survival within a macrophage was unaffected. These data are also in contrast to data generated using *S. aureus* where SCV formation was suggested to be a strategy for intracellular survival, and increased phagocytic uptake was observed (62). Furthermore and also in accordance with findings in other species (45, 63, 64), biofilm production of the SCV mutant was enhanced relative to the WT, though it is possible that different strain backgrounds will behave differently, thereby limiting generalizability. Taken together, these data suggest that SCV formation in GBS promotes persistence via associating with specific host cells, producing biofilms, and evading phagocytosis.

GBS is known to utilize a diverse set of tools to promote pathogenesis including specialized adhesins, immunomodulatory proteins, secreted proteases, and two-component systems to react to environmental stimuli. The addition of phenotypic diversification in the form of SCVs to this list results in another layer of complexity to the diagnosis and treatment of GBS mediated disease.

Materials and Methods

Bacterial Strain and Growth Conditions

GB00654, a serotype III, ST-17 strain isolated from a vaginal/rectal screen of a pregnant woman as described previously (12, 65) was the parent strain of the SCV. GBS was cultured in Todd-Hewitt broth (THB) or agar (THA) or on sheep's blood agar plates (BD) at 37°C with 5% CO_2. Supplementation with thymidine (Sigma) and hemin (Sigma) was performed using multiple concentrations, including 1, 2, and 5 µg/mL for hemin, and 1mM for thymidine. Growth curves were done in THB media under the above conditions with samples taken for determination of OD_{595} at timed intervals.

Modified Luria-Delbruck Fluctuation Test

As described previously, a modified Luria Delbruck Fluctuation assay was used to determine whether SCVs were induced or selected (50). Briefly, 100mL of fresh media was inoculated 1:50 from overnight culture and allowed to grow to OD_{595} = 0.4 (mid-log phase) at which point cultures were split into 4 - 10mL tubes and 1 - 60mL flask and selection agents, including penicillin and media adjusted to pH = 3.9 were added to specified concentrations to each vessel. Cultures were then grown for 24 hours before they were diluted and plated. SCVs were identified as those ~ 1/10 the size of WT colonies. Percentage of SCVs was determined as number of SCVs divided by total colonies.

Biofilm Assays and Microscopy

Overnight cultures inoculated from freezer stocks were grown in TH broth, and then diluted 1:20 in fresh TH supplemented with 1% glucose (THG). A total of 100 µl of the diluted culture was added to a 96-well plate with four technical replicates per strain. Cells were grown under static conditions at 37°C with 5% CO_2 for 20 hrs. Following incubation, unattached

bacteria were removed by washing twice with PBS (200ul), and attached bacteria were stained with 100 µl crystal violet for 10 minutes. Unbound crystal violet was removed by washing three times with PBS, and bound crystal violet was solubilized with 200 µl of 95% ethanol. Biofilm production was quantified through absorbance readings (OD_{595}) using a plate reader (Beckman Coulter, Inc.) and measurements were calculated as the sample value minus the media (blank) control. All assays were repeated at least three times.

Microscopy of colonies was performed by the Center for Advance Microscopy at MSU as previously described (66). Briefly, colonies were picked from THA plates following 24 hours of growth and mixed into PBS. These bacterial suspensions were mixed with an equal quantity of 4% glutaraldehyde buffered with 0.1m sodium phosphate at pH 7.4 before transfer to a poly-l-lysin (Sigma) covered coverslip where the solution was fixed and dried through a series of ethanol washes. Samples were critical point dried in a Leica Microsystems Model EM CPD300 dryer (Leica Microsystems), mounted on aluminum stubs using an epoxy glue (System Three Resins, Inc, Auburn, WA), and coated with iridium at an approximate thickness of 5.5 nm. Mounted and coated samples were examined in a JEOL JSM 7500F scanning electron microscope (JEOL Ltd, Tokyo, Japan).

Microscopy of biofilms was performed as previously described (67). Briefly, samples were fixed with 2.0% paraformaldehyde (Electron Microscopy Sciences), 2.5% gluteraldehyde (Electron Microscopy Sciences) in 0.05M sodium cacodylate (Electron Microscopy Sciences) buffer for 24 hours. After primary fixation, samples were washed three times with 0.05M sodium cacodylate buffer before sequential dehydration with increasing concentrations of ethanol. After dehydration, samples were dried at the critical point using a Tousimis Critical Point Dryer machine, mounted onto aluminum SEM sample stubs (Electron Microscopy Sciences), and sputter-coated with 5 nm of gold-palladium. Afterward, samples were painted with a thin strip of colloidal silver (Electron Microscopy Sciences) at the edge to facilitate charge dissipation.

Biofilms were imaged with an FEI Quanta 250 field-emission gun scanning electron microscope. Micrographs shown are representative of three biological replicates.

Association Assay

T-HESCs were decidualized as previously described (68). Briefly, cells were grown to approximately 50% confluence and treated with 0.5 mM 8-bromo-cyclic amp (cAMP) (Sigma) for 3 to 6 days. Decidualization was confirmed by examining the expression of prolactin and insulin-like growth factor (IGF)-binding protein 1, which are upregulated following decidualization. Assays were performed when cells reached 100% confluence. Bacterial strains were grown in THB overnight, washed once with phosphate-buffered saline (PBS), and resuspended in infection medium (HESC medium with 2% charcoal-treated FBS, no ITS+, and no antibiotics). Prior to infection, host cells were washed three times with PBS. They were then infected with GBS strains in the infection medium at a multiplicity of infection (MOI) of one bacterial cell per host cell. After 2 h of incubation at 37°C with 5% CO_2, wells were sampled, serially diluted, and plated to determine final growth. Then wells were washed three times with PBS to remove non-adherent bacteria, and host cells were lysed with 0.1% Triton X-100 (Sigma) for 30 min at 37°C. Lysates were subjected to gentle vortex mixing to further disrupt the host cells and liberate intracellular bacteria. After serial dilution, lysates were then plated on THA and incubated overnight at 37°C, and CFU were counted. All data were expressed as percentages of the total number of bacteria per well after the 2-h infection. Assays were run in triplicate at least three times.

Fibrinogen Binding Assay

Binding to fibrinogen was assessed as previously described (69). Briefly, wells of a flat bottomed 96 well plate were incubated for 18h at 4°C with 29.4nM human fibrinogen (Sigma). Overnight cultures were washed and diluted in phosphate buffered saline. 100uL, containing

$5x10^4$ to $5x10^5$ CFUs, was added to each well. Plates were incubated for 90 minutes at 37°C and 5% CO_2. Wells were then washed with PBS 3x to remove unbound cells and serine protease mixture (Sigma) was added and plates were incubated an additional 15 minutes before samples were taken, diluted, and plated on THB agar. Percent was calculated as the number of bound bacteria divided by the inoculums. Statistical significance was determined using a two-tailed Student's T-test assuming unequal variance between averages of 3 experiments.

Phagocytic Uptake and Survival in Macrophages

THP-1 cells were added to wells in a 24 well tissue culture treated plate and differentiated using Phorbol 12-Myristate 13-Acetate (PMA) as previously described(70). Briefly, PMA was added RPMI supplemented with 2% Fetal Bovine Serum to a final concentration of 100nM. THP-1 cells were centrifuged, washed with PBS, and resuspended in RPMI plus PMA to 1 x 10^6 cells per mL, and 1 mL per well was added and resulting plate was incubated for 24 hours at 37^0C at 5% CO_2. The next day, media was removed and adherent cells were washed 2x with PBS followed by the addition of 0.5mL RPMI lacking antibiotics and FBS to prepare for addition of GBS. Infection and phagocytic uptake was performed as previously described (71). Briefly, Infection inoculum was prepared from overnight GBS cultures, centrifuged, and resuspended in RPMI and added to each well at a MOI of 10 bacterial cells/ macrophage. Plates were incubated for 1 hour to allow phagocytosis, at which time bacteria-containing media was removed and cells were washed 3x before the addition of RPMI supplemented with 2% FBS and 100 μg/mL gentamicin and 5 µg/mL penicillin to kill extracellular bacteria. Phagocytic uptake was assessed at 2 hours post bacterial inoculation as previously described.

Antibiotic Susceptibility Testing

MICs were determined using E-test strips (Biomerieux, USA), as previously described (72). Strains were grown overnight in THB, diluted to 0.5 McFarland standard and spread onto

Meuller-Hinton Agar supplemented with 5% sheep's blood. E-Test strips were placed onto agar and plates were incubated until growth was visible and MIC was determined. Strains were considered susceptible for each antibiotic unless penicillin MIC was greater than 0.12 µg/mL and ampicillin MIC was greater than 0.25 µg/mL, and S. pneumoniae strain ATCC 49619 was used as a control in accordance with CLSI guidelines (73).

RNA Isolation and Sequencing

Bacterial RNA was isolated from cultures grown as described above. Samples were taken during log phase growth (OD595 = 0.4) and following 1 hour of macrophage exposure. Samples were extracted and DNA was removed as previously described (74), using RNAprotect Bacteria Reagent (Qiagen) and an RNeasy minikit (Qiagen). Briefly, samples were subjected to enzymatic and mechanical lysis and extracted following the RNeasy protocol. Following extraction, samples were treated with a Turbo DNA-free kit (Ambion) and checked for DNA contamination by PCR without prior reverse transcription (RT). For macrophage exposed samples, RNA was precipitated following Turbo DNase treatment and bacterial RNA was separated from mammalian following the standard protocol of a MICROBEnrich Kit (Ambion). Following microbial RNA enrichment, samples were quantitated with a Qubit fluorometer (Qubit) uising standard manufacturer protocols and subjected to rRNA removal with a Ribo-Zero rRNA removal for Gram-Positive bacteria (Epicentre). Samples were again quantitated with a fluorometer and analyzed using the Agilent RNA Pico 6000 kit and Agilent bioananalyzer (Agilent) to ensure high quality and purity. Following this, 100ng was added to a 1.5mL Eppendorf tube, dried, and resuspended into 5µL RNase-free H_2O for library preparation, sequencing, and analysis. RNA library was prepared and sequenced by the MSU Research Technology Support Facility using a modified standard protocol which skipped the oligo-dT bead step with a TruSeq Stranded mRNA library sample preparation kit (Illumina). Paired-end reads were done with an Illumina HiSeq 2500 Rapid Run flow cell (Illumina) with Rapid SBS reagents

and bases were called using Illumina Real Time Analysis v1.18.64 and converted to FastQ files by Illumina Bcl2fastq v.1.8.4.

Whole Transcriptome Analysis

Paired-end RNASeq data was generated using the Illumina HiSeq platform. Trimmomatic (version 0.3) was used to remove Illumina adapters from the raw sequence reads and for quality control: reads with average quality score less than 28 were dropped, and reads were clipped if average quality score in a window of 20 falls below 30. Tophat2 (version 2.0.11) was used to map the filtered reads to the GBSCOH1 bacterial reference genome as outlined in the gene feature files (.gff) from NCBI. Cufflinks (version 2.2) was used to identify differentially expressed genes from the reads mapped to reference transcriptomes, which gives the log_2 fold change in gene-expression. We then computed the p-values and the q-values (false discovery rates) for the log_2 fold change data and identified genes with FDR < 0.5 as the differentially expressed genes. P-values are calculated as the cumulative distribution function of the normally distributed z-scores for the log_2 fold expression change for each gene. Scripts for data analyses are available upon request.

ACKNOWLEDGEMENTS

This study was supported in part by the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) in collaboration with the Bill and Melinda Gates Foundation (project N015615). This funding agency did not play a role in the design, analysis, and interpretation of data or in the preparation of the manuscript. Student support was provided in part by the Graduate School and College of Natural Sciences at Michigan State University, the Thomas S. Whittam and the Rudolph Hugh Graduate Fellowships. We wish to thank both Carol Flegler of the MSU Center for Advanced Microscopy and Mr Ryan Doster and Dr Jennifer Gaddy of

Vanderbilt University for their assistance with SEM work, Dr Aditi Gupta and Mr David Knupp for their assistance in RNA Seq analysis, and Dr Pallavi Singh for helpful genomic discussions.

APPENDIX

APPENDIX

Table 4.1. Upregulated genes in mutant SCV relative to the WT identified in whole transcriptome comparisons. COH1 Gene ID refers to Genbank Accession # GCA_000689235.1. Average fold change (Avg Fold Change) and standard deviation (StDev) was calculated from 2 separate transcriptome comparisons between SCV and WT. Product is the predicted gene product from GenBank, and function is designated by gene ontology (GO) analysis.

COH1 Gene ID	Avg Fold Change	StDev	Product	Function
1581	24.3	10.0	major facilitator family transporter	Transport
1580	11.5	3.8	universal stress protein family	Stress Response
1887	8.0	0.4	anaerobic ribonucleoside-triphosphate reductase	Protein Synthesis
1873	6.7	1.3	uridine phosphorylase	Metabolism
523	6.7	1.3	adenosine deaminase (purine metabolism)	Metabolism
1420	6.6	1.9	D-isomer specific 2-hydroxyacid dehydrogenase family protein	Metabolism
1193	6.3	1.4	5-nucleotidase family protein	Metabolism
1422	6.2	1.6	phosphoserine aminotransferase	Metabolism
1421	6.1	1.8	acetyltransferase, GNAT family	Metabolism
1419	5.1	0.9	D-isomer specific 2-hydroxyacid dehydrogenase family protein	Metabolism
1521	5.0	0.5	pyruvate, phosphate dikinase	Metabolism

Table 4.2. Downregulated genes in mutant SCV relative to the WT identified in whole transcriptome comparisons. COH1 Gene ID refers to Genbank Accession # GCA_000689235.1. Average fold change (Avg Fold Change) and standard deviation (StDev) was calculated from 2 separate transcriptome comparisons between SCV and WT. Product is the predicted gene product from GenBank, and function is designated by GO analysis.

COH1 Gene ID	Avg Fold Change	StDev	Poductr	Function
1900	-15.4	5.7	cold shock protein, CSD family (transcription factors)	DNA binding transcription factor
890	-14.9	4.0	Hypothetical	Hypothetical
1503	-14.2	0.4	glycerol uptake facilitator protein	transport
891	-12.1	4.1	Hypothetical	hypotheical
645	-11.9	1.0	Aa ABC transporter (putative glutamine transport system)	Transport
1501	-11.7	1.4	dihydroxyacetone kinase family protein	metabolism
662	-11.6	8.9	phage shock protein C, putative	Stress response
112	-11.4	3.9	Carbonic anhydrase	Transport
1848	-11.3	0.9	Hypothetical	transmembrane hypothetical
643	-10.9	3.7	Aa ABC transporter (putative glutamine transport system)	Transport
704	-10.4	1.7	ATP-dependent RNA helicase, DEAD/DEAH box family	translation
1500	-10.3	0.0	dihydroxyacetone kinase family (glycerolipid metabolism)	metabolisn
644	-10.1	3.6	Aa ABC transporter (putative glutamine transport system)	Transport

Table 4.2. (cont'd)

200	-9.9	3.0	oxidoreductase, putative	Metabolism
642	-9.3	0.6	Aa ABC transporter (putative glutamine transport system)	Transport
1281	-8.7	0.5	Signal peptidase I	Signaling
673	-8.3	0.0	riboflavin biosynthese protein RibD (riboflavin metabolism)	metabolism
30	-7.5	2.0	peptidase, M23 /M37 family	Extracellular Peptidase
992	-7.4	1.2	guanosine monophosphate reductase	metabolism
1893	-6.7	1.4	ArsC family subfamily	metabolism
319	-5.9	1.5	ribosomal subunit interface protein	Metabolism
1840	-5.8	1.4	PAP2 family protein	transport
862	-5.4	0.1	conserved hypothetical protein	conserved hypothetical

Figure 4.1. Frequency of SCVs after antibiotic and acid stress. A CC-17 isolate was exposed to penicillin or acidic pH overnight in a modified Luria-Delbruck fluctuation test. Dark blue bars represent SCVs in a single vessel while light blue bars represent SCVs in 3 separate vessels. Error bars represent standard deviation of 3 biological replicates.

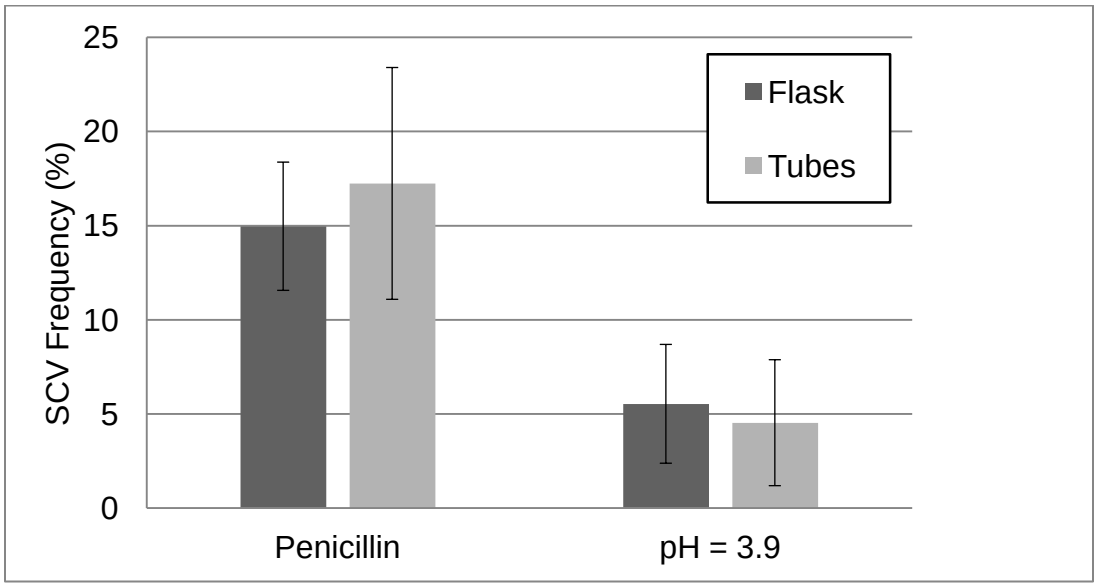


Figure 4.2. Dispersed growth, lack of extracellular debris, and reduced colony size of mutant SCV. A comparison of overnight growth between WT and SCV is shown in panels A & B, respectively. SEM microscopy comparing WT and SCV at single cell resolution is shown in panels C & D. Panel E shows a THA plate with both WT and SCVs and green arrows indicating examples of SCVs.

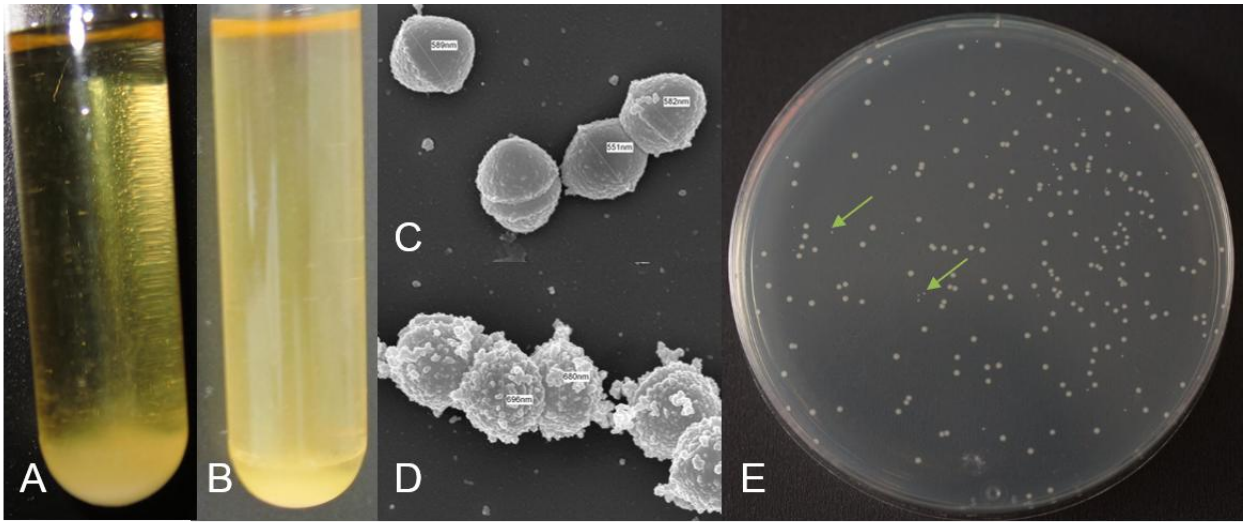


Figure 4.3. Decreased growth rate of the mutant SCV relative to the WT. Dark and light purple lines represent growth rate of WT and SCV, respectively, with the X and Y axes displaying hours of growth and OD_{595}. Error bars are indicative of the standard deviation between 3 separate biological replicates.

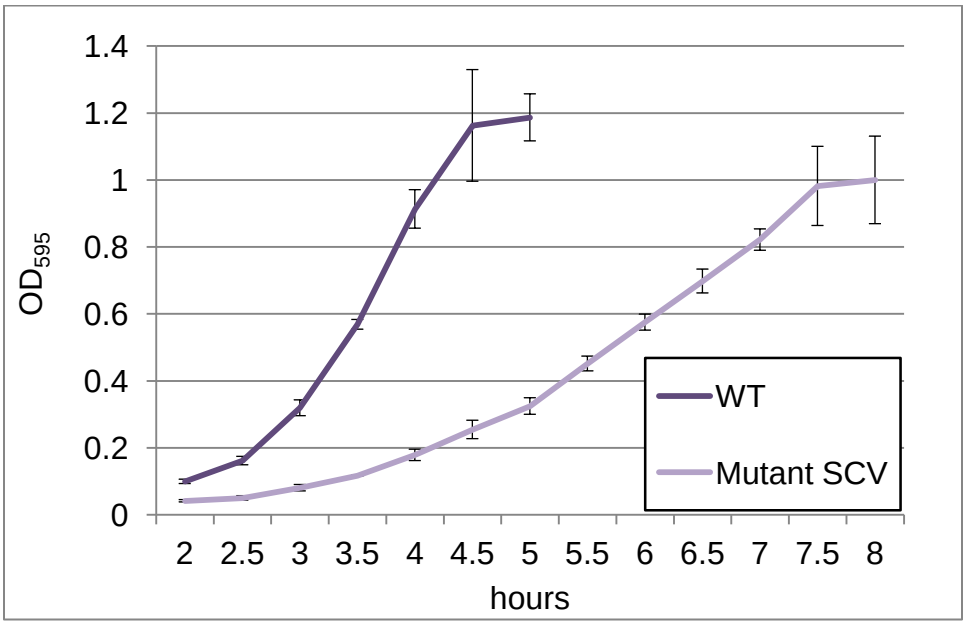


Figure 4.4. Enhanced biofilm production in the mutant SCV relative to the WT. Crystal Violet absorbance values of the WT and SCV mutant are shown in dark and light purple, respectively. Error bars represent the standard deviation of averages of 3 biological replicates. Star denotes significance (P-value =0.037

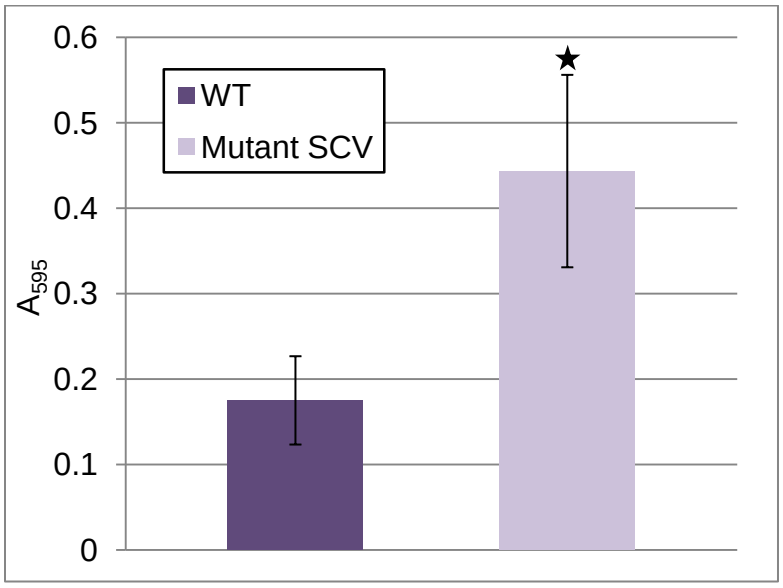


Figure 4.5. Biofilm of the mutant SCV lacks full development and contains fewer channels. Micrographs of the WT and SCV mutant biofilms. Images are representative of at least 3 replicates and were taken after 24 hours of biofilm growth on a cover slip.

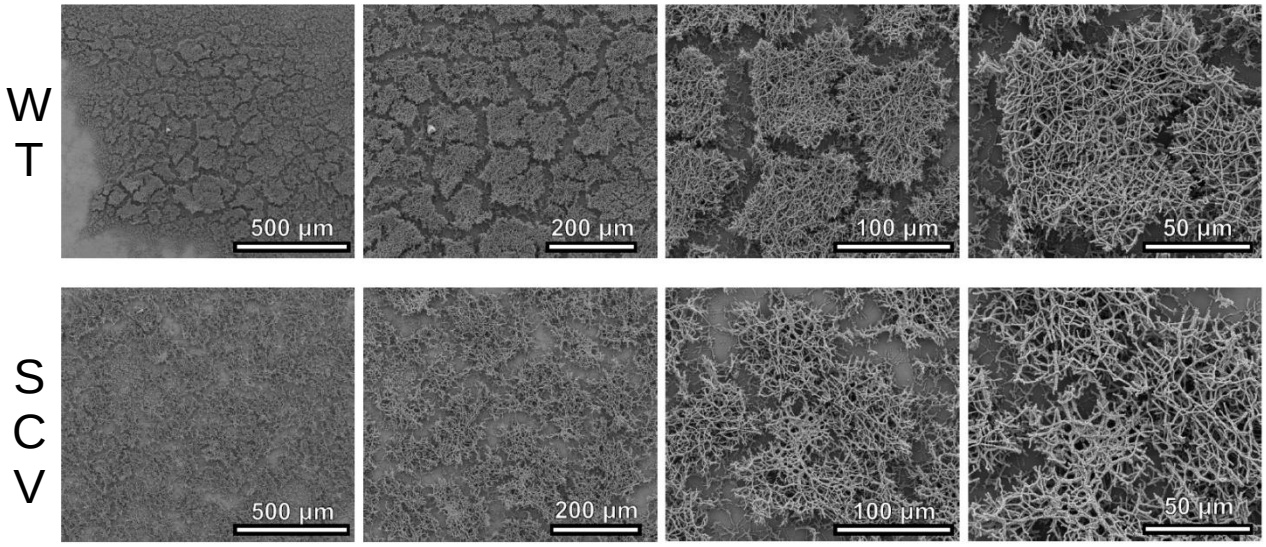


Figure 4.6. Adherence to fibrinogen in the mutant SCV relative to the WT. The SCV mutant had reduced adherence to immobilized fibrinogen on a polystyrene plate. Dark and light purple bars represent WT and SCV, respectively, and star denotes significance difference (P-value = 0.018).

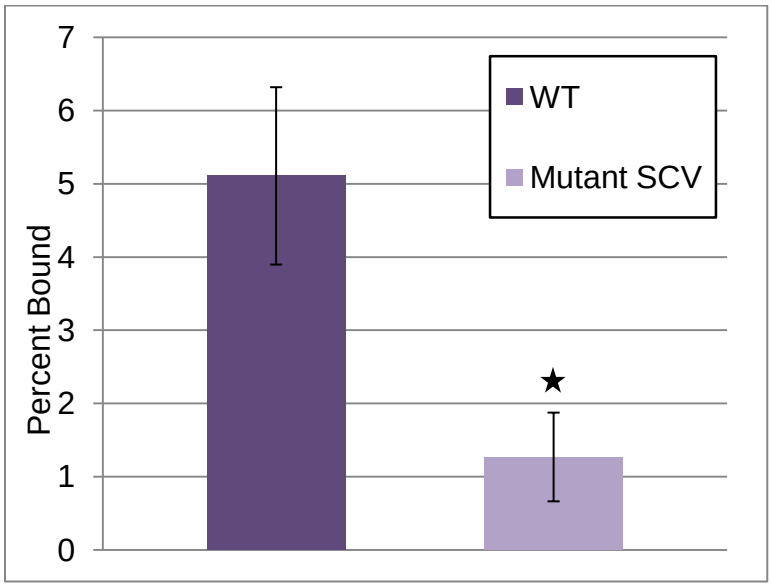


Figure 4.7. Association with decidualized T-HESCs in the WT and SCV mutant. There was no dtatistical difference between mutant SCV and WT in association with T-HESCs. The Y-axis represents the percent associated with T-HESCs. The bars and error bars represent the average and standard deviation of 3 experiments, respectively.

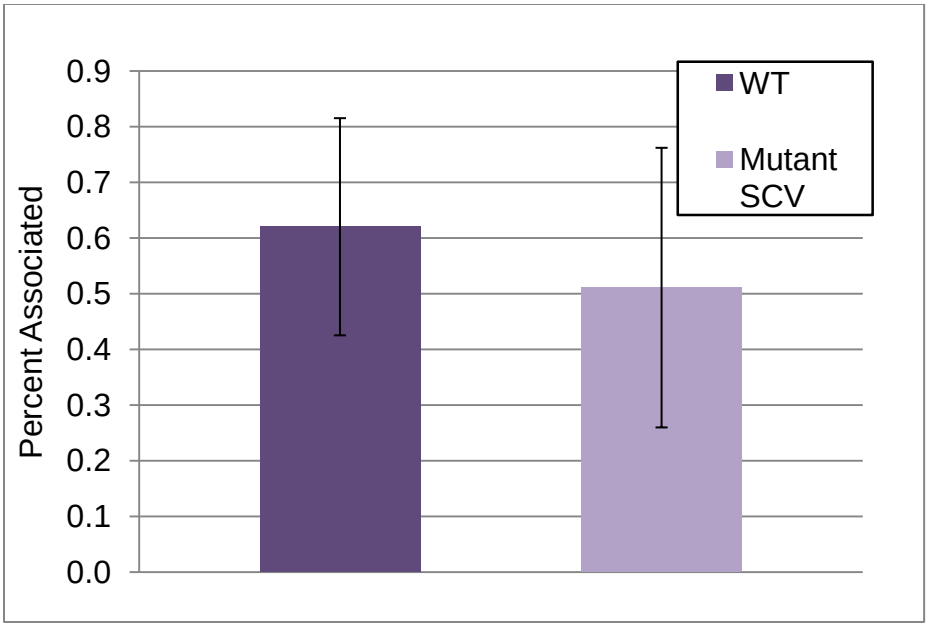
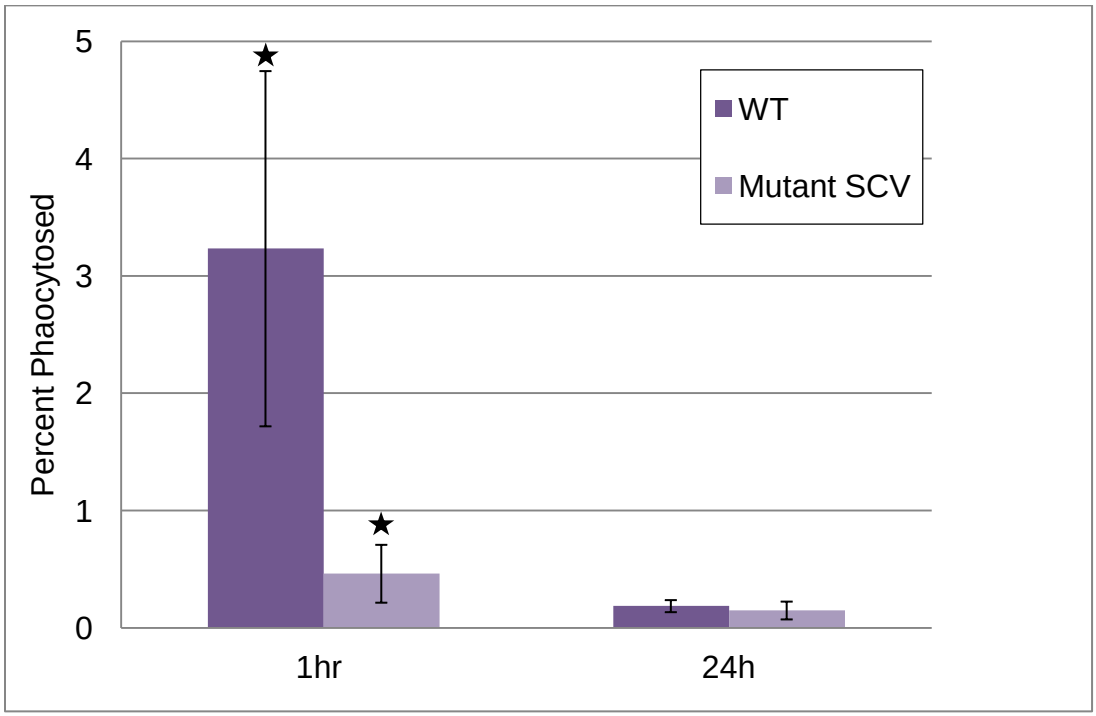


Figure 4.8. Phagocytic uptake and survival of the mutant SCV relative to the WT in THP-1 cells. At the 1hr time point, the bars represent the percent of cells that were phagocytosed while the 24hr time point represents the percent survival of those that were phagocytosed at 1 hr. The star indicates statistical significance between the marked data points (P-value = 0.03).



REFERENCES

REFERENCES

1. **Ferrieri P**, **Cleary PP**, **Seeds AE**. 1977. Epidemiology of Group-B Streptococcal carriage in pregnant women and newborn infants. J Med Microbiol **10**:103–114.

2. **Phares CR**, **Lynfield R**, **Farley MM**, **Mohle-boetani J**, **Harrison LH**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Stefonek KR**, **Albanese BA**, **Zell ER**, **Schuchat A**, **Schrag SJ**. 2008. Epidemiology of invasive Group B Streptococcal disease in the United States , 1999-2005. J Am **299**:1999–2005.

3. **Skoff TH**, **Farley MM**, **Petit S**, **Craig AS**, **Schaffner W**, **Gershman K**, **Harrison LH**, **Lynfield R**, **Mohle-Boetani J**, **Zansky S**, **Albanese B a**, **Stefonek K**, **Zell ER**, **Jackson D**, **Thompson T**, **Schrag SJ**. 2009. Increasing burden of invasive Group B Streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis **49**:85–92.

4. **Berardi A**, **Rossi C**, **Lugli L**, **Creti R**, **Reggiani MLB**, **Lanari M**, **Memo L**, **Pedna MF**, **Venturelli C**, **Perrone E**. 2013. Group B *Streptococcus* late-onset disease: 2003–2010. Pediatrics **131**:e361–e368.

5. **Imperi M**, **Gherardi G**, **Berardi A**, **Baldassarri L**, **Pataracchia M**, **Dicuonzo G**, **Orefici G**, **Creti R**. 2011. Invasive neonatal GBS infections from an a rea- based surveillance study in Italy. Clin Microbiol Infect **17**:1834–1839.

6. 2012. CDC - ABCs: 2010 GBS Surveillance Report - Active Bacterial Core surveillance.

7. **Ballard MS**, **Schønheyder HC**, **Knudsen JD**, **Lyytikäinen O**, **Dryden M**, **Kennedy KJ**, **Valiquette L**, **Pinholt M**, **Jacobsson G**, **Laupland KB**. 2016. The changing epidemiology of group B *Streptococcus* bloodstream infection: A multi-national population-based assessment. Infect Dis (Auckl) 1–6.

8. **Apostol M**, **Gershman K**, **Petit S**, **Arnold K**, **Harrison L**, **Lynfield R**, **Morin C**, **Baumbach J**, **Zansky S**, **Thomas A**. 2009. Trends in perinatal group B streptococcal disease-United States, 2000-2006. Morb Mortal Wkly Rep **58**:109–112.

9. **Hickman ME**, **Rench M a**, **Ferrieri P**, **Baker CJ**. 1999. Changing epidemiology of Group B Streptococcal colonization. Pediatrics **104**:203–9.

10. **Hansen SM**, **Uldbjerg N**, **Kilian M**, **Sørensen UBS**. 2004. Dynamics of *Streptococcus agalactiae* colonization in women during and after pregnancy and in their infants. J Clin Microbiol **42**:83–89.

11. **Schuchat A**. 1998. Epidemiology of Group B Streptococcal Disease in the United States : Shifting Paradigms. Clin Microbiol Rev **11**.

12. **Manning SD**, **Lewis M**, **Springman AC**, **Lehotzky E**, **Whittam TS**, **Davies HD**. 2008. Genotypic diversity and serotype distribution of Group B *Streptococcus* isolated from women before and after delivery. Clin Infect Dis **46**:1829–37.

13. **Hansen SM**, **Uldbjerg N**, **Kilian M**, **Sørensen UBS**. 2004. Dynamics of *Streptococcus agalactiae* Colonization in Women during and after Pregnancy and in Their Infants. J Clin Microbiol **42**:83–89.

14. **Jones N**, **Bohnsack JF**, **Takahashi S**, **Oliver KA**, **Chan M-S**, **Kunst F**, **Glaser P**, **Rusniok C**, **Crook DWM**, **Harding RM**. 2003. Multilocus sequence typing system for group B *Streptococcus*. J Clin Microbiol **41**:2530–2536.

15. **Davies HD**, **Raj S**, **Adair C**, **Robinson J**, **Mcgeer A**, **Group AGBSS**. 2001. Population-based active surveillance for neonatal group B streptococcal infections in Alberta, Canada: implications for vaccine formulation. Pediatr Infect Dis J **20**:879–884.

16. **Bohnsack JF**, **Whiting A**, **Gottschalk M**, **Dunn DM**, **Weiss R**, **Azimi PH**, **Philips JB**, **Weisman LE**, **Rhoads GG**, **Lin F-YC**. 2008. Population structure of invasive and colonizing strains of *Streptococcus agalactiae* from neonates of six U.S. academic centers from 1995 to 1999. J Clin Microbiol **46**:1285–1291.

17. **Lin F-YC**, **Whiting A**, **Adderson E**, **Takahashi S**, **Dunn DM**, **Weiss R**, **Azimi PH**, **Philips JB**, **Weisman LE**, **Regan J**. 2006. Phylogenetic lineages of invasive and colonizing strains of serotype III Group B Streptococci from neonates: a multicenter prospective study. J Clin Microbiol **44**:1257–1261.

18. **Manning SD**, **Springman AC**, **Lehotzky E**, **Lewis MA**, **Whittam TS**, **Davies HD**. 2009. Multilocus sequence types associated with neonatal Group B Streptococcal sepsis and meningitis in Canada. J Clin Microbiol **47**:1143–1148.

19. **Foxman B**, **Gillespie B**, **Manning SD**, **Howard LJ**, **Tallman P**, **Zhang L**, **Marrs CF**. 2006. Incidence and duration of group B *Streptococcus* by serotype among male and female college students living in a single dormitory. Am J Epidemiol **163**:544–551.

20. **Patras KA**, **Rösler B**, **Thoman ML**, **Doran KS**. 2015. Characterization of host immunity during persistent vaginal colonization by Group B *Streptococcus*. Mucosal Immunol.

21. **Colicchia LC**, **Lauderdale DS**, **Du H**, **Adams M**, **Hirsch E**. 2015. Recurrence of group B streptococcus colonization in successive pregnancies. J Perinatol **35**:173–176.

22. **Hoogkamp-Korstanje JAA**, **Gerards LJ**, **Cats BP**. 1982. Maternal carriage and neonatal acquisition of group B streptococci. J Infect Dis **145**:800–803.

23. **Bisharat N**, **Crook DW**, **Leigh J**, **Harding RM**, **Ward PN**, **Coffey TJ**, **Maiden MC**, **Peto T**, **Jones N**. 2004. Hyperinvasive neonatal Group B *Streptococcus* has arisen from a bovine ancestor. J Clin Microbiol **42**:2161–2167.

24. **Al Safadi R**, **Mereghetti L**, **Salloum M**, **Lartigue M-F**, **Virlogeux-Payant I**, **Quentin R**, **Rosenau A**. 2011. Two-component system RgfA/C activates the *fbsB* gene encoding major fibrinogen-binding protein in highly virulent CC17 clone group B *Streptococcus*. PLoS One **6**:e14658.

25. **Springman AC**, **Lacher DW**, **Waymire EA**, **Wengert SL**, **Singh P**, **Zadoks RN**, **Davies HD**, **Manning SD**. 2014. Pilus distribution among lineages of Group B *Streptococcus*: an evolutionary and clinical perspective. BMC Microbiol **14**:159.

26. **D'Urzo N**, **Martinelli M**, **Pezzicoli A**, **De Cesare V**, **Pinto V**, **Margarit I**, **Telford JL**, **Maione D**. 2014. Acidic pH strongly enhances *in vitro* biofilm formation by a subset of hypervirulent ST-17 *Streptococcus agalactiae* strains. Appl Environ Microbiol **80**:2176–2185.

27. **Ho Y-R**, **Li C-M**, **Yu C-H**, **Lin Y-J**, **Wu C-M**, **Harn I-C**, **Tang M-J**, **Chen Y-T**, **Shen F-C**, **Lu C-Y**, **Tsai T-C**, **Wu J-J**. 2013. The enhancement of biofilm formation in Group B Streptococcal isolates at vaginal pH. Med Microbiol Immunol **202**:105–115.

28. **Koh KS**, **Lam KW**, **Alhede M**, **Queck SY**, **Labbate M**, **Kjelleberg S**, **Rice SA**. 2007. Phenotypic diversification and adaptation of *Serratia marcescens* MG1 biofilm-derived morphotypes. J Bacteriol **189**:119–130.

29. **Schloter M**, **Lebuhn M**, **Heulin T**, **Hartmann A**. 2000. Ecology and evolution of bacterial microdiversity. FEMS Microbiol Rev **24**:647–660.

30. **Suerbaum S**, **Josenhans C**. 2007. *Helicobacter pylori* evolution and phenotypic diversification in a changing host. Nat Rev Microbiol **5**:441–452.

31. **Hallet B**. 2001. Playing Dr Jekyll and Mr Hyde: combined mechanisms of phase variation in bacteria. Curr Opin Microbiol **4**:570–581.

32. **Van Der Woude MW**, **Bäumler AJ**. 2004. Phase and antigenic variation in bacteria. Clin Microbiol Rev **17**:581–611.

33. **Brunham RC**, **Plummer FA**, **Stephens RS**. 1993. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. Infect Immun **61**:2273.

34. **Proctor RA**, **Von Eiff C**, **Kahl BC**, **Becker K**, **McNamara P**, **Herrmann M**, **Peters G**. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol **4**:295–305.

35. **von Eiff C**. 2008. *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinicians. Int J Antimicrob Agents **31**:507–510.

36. **Häussler S**, **Lehmann C**, **Breselge C**, **Rohde M**, **Classen M**, **Tümmler B**, **Vandamme P**, **Steinmetz I**. 2003. Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia* complex. Eur J Clin Microbiol Infect Dis **22**:249–253.

37. **Häußler S**, **Tümmler B**, **Weißbrodt H**, **Rohde M**, **Steinmetz I**. 1999. Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. Clin Infect Dis **29**:621–625.

38. **Häußler S**, **Rohde M**, **Steinmetz I**. 1999. Highly resistant *Burkholderia pseudomallei* small colony variants isolated in vitro and in experimental melioidosis. Med Microbiol Immunol **188**:91–97.

39. **Morton HE**, **Shoemaker J**. 1945. The identification of *gonorrhoeae* by means of bacterial variation and the detection of small colony forms in clinical material. J Bacteriol **50**:585.

40. **Bates DM**, **von Eiff C**, **McNamara PJ**, **Peters G**, **Yeaman MR**, **Bayer AS**, **Proctor RA**.

2003. *Staphylococcus aureus menD* and *hemB* mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. J Infect Dis **187**:1654–1661.

41. **von Eiff C**, **Proctor RA**, **Woltz C**, **Peters G**, **Götz F**. 1997. A site-directed *Staphylococcus aureus hemB* mutant is a small-colony variant which persists intracellularly. J Bacteriol **179**:4706–4712.

42. **Singh R**, **Ray P**, **Das A**, **Sharma M**. 2010. Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant. J Med Microbiol **59**:521–527.

43. **Vaudaux P**, **Francois P**, **Bisognano C**, **Kelley WL**, **Lew DP**, **Schrenzel J**, **Proctor RA**, **McNamara PJ**, **Peters G**, **Von Eiff C**. 2002. Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. Infect Immun **70**:5428–5437.

44. **Tuchscherr L**, **Heitmann V**, **Hussain M**, **Viemann D**, **Roth J**, **von Eiff C**, **Peters G**, **Becker K**, **Löffler B**. 2010. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis **202**:1031–1040.

45. **Allegrucci M**, **Sauer K**. 2008. Formation of *Streptococcus pneumoniae* non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. J Bacteriol **190**:6330–6339.

46. **Allegrucci M**, **Sauer K**. 2007. Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. J Bacteriol **189**:2030–8.

47. **Ravins M**, **Jaffe J**, **Hanski E**, **Shetzigovski I**, **Natanson-Yaron S**, **Moses AE**. 2000. Characterization of a mouse-passaged, highly encapsulated variant of Group A *Streptococcus* in *in vitro* and *in vivo* studies. J Infect Dis **182**:1702–1711.

48. **Green MT**, **Heidger PM**, **Domingue G**. 1974. Proposed reproductive cycle for a relatively stable L-phase variant of *Streptococcus faecalis*. Infect Immun **10**:915–927.

49. **Zbinden A**, **Quiblier C**, **Hernandez D**, **Herzog K**, **Bodler P**, **Senn MM**, **Gizard Y**, **Schrenzel J**, **François P**. 2014. Characterization of *Streptococcus tigurinus* small-colony variants causing prosthetic joint infection by comparative whole-genome analyses. J Clin Microbiol **52**:467–474.

50. **Luria SE**, **Delbrück M**. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491.

51. **O'Neill E**, **Pozzi C**, **Houston P**, **Humphreys H**, **Robinson DA**, **Loughman A**, **Foster TJ**, **O'Gara JP**. 2008. A Novel *Staphylococcus aureus* Biofilm Phenotype Mediated by the Fibronectin-Binding Proteins, FnBPA and FnBPB. J Bacteriol **190**:3835–3850.

52. **von Eiff C**, **Bettin D**, **Proctor RA**, **Rolauffs B**, **Lindner N**, **Winkelmann W**, **Peters G**. 1997. Recovery of small colony variants of *Streptococcus aureus* following gentamicin bead placement for osteomyelitis. Clin Infect Dis **25**:1250–1251.

53. **Saluja SK**, **Weiser JN**. 1995. The genetic basis of colony opacity in *Streptococcus pneumoniae*: Evidence for the effect of box elements on the frequency of phenotypic variation. Mol Microbiol **16**:215–227.

54. **McEllistrem MC**, **Ransford J V**, **Khan SA**. 2007. Characterization of in vitro biofilm-associated pneumococcal phase variants of a clinically relevant serotype 3 clone. J Clin Microbiol **45**:97–101.

55. **Clements MO**, **Watson SP**, **Poole RK**, **Foster SJ**. 1999. CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis. J Bacteriol **181**:501–507.

56. **Baumert N**, **von Eiff C**, **Schaaff F**, **Peters G**, **Proctor RA**, **Sahl H-G**. 2002. Physiology and antibiotic susceptibility of *Staphylococcus aureus* small colony variants. Microb Drug Resist **8**:253–260.

57. **Buu-Hoï A**, **Le Bouguenec C**, **Horaud T**. 1990. High-level chromosomal gentamicin resistance in *Streptococcus agalactiae* (group B). Antimicrob Agents Chemother **34**:985–988.

58. **Baker CN**, **Thornsberry C**, **Facklam RR**. 1981. Synergism, killing kinetics, and antimicrobial susceptibility of group A and B streptococci. Antimicrob Agents Chemother **19**:716–725.

59. **Proctor RA**, **van Langevelde P**, **Kristjansson M**, **Maslow JN**, **Arbeit RD**. 1995. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin Infect Dis **20**:95–102.

60. **Courtney HS**, **Bronze MS**, **Dale JB**, **Hasty DL**. 1994. Analysis of the role of M24 protein in group A streptococcal adhesion and colonization by use of omega-interposon mutagenesis. Infect Immun **62** :4868–4873.

61. **Shenkman B**, **Rubinstein E**, **Tamarin I**, **Dardik R**, **Savion N**, **Varon D**. 2000. *Staphylococcus aureus* adherence to thrombintreated endothelial cells is mediated by fibrinogen but not by platelets. J Lab Clin Med **135**:43–51.

62. **von Eiff C**, **Becker K**, **Metze D**, **Lubritz G**, **Hockmann J**, **Schwarz T**, **Peters G**. 2001. Intracellular persistence of *Staphylococcus aureus* small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. Clin Infect Dis **32**:1643–1647.

63. **Drenkard E**, **Ausubel FM**. 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature **416**:740–743.

64. **Singh R**, **Ray P**, **Das A**, **Sharma M**. 2009. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. J Med Microbiol **58**:1067–1073.

65. **Davies HD**, **Adair C**, **McGeer A**, **Ma D**, **Robertson S**, **Mucenski M**, **Kowalsky L**, **Tyrell G**, **Baker CJ**. 2001. Antibodies to capsular polysaccharides of Group B *Streptococcus* in pregnant Canadian women: Relationship to colonization status and infection in the

neonate. J Infect Dis **184**:285–291.

66. **Klomparens KL**, **Flegler SL**, **Hooper GR**. 1986. Procedures for transmission and scanning electron microscopy for biological and medical science.

67. **Gaddy JA**, **Tomaras AP**, **Actis LA**. 2009. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic Cells. Infect Immun **77**:3150–3160.

68. **Brosens J**, **Takada S**, **Acevedo CH**, **Lewis MP**, **Kirby PL**, **Symes EK**, **Krausz T**, **Purohit A**, **Gellersen B**, **White JO**. 1996. Human endometrial fibroblasts immortalized by simian virus 40 large T antigen differentiate in response to a decidualization stimulus. Endocrinology **137**:2225–2231.

69. **Rosenau A**, **Martins K**, **Amor S**, **Gannier F**, **Lanotte P**, **van der Mee-Marquet N**, **Mereghetti L**, **Quentin R**. 2007. Evaluation of the ability of *Streptococcus agalactiae* strains isolated from genital and neonatal specimens to bind to human fibrinogen and correlation with characteristics of the *fbsA* and *fbsB* genes. Infect Immun **75**:1310–1317.

70. **Daigneault M**, **Preston JA**, **Marriott HM**, **Whyte MKB**, **Dockrell DH**. 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One **5**:e8668.

71. **Hunolstein V**. 1998. Group B Streptococci persist inside macrophages. Immunology **93**:86–95.

72. **Manning SD**, **Pearlman MD**, **Tallman P**, **Pierson CL**, **Foxman B**. 2001. Frequency of Antibiotic Resistance among Group B *Streptococcus* Isolated from Healthy College Students. Clin Infect Dis **33** :e137–e139.

73. **Wayne PA**. 2014. Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing: Twenty-fourth informational supplement, M100-S24. Clin Lab Stand Inst **34**.

74. **Korir ML**, **Knupp D**, **LeMerise K**, **Boldenow E**, **Loch-Caruso R**, **Aronoff DM**, **Manning SD**. 2014. Association and virulence gene expression vary among serotype III Group B *Streptococcus* isolates following exposure to decidual and lung epithelial cells. Infect Immun **82**:4587–4595.

CHAPTER 5.

CONCLUSIONS AND FUTURE DIRECTIONS

Streptococcus agalactiae remains a significant bacterial pathogen despite increased education and vigilance. Although much progress has been made due to the implementation of guidelines for the prevention of neonatal GBS disease, the rate of late onset disease remains unchanged and that of early onset disease has stagnated after initial declines (1). Furthermore, the only currently effective treatment for GBS infection is through the use of antibiotics, a limited long-term strategy given the rise in resistance rates for most pathogens (2). The focus of this dissertation has been the study of pathogenesis determinants with a focus on further reducing the GBS disease burden. Towards this end, the first study explored biofilm formation in a bank of diverse strains and identified associations between biofilms and epidemiological factors such as source, genotype, and pilus profile. Furthermore, we directly assessed the relationship between biofilm production and association with endometrial cells for representative subset of strains. This study detected differences in biofilm production among lineages that varied in pathogenic potential and demonstrated a strong biofilm phenotype for bovine isolates while human isolates varied widely The second study uncovered a regulatory role for a putative auto-inducing peptide (AIP) in a virulence-associated two component system, which was found primarily in the genomes of hypervirulent lineages. A mutant lacking the gene encoding the AIP showed decreased association with host cells, an increased ability to bind fibrinogen, and an altered transcriptome during log phase growth. This study offers a glimpse into the complicated regulation of virulence factors that can initiate colonizing bacteria to become invasive pathogens. Finally, the third study identified stress-induced microdiversity in a hypervirulent lineage of GBS. In this work, small colony variant (SCV) formation was found to be inducible through both antibiotic and acid stress, with increased frequency of SCVs when exposed to penicillin. Furthermore, characterization of a mutant SCV, which failed to revert to normal colony morphology upon passage, had increased biofilm production, tolerance to antibiotics, and decreased phagocytic uptake, indicating multiple roles in pathogenesis. Additionally, candidate genes important for this phenotype were identified through the use of RNA

sequencing via a whole transcriptome comparison between the WT and mutant SCV. This chapter identifies microdiversity in a single GBS strain and suggests SCV formation to be important for persistent colonization. Taken together, the findings presented in this dissertation are relevant from multiple perspectives and could aid in the detection, treatment, and prevention of GBS-mediated disease.

Future directions for the projects described herein include the identification and characterization of genes important to biofilm production. Particularly, given the variation noted in the predominant hyperinvasive lineage, the identification of biofilm determinants in specific genotypes could enable the development of novel therapeutics targeted at the most virulent of strains. With the identification of phenotypically-distinct strains presented here, genomic comparisons between a large collection of strong and weak biofilm producers could also identify novel candidates important for this trait. Additionally, biofilm production in GBS has been shown to be variable in response to the environment, and the work presented here serves to narrow down the selection of strains for comparison in other clinically-relevant conditions, such as in a flow cell, in a multi-species biofilm, or in the presence of antibiotics. Furthermore, the understanding of the importance of pili in GBS is an important direction and would best be accomplished through expression or mutagenesis studies during biofilm formation in host tissue association or invasion. The information gained through a study such as this should prove valuable towards the development of therapeutic tools for the clearance of infections.

Quorum sensing in GBS is vastly understudied. The work here identified *rgfD* as important for the regulation of the *rgf* two component system, though it is still not clear whether it represents a signal transduction system driven by quorum sensing. Nevertheless, the work described herein showed that the *rgf* system is likely regulated by more than one gene (*rgfD*), and the identification of additional regulators, or environmental cues, may inform future attempts to block cell to cell communication in GBS. Future work should involve the generation of a

complemented mutants expressing the full *rgf* transcript extra-chromasomally, and an assessment of the role of allelic variation in the *rgf* operon. For example, a characterization of the *rgfD* truncation we identified in ST-17 strains on gene regulation, for example, would also be worthwhile.

The identification of SCVs in GBS is the most intriguing prospect for future work. The transcriptome profiling offers a glimpse into the genes that may be responsible for this phenotype. Monitoring these genes in stressful conditions, or mutagenesis of those genes that were found to be differentially expressed will undoubtedly enhance our understanding of phenotypic heterogeneity in GBS, which may vary across lineages. In particular, a thorough assessment of tools used to identify GBS with a focus on recognizing SCVs would ensure that these variants are accounted for when preventative tests are administered and women are classified as GBS positive or negative. Furthermore, it would be interesting to attempt isolation of SCVs from human subjects, both from invasive disease cases and from those asymptomatically colonized, as has been done for other pathogens. Lastly, to understand the genetic mechanism of SCV formation, single molecule real time sequencing will provide the resolution needed to identify sequence alterations that may be important for this phenotype.

REFERENCES

REFERENCES

1. **Apostol M**, **Gershman K**, **Petit S**, **Arnold K**, **Harrison L**, **Lynfield R**, **Morin C**, **Baumbach J**, **Zansky S**, **Thomas A**. 2009. Trends in perinatal group B streptococcal disease-United States, 2000-2006. Morb Mortal Wkly Rep **58**:109–112.

2. **Neu HC**. 1992. The crisis in antibiotic resistance. Science **257**:1064–1073.