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DETERMINING KEY RESIDUES OF THE LYTR DOMAIN IN THE STREPTOCOCCAL CPSA PROTEIN

by

Mohammad Fazeel Hashmi

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Microbiology)

The Honors College

University of Maine

May 2019

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ABSTRACT

Streptococcus agalactiae or Group B Streptococcus (GBS), is a Gram-positive commensal bacterium that is harmless in healthy adults, yet causes systemic diseases in neonates, the elderly, and immunocompromised individuals. Neonates are at risk of GBS infection in utero or during delivery due to the colonization of the organism in the vaginal canal of between 15-30% of adult females. GBS can cause severe neonatal sepsis and meningitis, as well as chorioamnionitis, which can cause premature birth and stillbirth. GBS infection is greatly facilitated by the presence of a bacterial capsule; a protective, polysaccharide matrix surrounding the cell that plays a key role in the pathogen's ability to evade host immune responses. Antibiotics are effective in reducing the chances of neonatal infection by GBS; however, they also increase the likelihood of the organism developing antibiotic resistance. An approach to manipulate GBS and reduce its functionality would be beneficial to counter the potential of antibiotic resistance developments, while avoiding the cytotoxic effects that antibiotics can impose on the host.

The GBS CpsA protein, a putative transcriptional regulator of the capsule locus within the GBS genome, plays a significant role in capsule production. Without CpsA, GBS displays reduced capsule production, and thus, reduced virulence. In this study Aspartic Acid-375 and Arginine-378 were targeted in the LytR domain of CpsA, a domain proposed to be responsible for the ligation of capsule to the cell wall of GBS. This work will provide insight into which amino acids are the key residues required for the function of CpsA.

ACKNOWLEDGMENTS

The completion of this thesis would not have been possible without the support, guidance, and contributions of many individuals. Foremost, I would like to express an immeasurable amount of gratitude to my thesis advisor, Dr. Melody Neely, for her knowledge, guidance, patience, and unwavering faith in my ability to conduct research. Through each research obstacle, Dr. Neely shared a heavy hand in teaching me how to think critically, and therefore grow as a researcher.

I would also like to thank the current members of the Neely lab for their contributions to my experimental techniques and several laboratory protocols. Thank you to Atefeh Rajaei, Ashley Soucy, Katie Patenaude, Caitlin Tetteh, Sydney Green, and Ben Tero.

Finally, I would like to thank my family and friends for the supportive roles they played in helping me to complete this thesis. Their encouragement and emotional support was vital to my development as a student.

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1 *Streptococcus agalactiae*

Streptococcus agalactiae, or Group B Streptococcus (GBS), is a Gram-positive commensal bacterium that normally grows in short chains of two to five cocci (1). In approximately 30% of healthy adults, GBS is an asymptomatic colonizer of the gastrointestinal tract, genitourinary tract, and additionally in women, the vaginal canal (2). Although asymptomatic and harmless in healthy adults, GBS is an opportunistic pathogen that can cause systemic diseases in neonates, the elderly, and immunocompromised individuals (3). Regardless of modern prophylactics, GBS is the leading cause of neonatal sepsis in high-income countries (4). In newborns, GBS can also cause meningitis, chorioamnionitis, and pneumonia (5). GBS infections are also a cause of preterm delivery, antepartum and postpartum stillbirth, and puerperal sepsis (6). Neonatal infection by GBS can be characterized as either being early-onset or late-onset. Early-onset GBS infections (0-6 days) stem from vertical transmission of the pathogen from mother to child before or during delivery through bacterial particles present within the amniotic fluid. Late-onset GBS infections (7-89 days) occur after delivery and are acquired from the mother or the environment (7). Current practices to reduce transmission of GBS to newborns include screening and intrapartum antibiotic prophylaxis using penicillin (8). Despite being effective, antibiotic administration can pose major threats to the development of both the newborn's gut microbiota as well as their immune system (9).

1.2 Peptidoglycan

Gram positive organisms, such as GBS, contain a cell wall consisting of a thick peptidoglycan layer, cell wall-associated teichoic acids, capsular polysaccharide (CPS) and other associated proteins. The peptidoglycan of the cell wall is made up of repeated N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), with several peptides and proteins attached via cross-linking or covalent and non-covalent interactions. The thick peptidoglycan layer also plays a major role in protecting the bacterial cell membrane. Importantly, anionic teichoic acids are scattered throughout the peptidoglycan, functioning to provide cell wall integrity, as well as granting the cell surface an overall negative charge (10). The peptidoglycan primarily acts as a scaffold for adherence and/or anchoring for several proteins and peptides. Many of these cell wall-associated proteins interact with the extracellular environment, working to maintain the functionality of the cell, while also protecting the cell from external threats. A major player in the protection of GBS is the capsular polysaccharide (CPS), which acts as the outermost layer of the cell surface, providing the cell with additional protection and structural integrity (11).

1.3 Group B Streptococcus Capsule

GBS infection is primarily facilitated by the presence of a bacterial capsule, a protective, polysaccharide matrix surrounding the cell that plays a key role in the ability of the pathogen to evade host immune responses, to bind to host cells, and to penetrate host tissues. The GBS capsule consists of capsular polysaccharides (CPS) which are covalently bound to the N-acetylglucosamine peptides of peptidoglycan (10). The enzyme(s) that covalently add and ligate CPS to the N-acetylglucosamine peptide have

not been determined in GBS and therefore elucidating the enzymatic process is one of the key objectives of this study. There are approximately nine antigenically distinct serotypes of GBS CPS, each consisting of various arrangements of galactose, glucose, GlcNAc, and sialic acids, such as *N*-acetylneuraminic acid (12). *N*-acetylneuraminic acid, commonly found in human and mammalian cells, allows for GBS to employ host-cell molecular mimicry, a means of avoiding recognition and phagocytosis by host immune cells (13).

The production and regulation of GBS capsule is key to the virulence and survival of the organism. CpsA is a putative transcriptional regulator protein of the capsule locus and is likely responsible for GBS capsule synthesis and ligation to the outer cell wall (1).

1.4 CpsA Protein

CpsA is a putative transcriptional regulator of the capsule locus in the GBS genome and is encoded by *cpsA*. This protein is common to all serotypes of GBS and belongs to the LytR-CpsA-Psr (LCP) protein family. The LCP protein family is associated with cell wall maintenance, stability, and carbohydrate linkage (1). The CpsA protein contains an intracellular DNA-binding domain, three transmembrane domains, an extracellular accessory domain, and an extracellular LytR domain (Figure 1). The LytR domain is a putative phosphotransferase that is likely involved in capsular attachment and cell wall stability. The CpsA protein is highly conserved in other Streptococcus species, including *Streptococcus pneumonia* and *Streptococcus iniae*, and is shown to play a bifunctional role in capsule expression and cell wall stability (1).

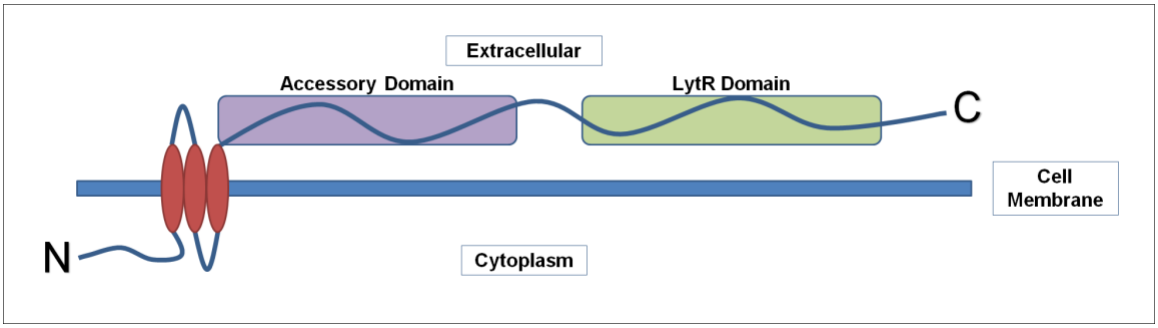

Figure 1. *CpsA protein structure.* Diagram displaying the DNA-Binding domain (N-terminus), three transmembrane domains (red), Accessory Domain (Purple), and LytR Domain (Green).

1.5 Previous Research

The CpsA protein has been demonstrated to regulate the transcription of the capsule locus (10), although the mechanism of this is not known. Previous research showed that the deletion of *cpsA* in *S. agalactiae* and *S. iniae* resulted in a myriad of pleiotropic effects (14). The deletion caused a decrease in levels of CPS production and microscopy analysis revealed that Δ*cpsA* GBS displayed a long-chain phenotype. Whether the long-chain phenotype is a result of decreased capsule is unknown, however it is predicted that CpsA-dependent changes to the cell wall are responsible for differences in chain lengths between wild-type and Δ*cpsA* GBS (1).

CpsA plays a vital role in the virulence of GBS. Rowe et al, demonstrated that in the absence of CpsA, GBS virulence was attenuated in a zebrafish model. Zebrafish were inoculated with a wild-type strain of GBS 515, and after six days post-infection only 8% survived. This was compared against Δ*cpsA* GBS 515-inoculated zebrafish, which after six days post-infection displayed a viability of 68% (1).

Cps2A is a *Streptococcus pneumoniae* protein analogous to CpsA. Both proteins are highly conserved (50% identical and 69% amino acid similarity), with exceptions to a

few differences in amino acids (1). Kawai et al, was able to characterize the Cps2A protein and solve the crystal structure to visualize the two extracellular domains (Figure 2). The goal of the study was to determine Cps2A functionality through the visualization of the folded protein structure and its amino acid residues (11). The crystal structure displays that both the Accessory and LytR domains fold independently of one another and that a buried decaprenyl-phosphate lipid is present in the LytR active site. The function of the buried lipid is not known; however, it may play an integral part in the ligation of CPS to the cell wall. Key residues that interact with the phosphate headgroup of the decaprenyl-phosphate lipid to maintain its stability are R267, R362, and R374 (Figure 3). These residues form key interactions with the phosphate oxygens to create a positively charged pocket in the surface of the protein (11). D371 and Q378 interact with the arginine residues to stabilize their conformations (Figure 3). In GBS, these residues are shifted slightly, but are predicted to maintain similar interactions.

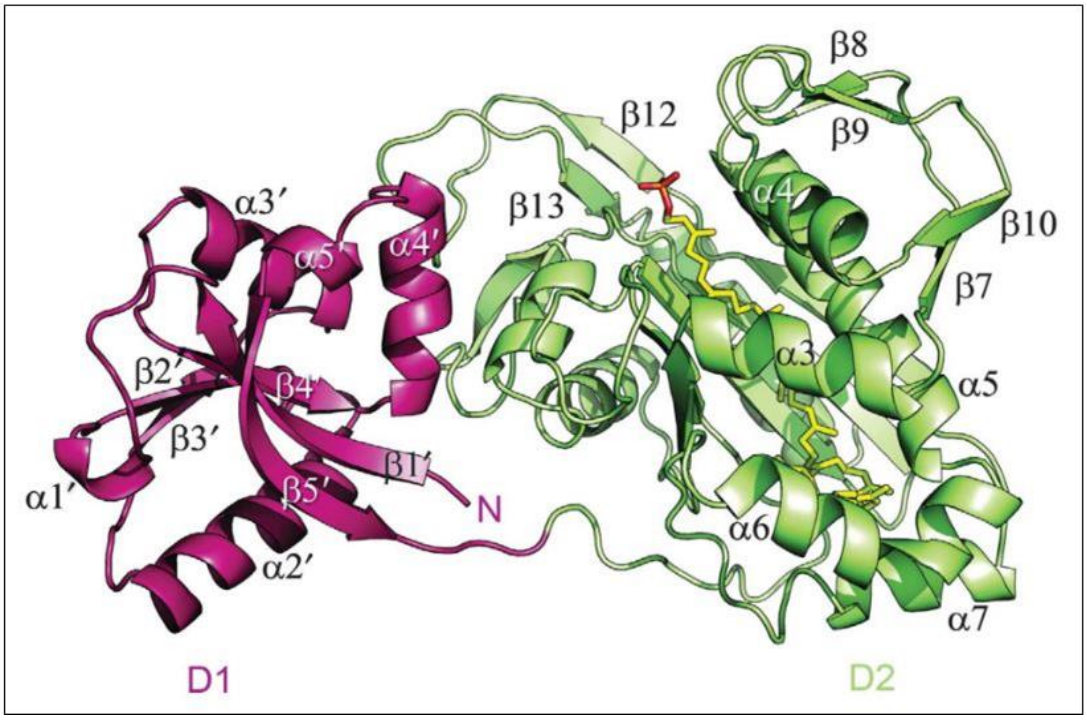

Figure 2. *Crystal structure of Cps2A extracellular domains in Streptococcus pneumoniae.* The Accessory Domain (red) folds separately from the LytR Domain (green). The LytR Domain displays a buried decaprenyl-phosphate (carbon atoms are yellow, phosphorous is orange, and oxygen is red) (11).

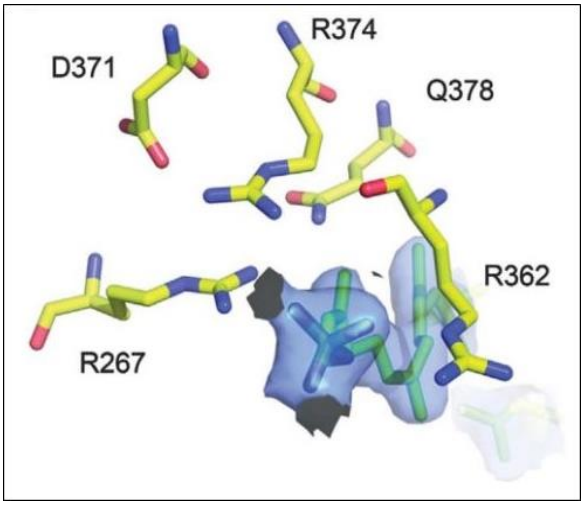

Figure 3. *Lipid binding by the LCP domain of Cps2A in Streptococcus pseudomonas.* Figure displays the decaprenyl-phosphate binding to Cps2A, as well as the two key residues of this study: D371 and R374 (D375 and R378 in GBS) (11).

1.6 Research Objective

The primary objective of this study is to gain insight into the functions of CpsA through LytR mutations. To test the hypothesis that there are key amino acid residues of the LytR Domain essential to the functionality of CpsA, point-mutations at Aspartic Acid-375 and Arginine-378 (correlating to D371 and R374 in *S. pneumoniae*) of the LytR domain will be generated in GBS. The effects of these point mutations will help elucidate the functions of key residues within the LytR domain and help determine if they are essential for GBS capsule production or capsular attachment to the cell wall. The findings of this study may contribute to future medical approaches to treating and preventing GBS infections. In determining which amino acids are essential to CpsA and capsule regulation, researchers could develop a targeted therapy that reduces GBS virulence and promotes the normal function of the innate immune system.

2. MATERIALS AND METHODS

2.1 Plasmid Construction

2.1.1 Plasmid Preparation

The pLZ12-*rofA*-pro shuttle vector (can replicate in *E. coli* and *Streptococci*) containing a Chloramphenicol resistance marker was provided by Dr. Melody Neely (University of Maine, Orono, Maine, USA). The plasmid underwent a restriction enzyme digestion in order to prepare it for ligation. The plasmid was digested with Bam*HI* and *Pst*I and was verified to be ~4500bp by gel electrophoresis on a 0.8% agarose gel. The digested plasmid was then gel isolated using a GeneJET Gel Extraction Kit (ThermoFisher Scientific).

2.1.2 Splicing by Overlap Extension – Polymerase Chain Reaction (SOE-PCR)

cpsA mutations D375A and R378A were created in the *cpsA* gene of GBS using a three-reaction PCR procedure (SOE-PCR) to create a mutant *cpsA* fragment. The first PCR used GBS genomic DNA as template with primers 5' GBS-CpsA-RBS-BamHI and 3' CpsA-D375A-R378A and Q5 High-Fidelity DNA Polymerase. Following the PCR, the reaction was verified to be ~1145bp using gel electrophoresis on a 0.8% agarose gel and the band was extracted and purified using a GeneJET Gel Extraction Kit (ThermoFisher Scientific). The purified mutant *cpsA* DNA fragment (Fragment 1) was stored in a 1.5mL microcentrifuge tube at 4℃.

The second PCR used GBS genomic DNA as template, primers 5' CpsA-D375A-R378A and 3' GBS-cpsA-full-PstI and Q5 High-Fidelity DNA Polymerase (New

England BioLabs). Following the PCR, the reaction was verified to be ~350bp using gel electrophoresis on a 0.8% agarose gel and the band was extracted and purified using a GeneJET Gel Extraction Kit (ThermoFisher Scientific). The purified mutant *cpsA* DNA fragment (Fragment 2) was stored in a 1.5mL microcentrifuge tube at 4℃.

In the final PCR, a SOE reaction was performed to create the full mutant *cpsA*. Fragment 1 and Fragment 2 were used as template with outside primers 5' GBS-CpsA-RBS-BamHI and 3' GBS-cpsA-full-PstI and Q5 High-Fidelity DNA Polymerase (New England BioLabs). Following the PCR, the reaction was verified to be ~1470bp using gel electrophoresis on a 0.8% agarose gel and the band was excised and purified using a GeneJET Gel Extraction Kit (ThermoFisher Scientific). Once purified, the full-length mutant *cpsA* fragment was digested with *Bam*HI and *Pst*I using the same protocol as listed in *2.1.1 Plasmid Preparation*. The digested mutant *cpsA* DNA fragment was then ligated with the digested pLZ12-rofA-pro vector from protocol *2.1.1.* In addition, a negative control consisting of just the digested vector and no mutant *cpsA* was also ligated. The ligation reaction mixtures were incubated at room temperature for 30 minutes. After the ligation reactions, any remaining salts in the products were removed using butanol precipitation.

2.1.3 Electrotransformation of *Escherichia coli*

Competent *Escherichia coli* were provided by Dr. Melody Neely (University of Maine, Orono, Maine, USA) and used as a primary means of transformation to complete the previous ligation reactions and seal any DNA nicks. 40 microliters of electro-competent *E. coli* cells were mixed with 5uL of the ligated mutant *cpsA* plasmid

Electrotransformation was performed using a Gene Pulser II Electroporation System (BIO-RAD) set to 2.5µm, 2.5kV, and 200Ω. The electroporated cells were immediately transferred into 1 mL of SOC medium and incubated at 37℃ for 1.5 hours in a shaking water bath. Following the incubation, the transformant groups and negative control were plated on LB agar plates supplemented with Chloramphenicol (20µg/mL) to select for transformants.

2.1.4 Colony PCR and Plasmid Analysis

Several transformed colonies were selected and used in a colony PCR to confirm the presence of the *cpsA* gene using Dreamtaq Green PCR Mastermix (ThermoFisher Scientific) and primers 5' GBS-CpsA-RBS-BamHI and 3' GBS-cpsA-full-PstI. After performing the colony PCRs, the reactions were verified for size through gel electrophoresis on a 0.8% agarose gel. Band length results were compared against a 1kb ladder, and a positive and negative control. Colonies that were verified to contain the mutant *cpsA* plasmid were inoculated into Luria Broth (LB) with Chloramphenicol (20ug/mL) and used in a plasmid preparation using the Hi-Pure Plasmid Midiprep Kit (ThermoFisher Scientific). Following plasmid isolation, the concentration of each plasmid DNA was analyzed using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). The products were then sequenced by Patty Singer (University of Maine Sequencing Facility, Orono, Maine, USA) to verify that the mutant plasmid contains the D375A and R378A mutations and no other mutations in the *cpsA* sequence.

2.2 Electrotransformation of *Streptococcus agalactiae*

Wild-Type and ΔCpsA Group B *Streptococcus* 515 cultures were grown overnight at 37℃ in Todd-Hewitt Yeast Broth (THYB) supplemented with 80 mM of Glycine. The overnight cultures were then diluted into 25mL of THYB supplemented with 80 mM of Glycine at a dilution rate of 1:20. The subcultures were grown in a shaking water bath at 37℃ to an OD600 of 0.400 and then harvested by centrifugation, washed three times with 10 mL off chilled 10% glycerol, and resuspended in 1mL of chilled 10% glycerol, followed by incubation on ice. The mutant plasmid was transformed into Wild-Type GBS 515 and into ΔCpsA GBS 515 using 3uL of the plasmid DNA and 200uL of the GBS cells. Electroporation was performed with a Gene Pulser II Electroporation System (BIO-RAD) at 25μm, 2.0kV, and 400Ω. The electroporated cells were immediately transferred to 10mL of fresh THYB medium and allowed to recover for 90 minutes at 37℃ before plating on THY agar with 3ug/mL of Chloramphenicol. Two negative controls were also electroporated, consisting of either Wild-Type or ΔCpsA GBS 515 cells and no added plasmid.

2.3 Microscopy

Light microscopy was performed on Wild-Type GBS 515, ΔCpsA GBS 515, Wild-Type GBS 515 + pCpsA Mutant, and ΔCpsA GBS 515 + pCpsA Mutant strains using a compound microscope (Zeiss Axiostar), visualized at a magnification of 1000X. Five fields of view for each strain were saved in JPEG format and the average cocci per chain was calculated manually.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Overnight cultures of each experimental strain (Wild-Type GBS 515, ΔCpsA GBS 515, Wild-Type + mutant plasmid, and ΔCpsA + mutant plasmid) were grown in 10mL THYB supplemented with Chloramphenicol(3ug/mL).

Pre-absorption of the secondary antibody was performed by mixing 2.5mL of both Wild-Type and ΔCpsA GBS 515, followed by centrifugation and resuspension in 1mL of Tris-Buffered Saline (TBS). Secondary antibody (goat, anti-rabbit IgG conjugated to alkaline phosphatase) was added at a 1:100 dilution and incubated at 4℃ for 1 hour on a rotator. After the incubation, cells were pelleted by centrifugation and the supernatant was collected and filtered with a 0.22µm syringe filter. This resulted in obtaining ~1mL of 1:100 pre-adsorbed secondary antibody.

The remaining overnight cell cultures were normalized to an OD600 of 0.750. Normalized samples were then pelleted via centrifugation, resuspended in 1mL TBST, and vortexed. The samples were pelleted again via centrifugation and resuspended in mL TBST. Primary antibody (rabbit - anti-serotype 1a) at a final dilution of 1:20,000 was added and incubated at 4℃ on a rotator for 1 hour. Cells were pelleted via centrifugation and washed three times with TBST, vortexing after each wash. The cells were resuspended in 1mL of TBS-T. Next, the pre-adsorbed secondary antibody was added to each cell sample to a final dilution of 1:5,000. The cell samples were then incubated at 4℃ for 1 hour on a rotator. Following the incubation, cell samples were pelleted via centrifugation and then washed three times with TBST. The cells were then resuspended in 1mL of TBS-T. Following this, an alkaline phosphatase assay was performed on all reactions in a 96-well plate, using pNPP as substrate and 50uL of cells per well. Assays

were performed in triplicate at no dilution, a 1:2 dilution, and a 1:4 dilution. Following the addition of cells and pNPP to each well, the contents of the 96-well plate were incubated in the dark at room temperature for 1 hour. The plate was then read at OD405 and OD600 using a 96 Microplate Reader (VersaMax).

2.5 Fluorescent Vancomycin Assay

Overnight cultures of negative control groups (Wild-Type GBS 515 and ΔCpsA GBS 515) and mutant plasmid *cpsA* in GBS 515 Wild-Type and ΔCpsA, were grown in THYB with 3ug/mL of chloramphenicol). Overnight cultures were subcultured into THYB+Chloramphenicol (3ug/mL) and grown to mid-log phase (OD600=0.300). The cultures were then concentrated to an OD600 of 1.0. Ten microliters of the cultures were incubated with 1ug/uL of BODIPY_FL Vancomycin (ThermoFisher Scientific) at 37℃ to allow for the incorporation of the fluorescent stain into newly formed cell walls. The cell cultures were then washed three times in Phosphate-Buffered Saline (PBS) and then resuspended in 3uL PBS. Cells treated with fluorescent vancomycin were observed as a wet mount at 1000X on a fluorescent microscope (Zeiss Axiostar).

2.6 Zebrafish Immune Response Assay

2.6.1 Preparation

Zebrafish were bred and 2-day old embryos were used for infections. The day before injecting zebrafish larvae, overnight cultures of Wild-Type GBS 515 with pLZ12-*rofA*-pro vector, ΔCpsA GBS 515 with pLZ12-*rofA*-pro vector, and ΔCpsA GBS 515

with the mutant *cpsA* plasmid were grown in THYB broth supplemented with Chloramphenicol (3ug/mL).

2.6.2 Bacterial Dosage

Overnight bacterial cultures were subcultured at a dilution of 1:20 into THYB supplemented with Chloramphenicol (3ug/mL). Subcultures were incubated at 37℃ until the culture reached the mid-log phase of growth. Subcultures were normalized to an OD600 of 0.225. Following normalization, 1 mL of each cell sample was pelleted via centrifugation at 14,000 RPM for 5 minutes. The supernatant was then removed, and the pellet was resuspended in 1 mL of sterile THYB. The sample was pelleted and resuspended again in the same manner and then placed on ice. The cell samples should now be at a concentration of 1 x 10^8 CFU/mL.

2.6.3 Inoculum Dose Verification

Each of the tested groups were serially diluted out to 10^-7 and 100uL of the 10^-6 and 10^-7 dilutions were plated on THY Agar-Chloramphenicol (3ug/mL) plates. The plates were incubated for 24 hours at 37℃ in 5% CO2 and then counted to verify that the concentration of the cell samples were 1x10^8 CFU/mL.

2.6.4 Microinjections into the Yolk Sac

Dechorionated embryos were anesthetized by placing them in 25 mL of fish embryo water supplemented with 2mL of Tricaine. A microinjector needle was cut and

calibrated to release ~1nL of the cell suspension per injection. Fish were injected with 100 CFU (1nL of 1x10^8 CFU/mL) of each strain or with sterile media as a negative control. Once the needle was loaded with the correct dose + 0.1% phenol red, anesthetized fish embryos were stabilized in methylcellulose on an agar injection plate. The fish were then injected in the lower part of their yolk sacs and washed into fresh fish water without tricaine to rinse off the methylcellulose. The fish were then immediately placed into a well of a 6-well plate. This procedure was repeated for each of the tested groups, using 20 zebrafish larvae for each experimental group and 10 zebrafish larvae for the negative control group (sterile media). The zebrafish were then incubated at 27-29℃ and monitored for survival over a period of 72 hours, removing any dead larvae as the monitoring progressed.

3. RESULTS

3.1 Chain Length Analysis using Microscopy

GBS chain lengths are dependent on cell wall interactions (14). For this reason, if the LytR Domain mutations influence cell wall stability and CpsA ligation to the cell wall, then a change in chain length phenotype may be displayed in the mutant GBS strain. Analysis of the chain lengths of each experimental strain of GBS 515 revealed that the wild-type chain length frequency (Figure 4A) remained consistent with the expected short-chain phenotype. As demonstrated in previous research (1), the *ΔcpsA* GBS chain length frequency (Figure 4B) remained consistent with the expected long-chain phenotype. Wild-Type-pmutant*cpsA* (Figure 4C) and *ΔcpsA*-pmutant*cpsA* (Figure 4D) strains exhibited short-chain phenotypes. A comparison of the average chain length between experimental groups (Figure 5E) displayed that the wild-type GBS strain had an average chain length of ~2.79 cocci, which is consistent with the normal amount of cocci seen in wild-type GBS (1). The *ΔcpsA* GBS strain had an average chain length of ~9.92 cocci, consistent with the long chain phenotype seen in the *cpsA* deletion strain (8). Wild-Type-pmutant*cpsA* strain had an average chain length of ~3.84 cocci and the *ΔcpsA*-pmutant*cpsA* strain had an average chain length of ~2.9 cocci. Statistical analysis of the data reveals that the average chain length of the *ΔcpsA*-pmutant*cpsA* strain complimented the average chain length of the wild-type GBS strain. Additionally, the data revealed that the increase in average chain length of the Wild-Type-pmutant*cpsA*, as compared to the wild-type strain, are statistically significant; However, with an average chain length of 3.84, the Wild-Type-pmutant*cpsA* strain has a chain length of between 2 and 4 cocci,

which is in the range of a normal, short-chain phenotype as exhibited by wild-type in a previous study (1). The short-chain phenotypes demonstrated by the mutant *cpsA* strains of GBS suggest that the D375A and R378A mutations are not effective in altering the functionality of the LytR Domain and therefore the cell wall interactions of GBS.

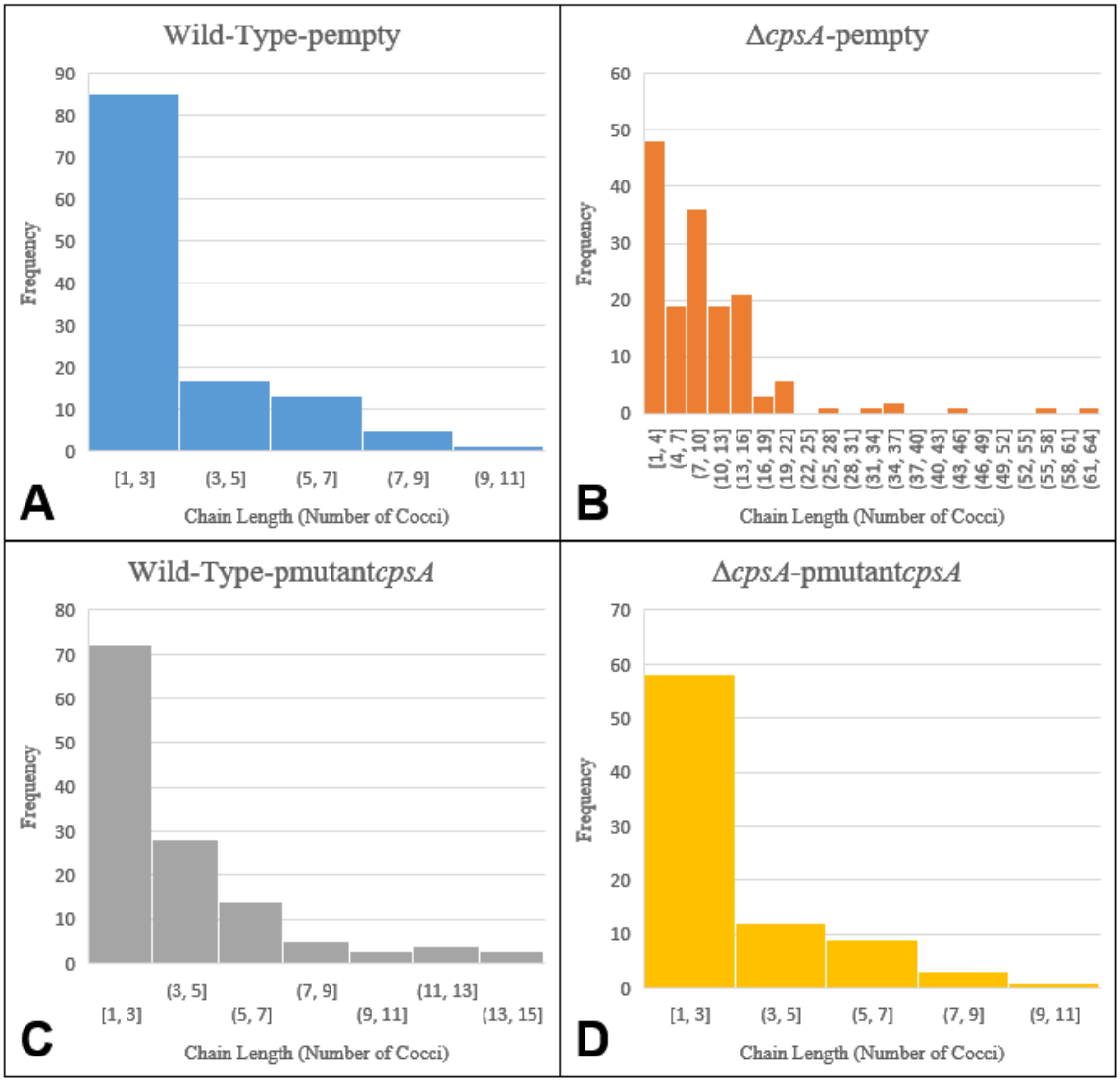

Figure 4. *Chain length frequency of experimental GBS 515 strains.* Five fields of view per group were analyzed to calculate the chain length distribution of each experimental group. A) Chain length frequency of Wild-Type GBS 515 strain containing an empty plasmid. B) Chain length frequency of *ΔcpsA* GBS 515 strain containing an empty plasmid. C) Chain length frequency of Wild-Type GBS 515 strain containing a mutant

cpsA plasmid. D) Chain length frequency of the *ΔcpsA* GBS 515 strain containing a mutant *cpsA* plasmid.

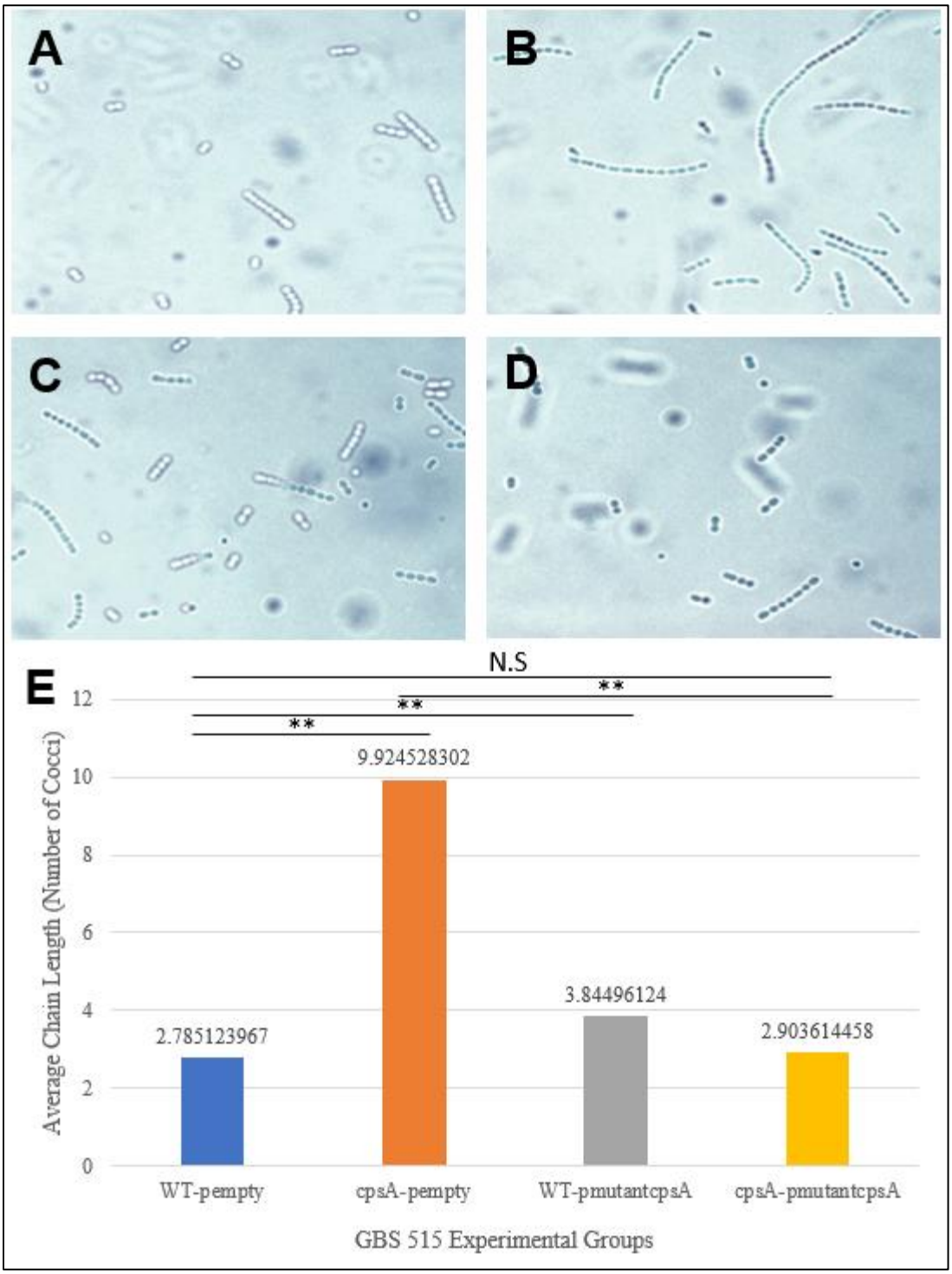

Figure 5. *Effect of mutations on chain length*. Analysis of overnight GBS cultures of Wild-Type with an empty plasmid (A), Δ*cpsA* with an empty plasmid (B), Wild-Type with a mutated Δ*cpsA* plasmid (C), and Δ*cpsA* with a mutated Δ*cpsA* plasmid (D) by

microscopy was performed. Five fields of view for each experimental group were visualized and the average number of cocci per chain was calculated (E). WT-pempty has an average chain length of 2.79 cocci, Δ*cpsA*-pempty has an average chain length of 9.92 cocci, WT-pmutant*cpsA* has an average chain length of 3.84 cocci, and Δ*cpsA*-pmutant*cpsA* has an average chain length of 2.90 cocci. Error bars represent the standard error. **, P<0.01. N.S., P=0.695.

3.2 Capsule Quantification through an Enzyme-Linked Immunosorbent Assay

An Indirect Enzyme Linked Immunosorbent Assay (ELISA) was used to quantify the amount of capsule expressed in the experimental GBS 515 groups. If the D375A and R378A mutations were effective in reducing capsule production or capsular ligation to the cell wall, then there should be an overall decrease in capsule expression when comparing the mutant strains of GBS to the wild-type strain. ELISA results of each experimental GBS 515 group revealed that Wild-Type-pempty has significantly more capsule present than *ΔcpsA*-pempty (Figure 6). The Wild-Type-pmutant*cpsA* GBS strain displayed an expression of 5992.29 capsular units and the *ΔcpsA*-pmutant*cpsA* GBS strain displayed an expression of 6280.153 capsular units. In comparison to the Wild-Type-pempty strain, which expressed 6514.8 capsular units, the capsular levels of the Wild-Type-pmutant*cpsA* and the *ΔcpsA*-pmutant*cpsA* strains were decreased, however the decreases were not statistically significant to confirm a correlation by the effects of the mutations. These results suggest that the D375A and R378A mutations are not effective in decreasing the amount of capsule that is expressed on the cell wall of GBS. The results of this data are based on a single experiment consisting of three dilutions, with each dilution performed in triplicate, and therefore further ELISA trials are required to confirm the accuracy of the presented data.

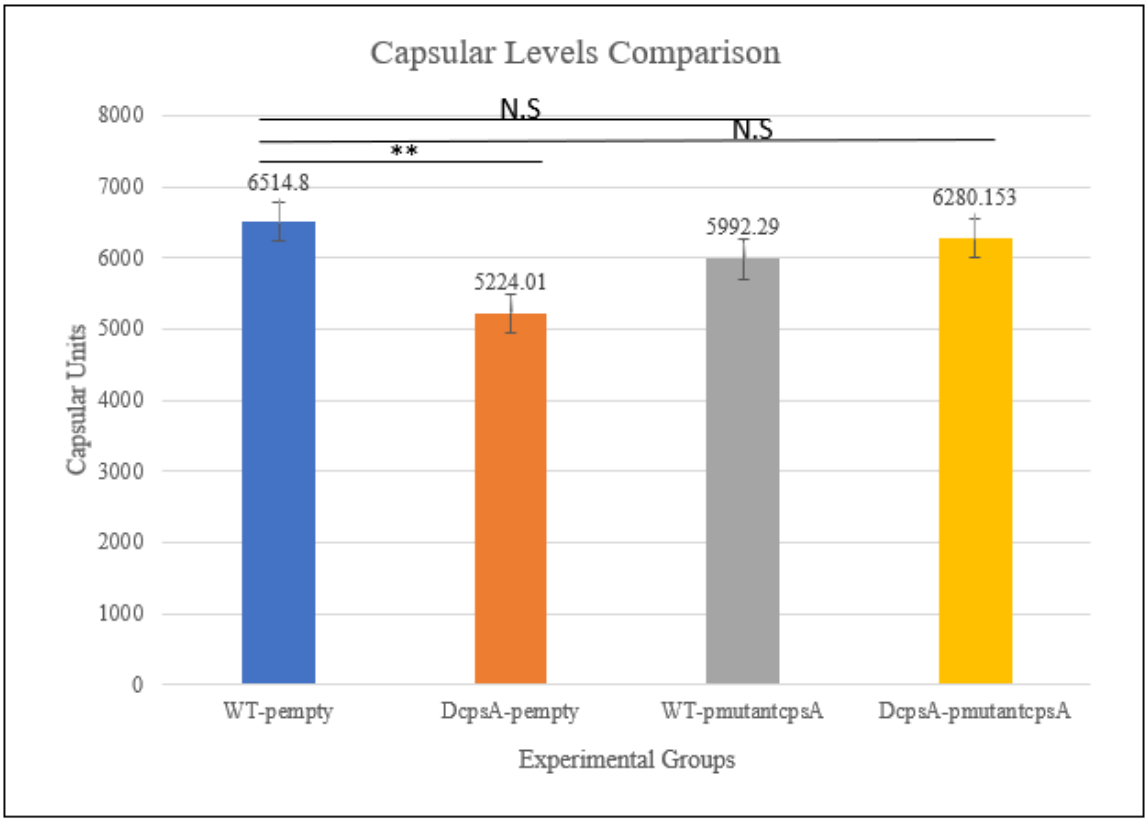

Figure 6. *Comparison of levels of capsule present in GBS experimental groups.* Capsule levels were calculated using the average of nine ELISA results per group, after subtracting background quantities. Error bars represent the standard error. **, P<0.02. N.S., P>0.10.

3.3 Changes in Cell Wall Morphology

The Fluorescent Vancomycin Assay was performed to determine the effect that the D375A and R378A mutations have on the cell wall morphologies of GBS. If D375 and R378 play a significant role in cell wall stability, then the mutant strains of GBS should display as having abnormal cell wall morphologies. Results of the Fluorescent Vancomycin Assay show that the cell wall morphologies of the Wild-Type-pempty GBS 515 strain are relatively stable, indicated by a bright ring of green fluorescence surrounding the individual cocci (Figure 7A). A *cpsA* deletion in GBS 515 causes cell wall abnormalities and instability, demonstrated by cocci that are equally fluorescent throughout, indicative of a leaky cell wall (Figure 7B). The Wild-Type-pmutant*cpsA* and

Δ*cpsA*-pmutant*cpsA* GBS 515 strains display varying results, with most cells in the observed fields of view displaying stable cell walls, similar to the results demonstrated by the Wild-Type-pempty GBS 515 strain (Figures 7C and 7D). Further experimentation and analysis is necessary to accurately quantify the overall cell wall stability of the GBS strains.

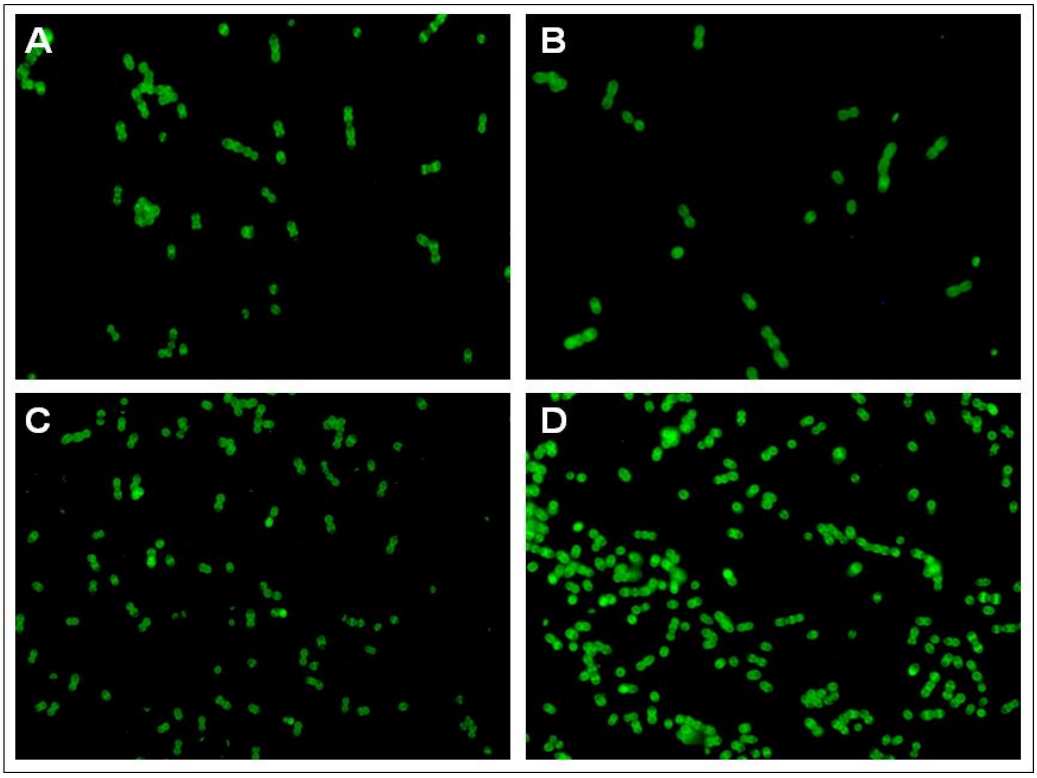

Figure 7. *Effects of mutations on GBS cell wall morphology.* The cell wall morphologies of Wild-Type-pempty (A), ΔcpsA-pempty (B), Wild-Type-pmutantcpsA (C), and ΔcpsA-pmutantcpsA (D) were observed by fluorescent microscopy after incubating with 1ug/uL BODIPY_FL Vancomycin (ThermoFisher Scientific).

3.4 Zebrafish Survivability Assay

Previous research shows that a *cpsA* deletion strain of GBS has an attenuated virulence in a zebrafish model of infection (1). Therefore, cpsA expression and

functionality is shown to be a key determinant in the infectivity of GBS. To test if the D375A and R378A mutations are effective in reducing the virulence of GBS, zebrafish larvae were infected with ~100 CFU of the experimental GBS strains and monitored for their survivability. If the D375A and R378A mutations reduce GBS virulence, then there should be an increased survivability compared to wild-type GBS infected zebrafish. Zebrafish infection results reveal that the Δ*cpsA*-pmutant*cpsA* GBS 515 strain is comparable to the Δ*cpsA*-pempty GBS 515, yet to a lesser extent (Figure 8). Twenty-Four hours post-infection, 70% of Wild-Type-pempty, 95% of Δ*cpsA*-pempty, and 75% of the Δ*cpsA*-pmutant*cpsA* injected zebrafish were alive. However, 48 hours and 72 hours post-infection, while all the Wild-Type-pempty GBS 515-infected fish had succumbed to the infection, 30% of the Δ*cpsA*-pempty, and 25% of the Δ*cpsA*-pmutant*cpsA* groups had survived the infection. These results demonstrate that the mutations created in *cpsA* may influence GBS virulence. The results of this experiment were compiled from a single trial, using 20 zebrafish per experimental group, and therefore the experiment needs to be repeated to confirm the effects of the mutations. Further trials are required to determine if the data is statistically significant.

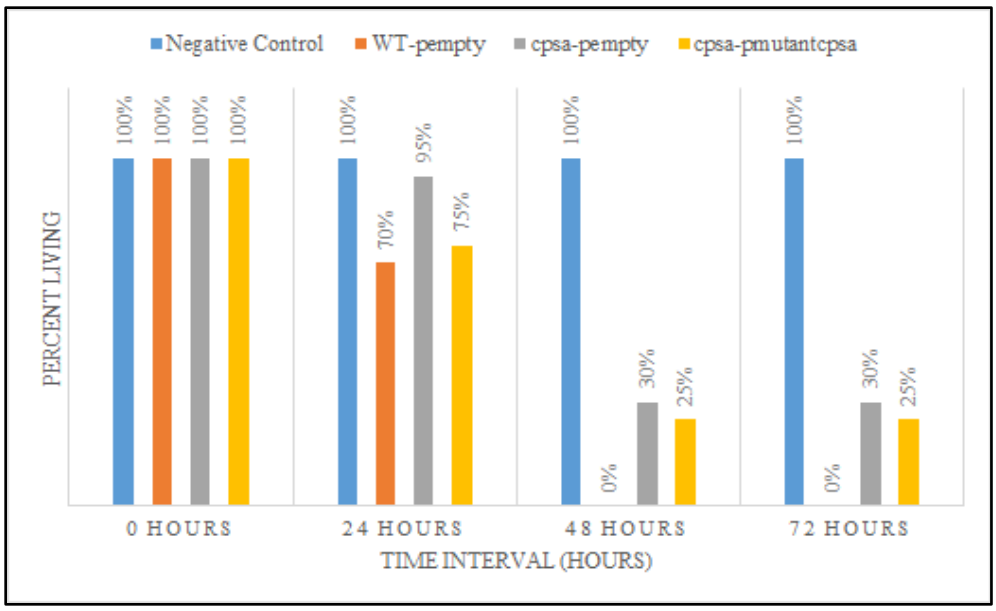

Figure 8. *Effects of mutations on GBS virulence.* Experimental strains were injected into zebrafish at a volume of 1nl, which was ~100 CFU. Every 24 hours, over a period of 72 hours, living fish were counted and a percentage of the surviving fish was recorded.

4. DISCUSSION

Despite medical advancements, such as pre-screening and intrapartum antibiotic prophylaxis (IAP), GBS infections remain a predominant cause of early-onset disease in neonates (15). Additionally, the incidence of invasive GBS infections in adults has increased over the past eleven years, most likely due to the increase in a prevalence of chronic diseases, such as diabetes and obesity (16). Current treatments for GBS infection include common antibiotics, such as penicillin, administered during labor, delivery, or after diagnosis of GBS infection. Antibiotics, however, are not a lasting treatment as antibiotic resistance becomes more prevalent in GBS populations (16). For this reason, new therapies targeting GBS virulence factors, such as capsular polysaccharide and β-hemolysin, are being researched (17).

The streptococcal CpsA protein is a member of the LytR-CpsA-Psr (LCP) family; a multifunctional family of proteins involved in attaching carbohydrates, capsular polysaccharide, and teichoic acids to the cell wall in various Gram-positive bacterial populations (1). Although the major functions of the LCP family have been identified, the mechanisms by which the LCP family functions is still mostly unknown.

CpsA is a putative transcriptional regulator of the capsule locus and is therefore an integral cog in the capsule-regulating machine. In a previous experiment, an in-frame deletion of *cpsA,* the protein-coding gene of CpsA, was performed in GBS which resulted in reduced levels of capsule (18), reinforcing the significance of the gene. The GBS capsule is one of the key virulence factors contributing to the pathogenicity of GBS. The capsule functions as a protective layer surrounding the cell, aiding in evasion of host immune responses and the binding of host cells. Hanson et al, demonstrated that a Δ*cpsA*

strain of GBS 515 had reduced levels of capsule, as well as a significantly reduced virulence in a zebrafish infection model (14).

The CpsA protein contains three major domains: an intracellular DNA-binding domain, an extracellular accessory domain, and an extracellular LytR domain. The LytR domain, predicted to be involved in capsular attachment and cell wall stability, was the central focus of this study. Kawai et al elucidated the crystal structure of the LytR domain in the CpsA protein of *Streptococcus pneumoniae* and determined which residues were potentially essential to its function (11). In their work, they found a lipid molecule buried in the LytR domain active site, leading them to suggest that this domain may play a key role in the ligation of CPS to the cell wall. Due to the significant homology between *S. pneumoniae* and GBS, the crystal structure solved by Kawai was used as a guide to discern which residues should be mutated in the GBS *cpsA* gene. Aspartic Acid-375 and arginine-378 in GBS, correlating to aspartic acid-371 and arginine-374 in *S. pneumoniae*, are predicted to interact with one another in order to form and stabilize a positively charged pocket containing the phosphate head group of the buried lipid (11). Aspartic Acid is a negatively charged, polar amino acid, and arginine is a positively charged, polar amino acid. Due to the properties of these amino acids, along with the proximity between the two residues as displayed in the Cps2A crystal structure (Figure 3), it is predicted that there may be an attraction between D375 and R378, contributing to the protein-folded structure of CpsA in GBS (11). In this study, D375 and R378 were both mutated to alanine molecules. Alanine is a small, neutral, and hydrophobic molecule. In replacing the two oppositely-charged amino acids with neutral molecules, potential interactions between the original two residues may be decreased, thus reducing the stability of the

phosphate head group interaction with the positively-charged pocket within the LytR active site. In doing so, experimental analysis of the mutant *cpsA* strains can be used to elucidate the mechanism behind the functions of the buried lipid within the LytR domain.

The chain length of GBS is determined by various factors, such as the production of autolysins. All the factors contributing to GBS chain length are not known, however capsular levels have been demonstrated to also be a factor (14). Due to the role that CpsA plays in capsular levels of GBS, microscopy analysis of the *cpsA* mutant strains of this study were performed (Figure 4). Wild-Type-pempty and Δ*cpsA*-pempty GBS 515 strains were significantly consistent with the respective short-chain and long-chain phenotypes demonstrated in the literature (14), with a p-value of less than 0.01. In comparing the Wild-Type-pmutant*cpsA* and Δ*cpsA*-pmutant*cpsA* GBS strains to the empty plasmid strains, it was determined that the mutant strains were not consistent with the Δ*cpsA*-pempty GBS 515 strain. Additionally, the Δ*cpsA*-pmutant*cpsA* strain displayed as having an average capsular level like that of the Wild-Type-pempty GBS 515 strain (Figure 6). The comparable results of the Δ*cpsA*-pmutant*cpsA* strain to the Wild-Type strain may allude to the failure of the D375A and R378A mutations to significantly affect capsular-cell wall interactions within GBS. This, however, is contradicted in the comparison of the Wild-Type-pmutant*cpsA* strain to the Wild-Type-pempty strain. Microscopy results revealed that the average chain length of the Wild-Type-pmutant*cpsA* strain was still significantly less than that of the Δ*cpsA*-pmutant*cpsA* strain, but slightly larger than the Wild-Type-pempty strain. Although these results are variable, the differences are not substantial enough to resemble the Δ*cpsA*-pmutant*cpsA* strain and additionally, the results

of the chain length analysis are comparable to the expected chain length analysis results of wild-type GBS in previous research (1).

The Enzyme-Linked Immunosorbent Assay data of this study similarly demonstrates the ineffectiveness of the mutations to significantly affect capsular interactions that would result in reduced levels of attached capsule. As described in previous literature, Δ*cpsA*-pempty GBS 515 strain had significantly reduced levels of capsule when compared to the Wild-Type-pempty strain (Figure 6). Further ELISA trials are required to confirm the accuracy of the presented data.

The LytR domain is predicted to have functions in capsular attachment to the cell wall as well as in cell wall stability. If mutations in the LytR domain reduced the regulation of cell wall stability in GBS, then the cell wall of GBS would be abnormally formed, unstable, and non-uniform. A fluorescent vancomycin assay was performed as a means of visualizing the cell walls of each of the experimental groups (Figure 7). Fluorescently-tagged vancomycin binds to the cell wall of GBS and is unable to penetrate through the bacteria, unless the cell wall is unstable. This is demonstrated in a comparison between the Wild-Type-pempty and Δ*cpsA*-pempty GBS 515 strains. The Wild-Type-pempty strain displays as round cocci, with fluorescent green rings surrounding many of the bacteria (Figure 7A). This differs from the Δ*cpsA*-pempty GBS 515 strain which demonstrated as having variable results, with many of the cocci fluorescing all throughout, indicating a weak and leaky cell wall (Figure 7B). Results of the mutant strains also reveal varying phenotypes; however, many of the cocci are normal and resemble the Wild-Type-pempty phenotype. This data supports the previous evidence

that the D375A and R378A mutations do not have a significant, negative effect on capsular-cell wall interactions within GBS 515.

Ultimately, *cpsA* mutations are being constructed and analyzed to better understand the function of the GBS CpsA protein in expression of capsular polysaccharide. In doing so, mutated GBS with reduced levels of capsule are not as virulent in hosts that don't have a developed immune system (1). Larval zebrafish infections of mutant GBS were used in this study to test the virulence of the experimental GBS 515 strains. Larval zebrafish are excellent model organisms of infection to demonstrate the effect of pathogens on a host without an adaptive immune system, such as human neonates. In this study, experimental strains of GBS 515 were injected into twenty zebrafish per strain at a volume of 1nl, which was ~100 CFU. The GBS injections were performed in the yolk-sac of the zebrafish to create a systemic GBS infection. The zebrafish were monitored for survivability over a period of 72 hours. As predicted, over a 72-hour period, the Wild-Type-pempty GBS infections led to the complete death of all tested zebrafish and the Δ*cpsA*-pempty GBS infections led to a greater level of survivability at a 30% survival rate. Interestingly, the Δ*cpsA*-pmutant*cpsA* GBS strain displayed a 25% survival rate of the infected zebrafish population. The survivability assay was only performed once, and further trials are required to determine if the data is statistically significant.

The lack of significant changes in capsular levels, cell wall stability, and capsular interactions demonstrated in this study confirm that the D375A and R375A mutations were not an effective means of decreasing GBS 515 virulence. Future experimentation to further elucidate the role of the buried lipid in the LytR domain of GBS CpsA would be

to mutate other significant residues that interact to form the positively-charged pocket of the LytR domain. Additionally, mutating amino acid residues that have an attractive relationship (aspartic acid and arginine) to residues that have a repulsive relationship may have an increased effect on altering the folding and functionality of the LytR domain. Research is currently being conducted to further characterize the capsular interactions of GBS and the amino acid residues that are essential to the functions of CpsA (Melody Neely, University of Maine, Orono, ME, USA). In its entirety, the research performed in this study helps to provide additional insights to the function of the LytR domain of the CpsA protein. Specifically, it helps to specify which residues may be key to decreasing the virulence of GBS and may provide information beneficial to the development of future GBS infection treatments.

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AUTHOR'S BIOGRAPHY

Mohammad Fazeel Hashmi was born in Bangor, Maine on February 17th, 1997. He was raised in the town of Veazie, Maine, where he helped his family run their small, family-owned motel. Mohammad graduated from Bangor High School in 2015 and then attended the University of Maine, pursuing a degree in Microbiology and Molecular and Cellular Biology. While at the University of Maine, he maintained an active role in the campus community by serving as an officer for the *Student Heritage Alliance Council, Muslim Students Association, Iota Nu Kappa Multicultural Fraternity,* and the *Campus Activities Board.* Additionally, he participated in both local and widespread service projects with a goal to help better his community and the communities of others.

Upon graduation, Mohammad will continue to develop his research experience and will work as a part-time EMT. Additionally, he will be applying to medical schools, with the hopes of attending in the Fall of 2020.