Construction of a CpsA Double Mutant to Determine the Function of the Lyt-R Domain

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CONSTRUCTION OF A CPSA DOUBLE MUTANT TO DETERMINE THE
FUNCTION OF THE LYTR DOMAIN

by

Klarissa Klier

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
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ABSTRACT

*Streptococcus agalactiae*, otherwise known as Group B Streptococcus (GBS), is a zoonotic, Gram-positive, commensal and invasive bacteria which is the leading cause of neonatal bacterial infections. These bacterial infections include sepsis, meningitis, pneumonia, and bacteremia. In neonates, GBS is most commonly transferred to the child in utero or during birth when the child aspirates amniotic or vaginal fluids. GBS can also infect the child through the bloodstream while in utero, causing premature births or still births. Children who survive the initial infection develop severe morbidities which include mental retardation, cerebral palsy, and seizures. GBS can also affect immunocompromised adults, leading to urinary tract infections, skin, and soft tissue infections, bacteremia, pneumonia, arthritis, and endocarditis. While there are current antibiotic methods of treatment, these treatments can lead to drug resistance. Therefore, other methods for treating GBS infections are desired. In this research project, creating a GBS CpsA double mutant in the LytR extracellular domain was attempted to determine how the double mutant affects capsule production. Formation of the CpsA double mutant was difficult and confirmation that the mutant is the correct sequence was not reached. In the future, the double mutant will be sequenced to determine the amino acid sequence of CpsA with alterations in the correct sequence. Then, this strain will be utilized to determine the role of these specific residues on GBS capsule level through an ELISA and morphology assays, and virulence will be measured through a zebrafish systemic infection.
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INTRODUCTION

Microbial infections are a complicated series of dynamic interactions between the pathogen and its host (Phelps & Neely, 2005). The dynamic interplay between the host and the pathogen determines the outcome of the infections (Miller & Neely, 2004). The use of an animal model to study host-pathogen interactions where the response of both the host and the pathogen can be analyzed is vital to understanding virulence regulation and pathogenesis (Miller & Neely, 2004). In this study, a double mutant was generated in the amino acid sequence of the LytR domain of the CpsA protein in *Streptococcus agalactiae*, a pathogen infecting both neonates and adult throughout the world. The goal of the experiment was to alter the biochemical interactions of the CpsA LytR region, causing a decrease in the major virulence factor for *S. agalactiae*, the capsule. Once the CpsA mutant was correctly sequenced, a zebrafish model could be used to visualize the decrease in virulence due to an impairment in capsule production.

**Group B Streptococcus**

*S. agalactiae*, otherwise known as Group B Streptococcus (GBS), emerged as a major cause of bovine mastitis in 1887 (Emaneini, khoramian, et al., 2016). By 1970, GBS had emerged as a major cause of neonatal meningitis and sepsis (Emaneini, khoramian, et al., 2016). Today, GBS is recognized as a major public health problem causing invasive and non-invasive infections in pregnant and non-pregnant adults, especially the elderly and immunocompromised (Di Renzo et al., 2015; Dutra et al., 2014). Non-pregnant adults have become increasingly associated with mortality due to invasive infections (Dutra et al., 2014). GBS is a nonmotile, Gram-positive, β-hemolytic, normal constituent of the
microbiome as well as a common commensal of the gastrointestinal tract and genitourinary tracts of both males and females (Di Renzo et al., 2015; Dutra et al., 2014; Lamagni et al., 2013; Landwehr-Kenzel & Henneke, 2014; Vornhagen, Adams Waldorf, & Rajagopal, 2017). While colonization can be transient, intermittent or persistent and is usually asymptomatic, colonization during pregnancy increases the risk of premature delivery and perinatal transmission through ascending infection during birth when the neonate aspirates contaminated amniotic or vaginal fluids (Emaneini, Jabalameli, Mírsalehian, Ghasemi, & Beigverdi, 2016). Once the commensal has moved from the vagina, through the cervix and into the uterus, the fetus can aspirate GBS, allowing the bacteria to invade the lung, then enter the bloodstream until finally crossing the blood brain barrier (BBB) (Figure 1) (Rajagopal, 2009; Vornhagen et al., 2017). This pathway leads first to pneumonia, then sepsis and finally meningitis (Rajagopal, 2009).

**Figure 1**: Proposed mechanism for GBS infection in-utero. (A) GBS is a commensal of the genital tract. (B)
Invasion of GBS to the amniotic cavity can cause inflammation of the placental membranes (chorioamnionitis), leading to premature births and still births (Vornhagen et al., 2017). GBS infections can lead to early on-set disease (EOD) and late on-set disease (LOD) in the neonate. EOD is caused by exposure to GBS before or during the birthing process and is characterized by pneumonia or sepsis occurring within the first 6 days post birth (Emameini, Jabalameli, et al., 2016; Maisey, Doran, & Nizet, 2008). LOD is a bloodstream infection characterized as meningitis and bacteremia within 7-90 days post birth (Emameini, Jabalameli, et al., 2016; Rajagopal, 2009). Non-pregnant adults clinically manifest GBS invasive infections in a variety of conditions which include skin, soft tissue and urinary tract infections, bacteremia, pneumonia, arthritis and endocarditis (Rajagopal, 2009). Meningitis and most neonatal infections are most frequently linked to the type III serotype of GBS (Di Renzo et al., 2015). Morbidity in survivors is high and includes neurological sequelae, cortical blindness, deafness, uncontrolled seizures, hydrocephalus, hearing loss, and speech and language delays (Johri et al., 2006; Simonsen, Anderson-Berry, Delair, & Davies, 2014).

In 1996, the CDC introduced intrapartum antibiotic prophylaxis (IAP) as the method of reducing the risk of neonatal morbidity and mortality from invasive infection (Braye et al., 2018; Phares et al., 2008). The regimen of IAP begins with a pregnant woman in labor receiving penicillin or ampicillin continuously for four hours until delivery (Powers & Wirtschafter, 2010). If the mother is allergic to penicillin, a variety of antibiotics can be offered; cefazolin or clindamycin is administered continuously for eight hours until delivery, erythromycin is administered continuously for six hours until delivery, or vancomycin is administered continuously for twelve hours until delivery (Powers &
Wirtschafter, 2010). Due to 46% of serotypes being resistant to erythromycin, the CDC no longer recommends erythromycin as an acceptable alternative for IAP (Stoll et al., 2011; Verani, McGee, & Schrag, 2010). The goal of IAP is to achieve adequate levels of antibiotic in fetal circulation without achieving neurotoxic serum levels (Braye et al., 2018). The use of IAP has resulted in a substantial decline in EOD, but has not had the same effect on LOD or infections in non-pregnant adults (Phares et al., 2008; Schuchat, 1999). The risks of IAP include drug resistance, adverse maternal reactions, a potential increase in Gram-negative infections in preterm neonates, and a lack of knowledge on the long term effects on the microbial balance of the mother and the neonate (Braye et al., 2018; Simonsen et al., 2014). Alternative methods of treatment for GBS infections such as a chlorhexidine douche and pretreatment with probiotic lactobacillus species have shown limited success, but IAP is still the best method of treating EOD (Braye et al., 2018; Vornhagen et al., 2017).

**World-Wide Incidence, Screening, and Serotypes**

From 1995-2005, incidence of EOD in the United States remained the same while incidences of LOD and non-pregnant related disease increased (Lamagni et al., 2013; Phares et al., 2008). From 1991-2010, EOD, LOD, and non-pregnant related disease in England and Wales increased (Lamagni et al., 2013). From 2010-2011, incidence of LOD were significantly higher in the United Kingdom and Ireland than incidence of EOD with LOD exhibiting a correlation of low gestational age and low birth rate (Okike et al., 2014). In contrast, many developing countries exhibit a lack of significant clinical disease due to GBS (Johri et al., 2006; Schuchat, 1999). This is puzzling, but can be explained through the commonality of less invasive procedures at hospitals, at home births, and less virulent
strains being prevalent (Johri et al., 2006; Schuchat, 1999). The less invasive procedures could be limiting the risk of GBS infection while at home births increase the risk of death due to GBS infection, masking the detection of EOD (Johri et al., 2006; Schuchat, 1999). The apparent lack of invasive disease in developing countries as well as excluding GBS invasive infections, which cause spontaneous abortion or stillbirth from the incidence reports makes it difficult to determine the true incidence of GBS invasive infection throughout the world (Lamagni et al., 2013).

In the United States, the Center for Disease Control (CDC) recommends a universal screening approach which requires that all women receive vaginal and anal swabs at 35-37 weeks gestations and cultured to determine positive colonization (Verani et al., 2010). This approach is also used in Italy, Spain, France, Germany, Switzerland, Czech Republic, Belgium, and Poland (Rodriguez-Granger et al., 2012). In Europe, the risk-factor based screening approach is recommended due to the countries beliefs that their low national incidence will not decrease further and that introduction of universal screening will increase antimicrobial resistance and anaphylactic reactions (Di Renzo et al., 2015). This approach required use of IAP when a pregnant woman presents with labor at less than 37 weeks, a release of membranes for more than 18 hours, and a fever of more than 38.0°C. This approach is also used in the Netherlands, New Zealand, and Australia.

There are pros and cons to each approach, with the universal approach missing pre-term babies as well as causing false positives and negatives due to the transient nature of GBS (Braye et al., 2018). The risk factor based approach does not address the risk of ascending infection and can lead to sub-optimal administration of IAP due to time used identifying the risk factors and administering the antibiotics (Braye et al., 2018; Vornhagen
et al., 2017). Neither approach prevents LOD infections where vertical transmission is not the only mode of acquisition (Vornhagen et al., 2017). To combat the weaknesses of these approaches, point of care test for at birth through rapid real time PCR assays can be used to detect GBS colonization (Di Renzo et al., 2015). This method is advantageous for determining GBS colonization in preterm deliveries and decreasing the number of false positive or negative cultures, but does not allow antibiotic susceptibility testing and can be expensive (Di Renzo et al., 2015). A screening protocol which could be applied throughout the world may help maintain low percentages of EOD.

One of the world’s many ongoing public health challenges is to develop vaccines that are effective against all serotypes of the infectious disease Group B Streptococcus (GBS) (Johri et al., 2006). This is increasingly difficult because most vaccines are serotype specific and the ten serotypes vary throughout the world. The least common serotypes are VI, VIII, IV, and Ib. Serotypes VI and VIII are only predominant in Japan and serotype Ib is only predominant in the Netherlands while serotype IV is predominant in the both the United Arab Emirates and Norway (De Francesco, Caracciolo, Gargiulo, & Manca, 2012; Emaneini, khoramian, et al., 2016; Powers & Wirtschafter, 2010; Rojo-Bezares et al., 2016). Serotypes Ia, III, and V are arguably more predominant throughout the world. Serotype V is predominant in Italy, Lebanon and Gambia (De Francesco et al., 2012; Emaneini, Jabalameli, et al., 2016; Powers & Wirtschafter, 2010). Serotype Ia is predominant in Brazil and Portugal while being linked to early onset disease (EOD) in the United States and the United Kingdom (Dutra et al., 2014; Otaguiri et al., 2013; Phares et al., 2008; Powers & Wirtschafter, 2010). Serotype III is predominant in Iran, Poland, Taiwan, England, Wales, and Spain while being linked to late-onset disease (LOD) in the
United Stated and the United Kingdom (Emaneini, Jabalameli, et al., 2016; Emaneini, khoramian, et al., 2016; Lamagni et al., 2013; Phares et al., 2008; Powers & Wirtschafter, 2010; Rojo-Bezares et al., 2016). Erythromycin, clindamycin, and tetracycline resistance was common throughout the world with rates varying greatly among different countries (Emaneini, Jabalameli, et al., 2016). Resistance rates in the United States and Europe were much higher than rates in Iran and Brazil, reflecting differences in doses, public health policies, and the spread of particular clones (Emaneini, Jabalameli, et al., 2016; Otaguiri et al., 2013). Despite claims that penicillin non-susceptible GBS have been reported in the US and Japan, all serotypes mentioned in all parts of the world reported susceptibility to penicillin (De Francesco et al., 2012; Dutra et al., 2014; Emaneini, Jabalameli, et al., 2016; Lamagni et al., 2013; Phares et al., 2008) Because of the wide variety in predominant serotypes and the increases in antibiotic resistance throughout the world, the goal of research today is to exploit the GBS genomic sequences to establish a universally effective vaccine and eliminate the need for antibiotics (Johri et al., 2006).

**Virulence and Pathogenesis of *S. agalactiae***

Group B Streptococcus virulence is complex and multifactorial with several virulence factors involved in the adhesion to and invasion of the host cells as well as evading the immune system of the host (Dutra et al., 2014). These virulence factors are regulated through expression of signal transduction systems (STS) in response to the external environment (Rajagopal, 2009). Virulence factors include α/β antigens of the C protein, surface protein Rib, hyaluronate lyase, C5α peptidase, laminin binding protein (LMB) and pilus proteins (Di Renzo et al., 2015; Dutra et al., 2014; Emaneini, khoramian, et al., 2016). Cα proteins mediates adherence to epithelial cells while Cβ proteins
participate in invasion and resistance to phagocyte clearance (Emaneini, khoramian, et al., 2016; Johri et al., 2006). Resistance to proteases (Rib) is used to confer protective immunity and has been linked to almost all strains of type III that caused invasive infections in neonates (Emaneini, khoramian, et al., 2016; Johri et al., 2006). Hyaluronate lyase surface proteins cleave hyaluronan and facilitates the invasion and spreading of GBS through the host tissues (Emaneini, khoramian, et al., 2016). C5α peptidase is a surface localized serine protease which cleaves a component of the human complement system C5α, leading to reduced neutrophil chemotaxis and decreased opsonophagocytic killing (Emaneini, khoramian, et al., 2016; Johri et al., 2006). Laminin binding proteins are important to GBS translocation across the interstitial epithelial cells and the blood brain barrier (BBB) (Landwehr-Kenzel & Henneke, 2014). Pilus proteins are involved in the adhesion by promoting colonization of the epithelial cell surfaces which supports biofilm formation and facilitates translocation across the BBB (Landwehr-Kenzel & Henneke, 2014).

The most important virulence factor of GBS is the capsular polysaccharide (CPS) which acts as the outer most layer of the cell surface, provides additional protection from extracellular assaults, and helps bacterial invasion by interfering with phagocytic clearance (Di Renzo et al., 2015; Hanson & Neely, 2012). Interfering with the phagocytic clearance allows for GBS to disseminate through the blood-stream (Hanson et al., 2012). Capsular polysaccharides have differences in structure on the surface polysaccharide that enables the subdivision into 10 serotypes and are important targets for the development of vaccines (Dutra et al., 2014; Emaneini, Jabalameli, et al., 2016). Disturbances of the CPS can result
in pleiotropic effects that highlight the interdependence of each component to the overall function of the cell surface (Hanson & Neely, 2012).

Pathogenesis of GBS can first be traced to asymptomatic mucosal colonization by adherence and invasion of the host-cell surface through initial binding to the extracellular cell matrix (ECM) by adhesion factors such as fibrinogen, fibronectin, and laminin (Landwehr-Kenzel & Henneke, 2014; Maisey et al., 2008; Rajagopal, 2009). GBS may prevent adaptive immune responses at sites of colonization through limiting cyclic dinucleotide secretion at the mucosal epithelium (McFarland & Woodward, 2016). The cell surfaces of Gram-positive pathogens are composed of a single cell membrane and represents a complex association of glycopolymers that control cell division, homeostasis, immune evasion, tissue invasion and resistance to antimicrobials (Hanson & Neely, 2012).

As evident in Figure 2, the glycopolymers of the phospholipid bilayer include peptidoglycan cell wall, wall teichoic acids, lipoteichoic acids, and the major virulence factor capsular polysaccharides (Di Renzo et al., 2015; Hanson & Neely, 2012).

**Figure 2**: Simplified model of the cell surface of Gram-positive bacteria: lipid membrane (LM), peptidoglycan (PG), lipoteichoic acids (LTA), wall teichoic acids (WTA), capsular polysaccharide (CPS). (Hanson & Neely, 2012).
The thick peptidoglycan layer (PG) protects the membrane and acts as a scaffold for other components of the cell membrane (Hanson & Neely, 2012). Anionic teichoic molecules are interspersed through the PG, providing the cell wall integrity, are useful for the invasion of the host tissue, are useful for the regulation of cell division, and supplying the major portion of the overall negative charge (Hanson & Neely, 2012). These include the lipoteichoic acids (LTA) and the wall teichoic acids (WTA). The WTA is covalently linked to the N-acetylmuramic acid (NAM) structure of the PG while LTA is anchored to the membrane through a glycolipid (Hanson & Neely, 2012). Capsule polysaccharides (CPS), an important virulence factor, are essential for systemic dissemination during infection (Hanson, Lowe, & Neely, 2011). Regulation of the CPS is vital for bacteria to act as a commensal and a pathogen (Rowe et al., 2015). GBS CPS is attached to the N-acetylglucosamine (NAG) of the PG (Hanson & Neely, 2012). The connections of WTA and CPS to the PG are important to the final cell architecture and essential for proper cell wall functionality (Kawai et al., 2011). Because high levels of CPS inhibit adherence, GBS may facilitate alterations in the cell surface from CPS and immune evasions functions to WTA and LTA adherence and colonization functions through signals from the host (Hanson et al., 2011; Hanson & Neely, 2012). Following adherence and colonization, GBS can use invasions to promote bacterial entry and survival within host cells (Maisey et al., 2008). Intracellular uptake of GBS by alveolar epithelial and pulmonary endothelial cells involves activation of cytoskeletal rearrangements in the target cell through Rho family GTPases (Maisey et al., 2008). CPS prevents uptake and clearance by macrophages and neutrophil through preventing opsonophagocytic killing (Maisey et al., 2008).
**CpsA**

In Gram-positive bacteria, such as GBS, the CPS is covalently attached to the cell wall PG with members of the LtyR-Cps2A-Psr (LCP) family (Eberhardt et al., 2012). LCP are phosphotransferase enzymes used to catalyze the transfer of anionic cell wall polymers like capsular polysaccharide and teichoic acids from lipid-linked polymers to PG (Eberhardt et al., 2012). The LCP family has a role in the biogenesis of WTA and anionic polysaccharides as well as the maintenance of cell envelopes (Kawai et al., 2011; Rowe et al., 2015). Members of the LCP contribute to the maintenance of normal capsule levels and the retention of CPS to the cell wall (Eberhardt et al., 2012). CpsA is believed to act as the enzyme responsible for the attachment of CPS to the cell wall, direct the polymerization and export of the polysaccharide, or be involved in transcriptional regulation (Hanson et al., 2011; Rosini et al., 2015; Toniolo et al., 2015). CpsA exhibits the predicted topology exhibited in Figure 3 where there are three transmembrane domains and two extracellular domains which include the accessory domain and the LytR domain (Hanson et al., 2011). The accessory domain is specific to CpsA while the LytR domain is a shared domain among pathogenic bacteria including *Bacillus subtilis* (Hanson et al., 2012). LytR proteins have been associated with transcriptional attenuation of the *lytRABC* divergon which is believed to encode cell-wall modifying enzymes as well as potentially alter the function of the extracellular DNA polymerase processivity factor (DNA_PPF) domain (Hanson et al., 2012). The DNA_PPF is adjacent to the LytR domain and used in protein-protein interactions (Hanson et al., 2012). In contrast, the leucine zipper domain extends from the
cytoplasmic regions into the transmembrane regions and is used in DNA binding and dimerization (Hanson et al., 2011).

Figure 3: Diagram of the topology of cpsA.

As shown in Figure 3, CpsA is composed of a short N-terminal cytoplasmic tail, a small extracellular loop, a small cytoplasmic loop, and a large extracellular C-terminus (Hanson et al., 2011). The unique topology of CpsA suggests a novel mechanism of transcriptional regulation (Hanson et al., 2011). CpsA is the first gene in the CPS operon and acts as a cell-surface protease that cleaves the EMC protein fibrinogen, producing adherent fibrin like cleavage products which coat the bacterial surface and interfere with complement-mediated clearance (Hanson et al., 2011; Maisey et al., 2008; Rajagopal, 2009). Sequence analysis of the cytoplasmic regions highlight the high density of positively charged amino acids, indicating that CpsA may be able to bind directly to the negatively charged phosphate backbone of DNA (Hanson et al., 2011). CpsA regulates capsule expression and is adjacent to the putative capsule promotor (Hanson et al., 2011). Experiments completed on the CpsA regions have indicated that the full CpsA region must be present for full expression of the capsule, mutation in cpsA lead to unusually long chains of coccii, the large extracellular region and the cytoplasmic loops of CpsA affect the specificity with which CpsA can interact with DNA sequences, and the N-terminus
represented in Figure 3 is required for binding to DNA (Eberhardt et al., 2012; Hanson et al., 2012; Rowe et al., 2015).

To obtain insights into the LCP family of proteins and their likely enzymatic functions, Kawai et al. determined the crystal structure of the CpsA region of S. pneumoniae.

**Figure 4:** The crystal structure of S. pneumoniae CpsA. The purple region is the accessory domain, the green region represents the LytR domain, and the small yellow region represents the phospholipid. (Kawai et al., 2011)

As shown in Figure 4, the LCP domain has an α-β-α architecture with a 5-stranded β-sheet forming the core of the protein and alpha helices and two pairs of β-strands.
extending away from the protein core, forming the interface between the two domains (Kawai et al., 2011). Kawai et al., concluded that the accessory domain folded independently of the LCP domain, indicating separate functions for the two regions (Kawai et al., 2011). A polyisoproenoid phosphate lipid was located in the hydrophobic pocket between the β-sheet and α-helices (Kawai et al., 2011).

Figure 5: Diagram of lipid binding in the LCP domain with A, B showing decaprenyl-phosphate binding and C, D representing octaprenyl-pyrophosphatase binding. Amino acids of interest are R374 and Q378. (Kawai et al., 2011)

The polyisoproenoid phosphate lipid, shown in Figure 5, is lined with hydrophobic side chains from residues that are completely conserved in hydrophobic character across the entire LCP family of proteins (Kawai et al., 2011). Key interactions between the lipid and the protein are maintained in both structures (Kawai et al., 2011). Because deletion of the CpsA gene as well as deletion of the LytR domain have both been associated with a decrease in capsule production (Hanson et al., 2012), it was hypothesized that mutations in key amino acids of the LytR domain from Figure 5, specifically R374 and Q378, would lead to a decrease in capsule production.

**Zebrafish as a Model Organism**

Animal models are vital to understanding virulence regulation and host-pathogen interactions in the study of bacterial pathogenesis in vivo. In recent years, a need for an alternative vertebrate model for studying bacterial infections has arisen and been filled by the zebrafish (*Danio Rerio*). The zebrafish has been used to study both aquatic and human bacterial pathogens which include but are not limited to *Salmonella arisonae*, *Bacillus subtilis*, and *Streptococcus agalactiae* (Kim et al., 2015; Phelps & Neely, 2005). Zebrafish have several advantages for use as a model organism. Firstly, this model is inexpensive,
easy to maintain, store, and genetically manipulate, and produce an abundance of offspring (Phelps & Neely, 2005; Trede, Langenau, Traver, Look, & Zon, 2004). For decades, murine models were an ideal model for bacterial pathogenesis, but with the advent of target gene knockouts became too difficult, expensive, and time consuming to use (Miller & Neely, 2004). Compared to the murine model, zebrafish can provide a more natural route of infection for the invasion of a pathogen in the brain. (Phelps & Neely, 2005). The small size of zebrafish is beneficial for studying disease progression because a longitudinal section of the whole adult zebrafish can be mounted on a slide and used for histological analysis (Phelps & Neely, 2005). Additionally, zebrafish possess a fully developed immune system which can replicate the host-pathogen responses in humans and allows for the study of diseases that involve both systems (Miller & Neely, 2004; Phelps & Neely, 2005). The innate immune system, with a primary function of eliminating invading pathogens and initiating the adaptive immune system, may be the most rapid and efficient in responding to microbial exposure (Phelps & Neely, 2005; Trede et al., 2004). The adaptive immune system development is delayed, allowing for stepwise infection and study of specific innate and adaptive immune responses (Phelps & Neely, 2005). Lastly, the transparency of zebrafish for their first week of life allows for observation of developmental processes in real time through fluorescently labeled bacteria (Kim et al., 2015; Miller & Neely, 2004; Phelps & Neely, 2005). This technique was used to study early T cell development in a living organism (Trede et al., 2004). As a vertebrate genetic model, the zebrafish has proven itself as an alternative for the study of genetic modifiers to the immune response (Trede et al., 2004).
Zebrafish were established as being susceptible to *Streptococcus iniae*, leading to the 16S rRNA sequence of the bacteria revealing close genetic relation between *S. iniae* and *S. agalactiae* (Miller & Neely, 2004). *S. agalactiae*, the human-specific pathogen is characterized by sepsis after intravascular invasion (Miller & Neely, 2004). Similar to *S. agalactiae*, *S. iniae* cause systemic infections which disseminate into multiple organs including the heart, liver, and brain and rely on genes involved in β-hemolysis and capsule synthesis for virulence (Miller & Neely, 2004).

Zebrafish have emerged as a valuable tool for modeling *S. agalactiae* infections in both the adult and larvae (Kim et al., 2015). Adult zebrafish exhibit mortality, cerebral edema and bacteria in the brain while the larvae show susceptibility through bacteria crossing the blood brain barrier (BBB) and dose-dependent mortality (Kim et al., 2015). Hanson et al., demonstrated that mutated strains with a CpsA deletion (ΔCpsA) were not attenuated in human whole blood, despite the production of less capsule, which lead to in vivo assessment of virulence (Hanson et al., 2012). Results showed WT strains of GBS were highly virulent with 8% survival while ΔCpsA strains were attenuated with 68% survival (Hanson et al., 2012). The observed decrease in virulence of ΔCpsA in zebrafish lead researchers to conclude that disruption of CpsA may lead to deficiencies that are only observable in the context of systemic disease (Hanson et al., 2012). Therefore, after the CpsA double mutant is constructed, attenuation of virulence should be tested in the context of systemic disease through analysis in a zebrafish model.
MATERIALS AND METHODS

Preparation of Plasmid: The plasmid pLZ12-rofA-pro (strain #12E34) was digested first with BamHI and allowed to incubate at 37°C for 15 minutes. Then the plasmid was digested with PstI and allowed to incubate at 37°C for 30 minutes. The digested plasmid was separated on an 0.8% agarose gel to check for a band at 4500 bp and isolated from the gel using the GeneJet Gel Extraction Kit (Thermo-Fisher).

Polymerase Chain Reactions (PCR): S. agalactiae genomic DNA was used as a template to amplify regions of the DNA associated with the cpsA gene. The polymerase utilized was Q5 (New England Biolabs), a high-fidelity polymerase and the primers used were specific to each reaction. The long fragment reaction used primers 5’ GBS-CpsA-RBS-BamHI and 3’ GBS-cpsA-Q382A/ R378A (#1740, #1956) while the short fragment reaction used primers 3’ GBS-cpsA-full-PstI and 5’ CpsA-Q382/R378 (#1672, #1955). After PCR was completed at an annealing temperature of 63°C, both reactions were separated on an 0.8% agarose gel, to confirm DNA fragments of 1160 bp and 338 bp. The reactions were gel purified using the GeneJet Gel Extraction Kit (Thermo-Fisher).

PCR Splice Overlap Extension (SOE): The two PCR fragments were used as a template to SOE the fragments together, forming a full-sized fragment of ~1475 bp. The polymerase utilized was Q5 (New England Biolabs) and the two primers used were 5’ GBS-CpsA-RBS-BamHI and 3’ GBS-cpsA-full-PstI (#1740, #1672) at an annealing temperature of 65°C. The reaction was separated on an 0.8% agarose gel to confirm a final product of 1475 bp. The full-sized fragment was gel purified using the GeneJet Gel Extraction Kit (Thermo-Fisher) followed by a digestion with BamHI and PstI.
Figure 6: PCR SOEing model. The CpsA gene represented at the top will have mutations occur at the double arrows labeled 1. Reaction 1 and reaction 2 will SOE together at site 1, forming the completed fragment represented in reaction 3. Under Gel Electrophoresis, a band will be visible at ~1475 bp.

Ligation: In a 1.5 mL microcentrifuge tube, the digested pLZ12-rofA-pro plasmid was utilized as the vector backbone. The digested PCR SOEing fragment was inserted into the plasmid with the assistance of the T4 DNA ligase (New England Biolabs). The reaction was incubated at room temperature for 30 minutes. To inactivate the ligase, the reaction was incubated at 65°C for 10 minutes, followed by a butanol precipitation to remove excess salts.

Transformation into *E. coli*: The DNA ligation mixture was mixed with electro competent cells and transferred to a cold electroporation cuvette. The cells were electroporated with a Gene Pulser (Bio-Rad) set to 25 µF, 2.5 kV, and 200 Ω. The electroporated cells were immediately transferred to SOC medium and incubated at 37°C for 90 minutes with continual shaking. Cells were then plated onto Luria-Bertani (LB) agar plates supplemented with chloramphenicol at 20 µg/ml final concentration and incubated at
37°C overnight. A PCR on the colonies was performed to confirm that the PCR SOEing fragment was inserted into the *E.coli*.

**Plasmid Isolation**: In a 250 mL Erlenmeyer flask, 100 mL of (LB) medium supplemented with 20 µg/ml chloramphenicol was used to culture several positive colonies post transformation. Overnight, the cultures incubated at 37°C with continual shaking. Next day, a PureLink HiPure Plasmid Filter DNA Purification Kit (Invitrogen) was used to purify plasmid DNA. Then, an Isopropanol Precipitation was performed, and a Nanodrop was used to determine the concentration of DNA present.

**Sequencing Prep**: Three PCR reactions were run on plasmid prep colonies with good DNA concentrations before the CpsA double mutant was sequenced. DreamTaq Mastermix was combined in 0.2 mL microcentrifuge tubes with different primers per reaction and annealed at a temperature set to 55°C. The expected band size was 350 bp for Set A, 720 bp for Set B, and 700 bp for Set C.
RESULTS

Preparation of Plasmid: The pLZ12-rofA-pro plasmid was double digested using the restriction enzymes BamHI and PstI. To confirm that the digested plasmid was cut correctly, the digest was separated on an 0.8% agarose gel by electrophoresis. The results visualized a fragment at band size ~4500 bp, as expected (Figure 7). The fragment was then gel isolated and later utilized during the ligation.

![Figure 7: Gel Electrophoresis of plasmid exhibits correct band size. The first column of the 0.8% agarose gel is the ladder while the third column in the digested plasmid. A faint band is visible at the expected bp of ~4500.](image)

PCR: Genomic DNA was used as the template in 2 PCR reactions. Reaction 1 used primers 5’GBS-CpsA-RBS-BamHI and 3’ CpsA-Q382/R378. Reaction 2 used primers 5’GBS-CpsA-Q382/R378 and 3’GBS-CpsA-full-PstI. Following PCR, the fragments were separated by electrophoresis 0.8 % agarose gel. The results visualized the bands at bp
~1160 for reaction 1 and ~338 for reaction 2, as expected for each reaction (Figure 8). The fragments were gel purified and used in the PCR SOE.

![Gel Electrophoresis](image)

**Figure 8**: Gel Electrophoresis shows correct PCR band size. The first column represents the ladder. The third column shows the band for reaction 1, with the expected band size at ~1160 bp. The fourth column shows the band for reaction 2, with the expected band size at ~338 bp. Reaction 2 has a second band which indicated potential contamination, so this reaction was completed again.

**PCR SOE**: The purified PCR fragments from reactions 1 and 2 were used as the template with the outside primers in a PCR SOEing reaction to form a full-sized fragment. To confirm that a single full-size fragment was generated, the reaction was separated by electrophoresis on an 0.8% agarose gel. The results visualized the band at ~1475 bp, as expected (Figure 9). The fragment was gel isolated and digested using *Bam*HI and *Pst*I to prepare the fragment for ligation.
Figure 9: Gel Electrophoresis of PCR SOE shows correct band size. Starting from the left, the first column is the ladder and the third column is the PCR SOEing reaction. The band size at ~1475 bp is the correct band size for the reaction.

**Transformation:** The digested PCR SOEing reaction and the digested pLZ12-rofA-pro plasmid were ligated together, butanol precipitated and transformed into competent *E. coli* by electroporation. Transformants were then selected on LB-chloramphenicol agar plates. Any number between 1 and 1000 colonies could develop on these plates (Figure 10A). To purify the colonies, individual colonies were streaked onto a new LB-chloramphenicol agar plate with 8 colonies per plate and grown overnight (Figure 10B). The purified colonies were utilized in the colony PCR.
Figure 10: LB cam20 plates show colony growth and purification. In 10A, the transformations were plated at 50 µl, 150 µl, and 800 µl. After incubating overnight at 37°C, colonies were purified using the ‘pizza plate’ as shown in 10B. Individual growths were streaked onto a plate, forming a pizza shape and were later used to PCR the colonies.

Colony PCR: To confirm that the cpsA gene was present on the plasmid in E.coli, colony PCR was performed and using primers specific for the cpsA gene and reactions were separated by electrophoresis on an 0.8% agarose gel. The results visualized the band
at ∼1475 for positive colonies as expected (Figure 11). Negative colonies had no band present. Positive colonies were prepared for sequencing.

**Figure 11: Gel Electrophoresis of Colony PCR shows positive colony.** Starting from the left, columns are organized as followed: ladder, positive control (genomic DNA), negative control (ddH\_2O), and colonies. The positive control PCR was ineffective because no band is present. The negative control looks as expected with no band. The colonies all appear negative except for column 8. With a band at ∼1475bp, this colony is positive and was prepared for sequencing.

**Sequencing Prep:** After plasmids were purified from the positive transformants, an additional PCR was performed with the primers that were to be used for sequencing. Three sets of PCRs were performed with 5 primers and reactions were visualized on an 0.8% agarose gel. The bands for set B and C were visualized as expected at ∼720 bp and ∼700 bp respectively (Figure 12, column 6, and 9). Set A visualized 2 bands, one at the expected ∼350 and another at ∼700 bp (Figure 12, column 3). This second band indicated that
sequencing the CpsA double mutant would result in an incorrect sequence, so the mutant was not sequenced. The project was begun again from scratch because there was not enough PCR SOEing reaction to complete a new ligation.

![Gel Electrophoresis of Sequence Prep PCR](image)

**Figure 12: Gel Electrophoresis of Sequence Prep PCR indicated that the mutant has the incorrect sequence.** Starting from the left, the first column is the ladder, the third column is Set A, the fifth column is Set B, and the seventh column is Set C. Set B and C visualize expected bands at ~720 bp and ~700 bp respectively. Set A visualized 2 bands, one at ~350 bp, as expected, and a second band at ~700 bp. This second band indicates that the CpsA double mutant sequence will be incorrect.

**Construction of the CpsA Double Mutant:** Construction of the CpsA double mutant began by preparing the plasmid. The pLZ12-rof-A pro plasmid was double digested correctly, as shown in Figure 7. This step presented with no major complications. The next step involved creating 2 PCR reactions. Reaction 1 had no major complications when forming the reaction while reaction 2 presented with a correct band at ~338bp and an incorrect band at ~1500bp as visualized in Figure 8. Therefore, PCR reaction 2 was attempted again with the correct band at ~338 bp being the only band visualized. Both fragments were gel purified and used in the PCR SOEing reaction presented in Figure 9.
The PCR SOEing reaction generated a full-size fragment from reaction 1 and 2 with no major complications. The full-size fragment was then gel isolated and digested with BamHI and PstI to prepare the fragment for ligation.

The plasmid and the PCR SOEing reaction were ligated together, butanol precipitated, transformed into competent *E.coli*, and plated onto LB-chloramphenicol agar plates as shown in Figure 10. The purified colonies were then used for colony PCR to confirm positive colonies. These steps took 6 weeks to complete because positive colonies were not identified through colony PCR. During the first round of ligation, electroporation, plating and colony PCR, no positive colonies were located despite hundreds of colonies being grown. Because a pellet is not visible during the electroporation step, it was assumed that the electroporation was not completed correctly. The electroporation, plating and colony PCR was completed three times more with no positive colonies resulting. It was then determined that the reason no positive colonies were visible was due to the ligation being performed incorrectly. Therefore, the ligation, electroporation, plating and colony PCR was again repeated. This round of plates showed growth on the negative control plate, but after counting it, was determined that the number of colonies grown on the negative control plate were less than the number of colonies grown on the positive plate. Therefore, a colony PCR was performed looking for positive colonies. The results of the colony PCR are expressed in Figure 11 with column 8 representing a positive colony. Two positive colonies were found from these plates.

The plasmid DNA from the two positive colonies had the concentration of DNA examined using a Nanodrop. It was determined that one of the positive colonies did not have a high enough concentration of DNA present to sequence the *cpsA* gene.
electroporated into *E. coli*. Therefore, only one colony was prepared for sequencing. The sequence preparation using primers that were going to be used during sequencing is shown in Figure 12. Three sets of PCR reactions were performed with Sets B and C shown in columns 6 and 9 visualizing the correct bands at ~720 bp and ~700 bp respectively. Set A presented in column 3 visualized the expected band at ~350 bp as well as an unexpected band at ~700bp. The second band at ~700bp indicated that the *CpsA* sequence would not be correct so the colony was not sequenced. It was determined that the plasmid was the most likely cause of the incorrect band. Moving forward, a new pLZ12-rofA-pro plasmid was used beginning at the ligation step.

When the ligation was attempted again, there was not enough PCR SOEing reaction to complete the ligation and there was also no more reaction 1 or reaction 2 prepared to SOE together. Therefore, reaction 1 and 2 were re-prepared via PCR. After the PCR, both reactions were run on a gel with reaction 2 exhibiting the expected band at ~338bp. Reaction 1 did not exhibit any band. Reaction 1 was again prepared through PCR and run on a gel with no band exhibited again. To correct for any potential contamination, the ddH$_2$O was replaced and all primers were prepared with new water. Reaction 1, with the new ddH$_2$O and the new primers, was prepared via PCR and run on a gel with no band present. After the water was replaced, the Q5 Polymerase and Buffer were both replaced. The PCR with the new Q5 was run twice with no band being present. Then, the dNTPs were re-made with the new ddH$_2$O and PCR reaction 1 was again performed with the same no band result. The next step was to create more PCR SOEing using what was left of the original PCR SOEing reaction. This approach, when separated on a gel, again produced no band at ~1470bp. Then, the genomic DNA concentration was tested through a Nanodrop
and was determined to have good purity, indicating that the genomic DNA was not interfering in the PCR reaction. After the Nanodrop, the melting temperatures of 5’ GBS-CpsA-RBS-BamHI and 3’CpsA-Q382/R378 were double checked and it was discovered that the melting temperature of the 5’GBS-CpsA-BamHI was being prepared in the PCR machine at an annealing temperature that was too high. The reaction 1 was then prepared in the PCR at a lower annealing temperature. This again did not show a band at ~1470 bp. It was then determined that the issue was not the components of the PCR reaction but that the PCR machine was defective. The PCR machine was changed, and the reaction was prepared several times with a newer PCR machine with the same no band result. Despite the Nanodrop results of the genomic DNA being well within the expected range of purity, reaction 1 was prepared again using a new genomic chromosome as well as a new Q5 polymerase. This again resulted in no band being present when the reaction was separated on a gel. After nearly 2 months of the PCR reaction 1 not having a band present when the reaction was separated onto a gel, Dr. Neely attempted to prepare the PCR reaction and was successful. In response, the 0.5 microliters of the original PCR SOEing remaining was used to successfully create more PCR SOEing.

The newly formed PCR SOEing reaction was digested with BamHI and PstI, separated on a gel, and gel isolated to purify. Once digested, the ligation with the new double digested pLZ12-rofA-pro plasmid was completed. The ligation was electroporated into E.coli, plated onto LB-chloramphenicol plates, and a colony PCR was performed. The results of the colony PCR showed a faint positive band. To confirm whether a positive colony was present or if the faint band was due to contamination, the apparent positive colony was re-streaked onto a LB-chloramphenicol plate with the goal of performing a new
colony PCR and separating the reaction on a gel with the hope of visualizing a band at 
~1475 bp.
DISCUSSION

For millions of years, the bacterial pathogen *S. agalactiae* has survived as a commensal of the vaginal and gastrointestinal tracts. In recent years, Group B Streptococcus (GBS) had been established as a major cause of both bovine systemic infections, neonatal systemic infections and systemic infection in adults, especially the elderly and immunocompromised (Di Renzo et al., 2015). To prevent the spreading of GBS infections, pregnant women undergo universal screening and when positive for GBS colonization, intrapartum antibiotic prophylaxis (IAP) is administered ideally up to four hours before birth. Despite CDC recommendations, only 67% of pregnant-women who deliver term infants with GBS infections were screened and 81% of term infants with GBS infections were born to mothers who screened negative for GBS colonization (Braye et al., 2018). While a treatment does exist, the transient nature of the infection as well as the virulence factors designed evade the host immune system limit the effectiveness of the treatment, especially in late onset disease (LOD) and the elderly. The most common virulence factor, capsule polysaccharide (CPS) is a novel target for the treatment of GBS systemic infections. Analysis of CPS led to the discovery of five highly conserved genes of the capsule operon with CpsA being proposed as a transcriptional regulator of capsule. The crystal structure of the *S. pneumoniae* CpsA region was recently determined and revealed the amino acids R374 and Q378 as potential targets for creating a mutation in the LytR domain of CpsA to decrease capsule production. The construction of a double mutant in the R378 and Q382 regions of *S. agalactiae* was attempted and unfortunately not achieved. In the future, the double mutant will be correctly sequenced, capsule level will be determined
through an ELISA, and the zebrafish model will be used to determine whether virulence was attenuated due to a decrease in capsule production.
FUTURE DIRECTIONS

If a band is present at ~1475 bp, a Nanodrop will be performed on the positive colony to confirm the concentration of DNA present in the colony. When the Nanodrop is complete, the colony will be sequence prepared using the 5 primers that will be used during sequencing. With bands present at ~350bp, ~720bp, and ~700bp, the \(CpsA\) will be sequenced to confirm the mutations were made and the rest of the sequence is correct. Once the sequence confirmed correct, the \(CpsA\) will be transformed into wild type GBS and \(\Delta CpsA\) through electroporation, plated onto THY-chloramphenicol agar plates, and PCR to confirm the presence of the plasmid. Once the presence of plasmid has been confirmed, assays will be completed to determined capsule production.

The first assay completed will be and enzyme-linked immunosorbent assay (ELISA). An ELISA will detect capsule production through the highly specific anti-body antigen interaction. It is expected that the \(\Delta CpsA\) strains will produce less capsule than the wild type strain, indicating that the antibody will detect less capsule. Once a decrease in capsule production has been confirmed, a colony morphology will be analyzed using microscopy to determine the length of cocci due to the capsule. Virulence will be measured through the number of zebrafish alive three days post infection. Because \(\Delta CpsA\) is anticipated to produce less capsule and capsule is an important virulence factor, it is expected that GBS \(\Delta CpsA\) transformants will show a decrease in virulence when compared to the GBS wild type transformants. Once performed, these assays will provide evidence that either the amino acids mutated are unique targets for the treatment of GBS systemic
infections by decreasing capsule production and attenuating virulence or the assays will indicate that new amino acids should be targeted.
REFERENCES


Klarissa Klier was born in Methuen, Massachusetts on July 18, 1997 to a loving and supportive family. She and her family live in Methuen, Massachusetts in the same home she has lived in her entire life. She graduation fourth in her class from Presentation of Mary Academy in 2015. Klarissa began her collegiate career at the University of Maine fall of 2015 where she would join an array of extracurricular activities which include Mock Trial and secretary of Figure Skating Club. On May 11, 2019 she graduated with Honors with a Bachelor of Science in Zoology with a Pre-medical Concentration and minors in Neuroscience and Psychology. Klarissa plans to take a year or two off to work as a certified nurse’s assistant (CNA) with the goal of applying to Physician’s Assistant graduate programs. She is excited for the future and hopes to spend the next several decades exploring the world and serving the community she loves as a Physician’s Assistant.