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The Role of Accessory Domain in CPSA Function and Capsule Production in Group B Streptococcus

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THE ROLE OF THE ACCESSORY DOMAIN IN CPSA FUNCTION AND CAPSULE PRODUCTION IN GROUP B *STREPTOCOCCUS*

by

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ABSTRACT

Group B Streptococcus (GBS) can be found in the vaginal and genitourinary tract of females, as well as the genitourinary tract of males, where it behaves as a commensal organism. However, as an opportunistic pathogen, GBS has the capability to infect the immunocompromised, making it a major threat to neonates and fetuses. The pathogen can be passed from mother to baby either in utero or during birth. The capsule, which is a polysaccharide coating on the outside of the cell, is considered the most important virulence factor in GBS. Expression of capsule plays a role in evasion of the host immune response to GBS infection. The presence of capsule on GBS depends on the CpsA protein, which is involved in the attachment of capsule to the cell wall. CpsA is a multi-functional protein containing an intracellular domain and two extracellular domains including the accessory and the LytR domains. Previous data demonstrates a small region within the accessory domain of CpsA that, when expressed separately, can have a negative effect on the amount of capsule on the cell. In this study, cell morphology analysis and capsule assays were used to determine the role of the accessory domain on CpsA function and capsule production. The data collected in this study suggest that the accessory domain is important in capsule expression, and without the accessory domain capsule expression cannot be complemented back to WT CpsA levels. In addition, this data shows that the intradomain region plays an important, but unknown role in CpsA function. Finally, for the first time, short chain morphologies were associated with decreased capsule expression.

ACKNOWLEDGEMENTS

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Group B Streptococcus (GBS) can be found in the vaginal and genitourinary tract of females, as well as the genitourinary tract of males, where it behaves as a commensal organism. However, as an opportunistic pathogen, GBS has the capability to infect the immunocompromised, making it a major threat to neonates and fetuses. The pathogen can be passed from mother to baby either in utero or during birth. The capsule, which is a polysaccharide coating on the outside of the cell, is considered the most important virulence factor of GBS [9]. Expression of capsule plays a role in evasion of the host immune response to GBS infection. The presence of capsule on GBS depends on the CpsA protein, which is involved in the attachment of capsule to the cell wall [9]. CpsA is a multifunctional protein containing an intracellular domain and two extracellular domains including the accessory and the LytR domains [8]. Previous data demonstrates a small region within the accessory domain of CpsA that, when expressed separately, can have a negative effect on the amount of capsule on the cell [17]. In this study, the deletion of the extracellular accessory domain of CpsA will help to determine the role of this domain on CpsA function and capsule production. Cell morphology analysis, capsule assays, and virulence studies will all be used to analyze results of a deletion of the accessory domain.

Streptococcus agalactiae

Streptococcus agalactiae, commonly referred to as Group B *Streptococcus* (GBS), is the leading cause of neonatal infection, premature birth, and stillbirth [20]. Research has suggested that GBS associated infections affect 2,000 live births per year in the United States [15]. GBS are Gram positive, beta-hemolytic, chain forming cocci that frequently colonizes the vaginal and genitourinary tract of females and the genitourinary tract of males [15,20]. They are normal colonizers of the vaginal microflora in 25% of healthy women, where it behaves as a commensal organism, leaving healthy individuals unimpacted [20]. However, as an opportunistic pathogen, GBS has the ability to infect the immunocompromised. The colonization of the vaginal and genitourinary tract of females makes GBS a serious threat to neonates and fetuses because the pathogen can be passed down from mother to baby, either in utero or during birth [17]. This pathway

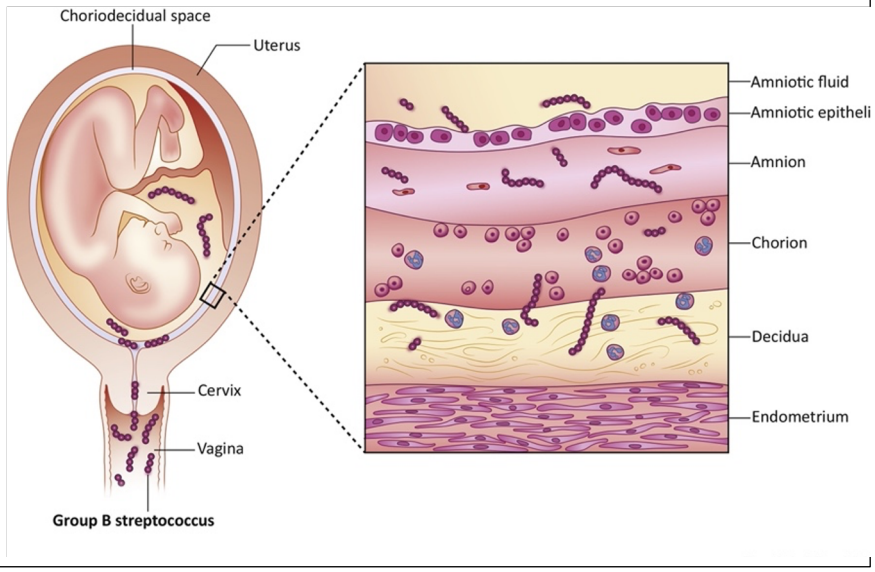


Figure 1. Ascending Group B *Streptococcus* (GBS) Infection. GBS can be passed down from mother to baby either in utero or during birth. Ascending infection during pregnancy involves bacterial colonization of the vagina, placental membranes, and amniotic cavity which increase the risk of fetal infection, injury, premature or stillbirths. (Vornhagen, *et. al.,* Trends in Microbiology. 2017. 25:919-31)

allows GBS to move from the vagina, to the cervix and into the uterus, where the fetus can become infected (Figure 1) [17]. In utero, the fetus can aspirate contaminated vaginal fluid, allowing GBS to cross the blood brain barrier and cause meningitis [5].

Worldwide, it is estimated that 18% of women are colonized, with approximately 22 million pregnant women affected worldwide [18]. A recent meta-analysis performed in 2015 suggested that there were at least 409,000 maternal, fetal and infant cases of GBS infection annually [18]. While Africa makes up only 13% of the global population, it accounts for 54% of the worldwide estimated cases, and 65% of all GBS related fetal and infant deaths [18]. Early-onset infection is defined as occurring in less than 7 days after birth and late-onset infection is defined as occurring 7 days or later [20]. The same study performed in 2015 also estimated 205,000 infant cases with early-onset disease, and 114,000 infant cases with late-onset disease [18]. Early-onset GBS infection is capable of causing premature and still births and is known to be the leading cause of neonatal meningitis and sepsis [17]. Other devastating effects of early-onset GBS infection are encephalopathy and immune impairments which can make it hard to fight other infections later in life [18]. Even individuals who overcome neonatal GBS infection can still have neurodevelopmental impairments later on in life [18]. Late onset GBS infection can also cause hearing and vision loss, learning disabilities, and seizures [18]. Some mothers and babies can recover from GBS infection, but with a mortality rate of 6%, even if they do recover there might be some impairments later on in life [18].

The current treatment recommendations for infection prevention in neonates consists of late gestation screening and intrapartum antibiotic prophylaxis (IAP) [20]. Late gestational screening indicates whether or not a pregnant female is positive for GBS

infection. IAP is administered intravenously during labor in an effort to prevent the pathogen from infecting the newborn as it passes through the birth canal. Although IAP is effective in preventing neonatal infection, the rates of stillbirths and prematurity due to GBS has not decreased [20]. While these treatments are effective in reducing the incidence of GBS infection in the first week of life, they are not ideal because treatment with antibiotics in newborns depletes normal gut flora, which are important in the development of the immune system [4]. A recent longitudinal study compared the association of infant antibiotic exposure with childhood health outcomes [2]. This group found that exposure to antibiotics within the first two years of life was associated with an increased risk of childhood-onset asthma, allergic rhinitis, atopic dermatitis, celiac disease, overweight, obesity, and attention deficit hyperactivity disorder (ADHD) [1]. Penicillin was one of the most prescribed antibiotic classes in this study and is also the standard antibiotic used for IAP in pregnant women, showing the devastating effects of infant antibiotic use [2]. Recent research also suggests that intrapartum antibiotic exposure may increase risk of sepsis from other bacteria colonizing the genitourinary tract [3]. IAP use also affects the mother, potentially increasing the risk of antibiotic resistance and adverse maternal affects such as allergic reactions [3]. While the use of IAP is not ideal for mother or baby, its use has doubled in the past 30 years, since no other ideal treatment options have emerged [3]. Late gestational screening and IAP are the only current effective treatment for GBS infection in neonates and there is no current vaccine.

Virulence Factors of GBS

GBS encodes many virulence factors that aid in its pathogenicity. Some virulence factors that promote immune evasion include superoxide dismutase, C5a peptidase, serine protease, and penicillin-binding proteins [11]. Two major virulence factors that play an important role in GBS dissemination into host cells are pore-forming toxins and sialic acid containing capsular polysaccharide [11]. Genomic sequencing data has suggested 80% similarity among GBS strains, showing the high conservation of virulence factors [11].

GBS encodes superoxide dismutase to resist reactive oxygen species from the host [11]. These enzymes convert superoxide anions to molecular oxygen and peroxide, which can then be metabolized by other enzymes like catalases or peroxidases [11]. In order to cleave and inactivate human complement component C5a, GBS encodes a C5a peptidase [11]. This aids in immune evasion because C5a plays a role in neutrophil recruitment [11]. Serine proteases cleave the extracellular matrix protein fibrinogen to fibrin like products, ultimately preventing pathogen recognition and phagocytic uptake of GBS [11]. Penicillin binding proteins are utilized by bacteria to synthesize peptidoglycan and resist phagocytosis [11, 20].

Pore-forming toxins are common in many pathogenic bacteria because they allow the entry of the pathogen into host cells [11]. GBS encodes at least two pore-forming toxins, which are denoted β-hemolysin/cytolysin (β-H/C) and Christie Atkins Munch Peterson (CAMP) factor [11]. β-H/C promotes GBS infection by allowing for the invasion of host epithelial and endothelial cells of the lung and blood brain barrier [11]. This toxin also affects the entire host by promoting liver failure and induced

inflammatory responses that can cause neurological damage [11]. On the other hand, CAMP factor is a secreted protein which is known to oligomerize and form small pores on susceptible target host cells [11]. It is hypothesized that these pore-forming toxins have compensatory mechanisms in the absence of one of them, which aids in causing a systemic infection in the host [11].

While the virulence factors described above are all important in immune evasion, sialic acid rich capsular polysaccharide is understood to be the most important virulence factor of GBS infection [8]. The capsule of GBS contains sialic acid residues that mimic host cells (Figure 2). Sialic acids are nine carbon sugars that are also typically found on glycans of host cells [11]. Utilizing this molecular mimicry, the pathogen evades the immune system and the host fails to recognize GBS as a foreign substance [11]. As a result of this evasion, the capsule also prevents complement factor C3b from being activated and consequently prevents phagocytosis of the pathogen [11].

Capsule in *Streptococcal* Species

GBS is not the only *Streptococcal* species that requires capsule for survival in host epithelial cells. The capsule operon is highly conserved among *Streptococcal* species and other Gram-positive organisms [7]. The first gene on this locus is the *cpsA* gene, which encodes one of the transcriptional regulators of the capsule operon, the CpsA protein [7]. The CpsA protein is understood to directly aid in the production of capsule and virulence of the bacteria [8]. This protein has been the main focus of much of the research regarding capsule expression and production in Gram positive organisms and is

the focus of the GBS research being performed in the Neely lab at the University of Maine.

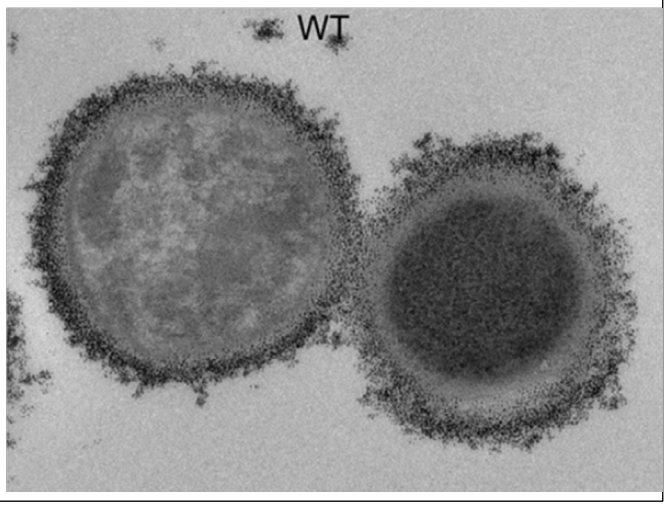


Figure 2. Electron micrograph of the capsular polysaccharide of Group B *Streptococcus.* (F.D. Xia, *et. al.* (2015). Microbes and Infection *17.* 71-76.)

In *Streptococcus pneumoniae* and *Streptococcus suis*, the presence of capsule had a significant effect on the minimal bactericidal activity of many cell wall reactive agents [6]. The first four genes of the capsule operon in *S. iniae* and GBS have 70% amino acid similarity, suggesting that CpsA is highly conserved as a control mechanism for virulence in *Streptococcal* species [8]. This observation suggests that capsule is mediated by the CpsA protein [6]. Further research demonstrates that the purified CpsA protein specifically binds to the capsule promoter region of DNA [8].

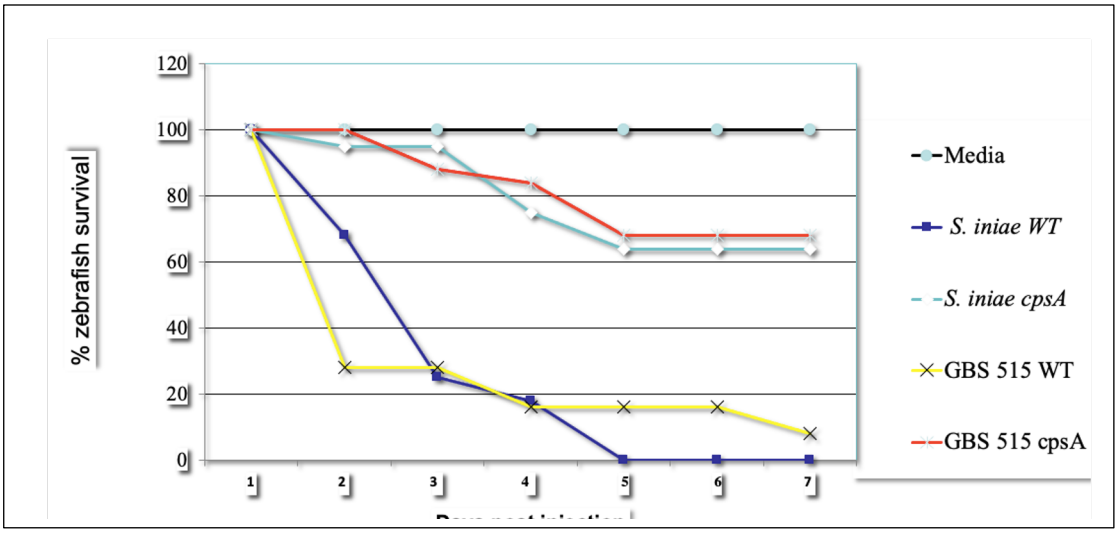


Figure 3. Percent zebrafish survival after 7 days post injection. Media was used as a negative control and 100% of zebrafish survived after 7 days post injection. In the CpsA mutant GBS and *S. iniae*, 40% of zebrafish died after 7 days post injection. In the GBS and *S. iniae* WT, 100% of zebrafish died after 7 days post injection. This data indicates that the mutant CpsA strains of *Streptococcal* species are less virulent than WT strains, suggesting that CpsA is an important virulence factor for infection. (Neely Laboratory).

Streptococcus iniae, a zoonotic pathogen that is able to infect both animals and humans, also contains the CpsA protein. Previous research of *S. iniae* infection in zebrafish showed that CpsA mutants were more susceptible to phagocytosis than WT, confirming that CpsA plays a role in capsule production and thus virulence (Figure 3) [9]. In this study, small doses of WT *S. iniae* were injected into the dorsal muscle of adult zebrafish. This induced infection resulted in a high mortality rate and large bacterial burden [9]. Injection of CpsA mutant *S. iniae* reduced lethality, however, and there was no increase in bacterial burden, suggesting that the mutated CpsA protein decreases the virulence of GBS [9]. This research summarizes that bacterium-phagocyte interaction are different between the WT and CpsA mutant, suggesting that WT is pathogenic, and the CpsA mutant is non-pathogenic, confirming its clinical relevance in the treatment of GBS infection [9]. The capsule of *S. iniae* is closely related to GBS, suggesting that these data may be generalized to include GBS [9].

CpsA Protein of GBS

CpsA has an intracellular N-terminal domain, three transmembrane domains, and an extracellular domain, including the C-terminal domain (Figure 4). The extracellular portion of the protein is broken into two domains, the accessory and LytR domains [9]. These two domains are connected by an intradomain region of uncharacterized structure and function. The extracellular domains of CpsA are known to fold separately and have separate functions (Figure 5) [9]. While the CpsA protein is a member of the LytR_CpsA_Psr protein family, it has several differences compared to other members of the family [8]. The CpsA protein is the only member of its family that possesses an extracellular accessory domain, and it also has three transmembrane domains instead of one [12].

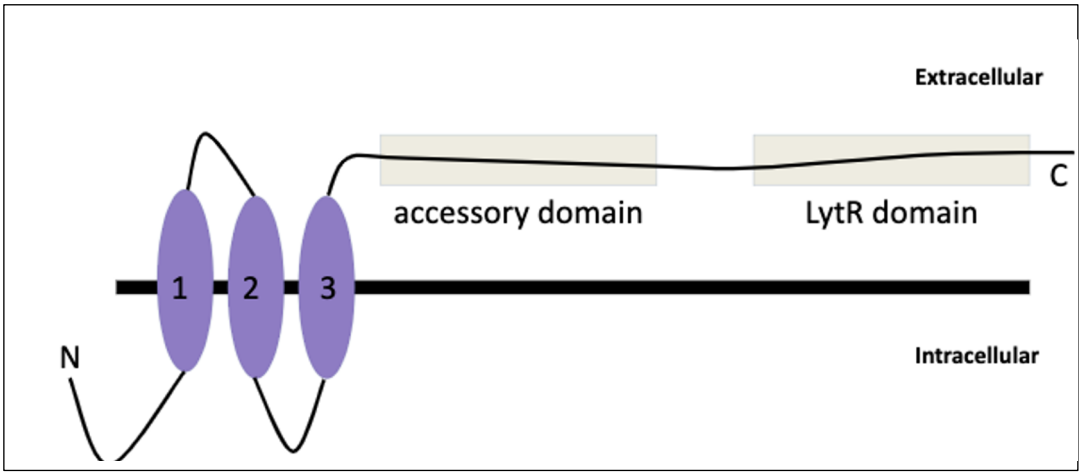


Figure 4. CpsA protein topology embedded within the cell membrane, including the N terminus, three transmembrane domains, two extracellular domains, and C terminus. (No citation, made by me)

Intracellular CpsA Domain

The intracellular N-terminal domain of CpsA is a small, cytoplasmic region that is highly conserved in both GBS and other Gram-positive organisms [8]. While the

complete function of this domain is not understood, research suggests that it does contain a DNA binding region [8]. The high density of positively charged amino acids allows this region to bind its own promoter, attracting the negatively charged backbone of DNA to bind [8].

Previous research on the intracellular domain of the CpsA proteins of *S. pneumoniae* and GBS suggests that there is a leucine zipper domain which extends into the transmembrane region and is commonly associated with DNA binding through dimerization [6]. Regardless of the similarity between the intracellular domains of GBS and *S. pneumoniae*, no data has supported similarity between these regions of *S. iniae* and GBS, suggesting the need for further research of this leucine zipper motif [6]. Although there are genomic differences between this region in *Streptococcal* species, the GBS and *S. iniae* CpsA proteins do bind DNA in vitro, however, the mechanism is unknown [7].

Extracellular Domains

The focus of this research is understanding how the accessory domain of CpsA affects capsule production and expression. It is important to understand the structure of the accessory and LytR domains of CpsA and how they function extracellularly because CpsA is the only protein in its family that carries the accessory domain. As mentioned previously, these domains fold separately and have separate functions (Figure 5) [12]. This data suggests many alpha helices in each domain, as well as an active site in the

LytR domain [12]. This region shows little structural homology to any other protein, only having 11% sequence identity with an uncharacterized peptide binding protein [12].

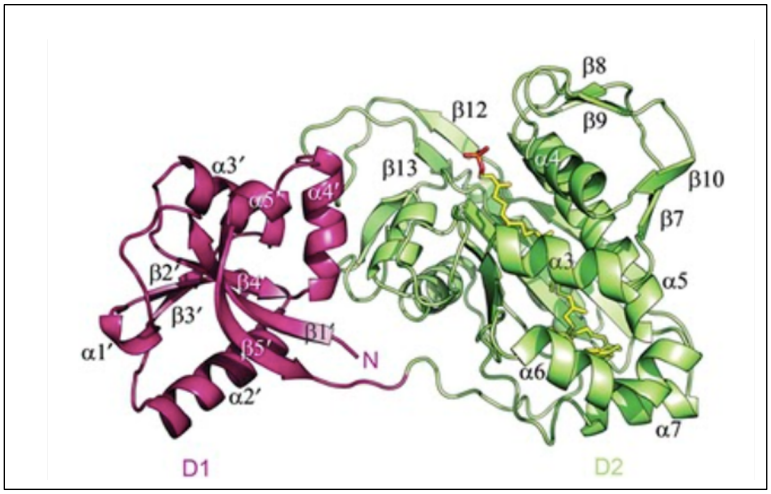


Figure 5. Crystal structure of the extracellular domains of CpsA in *S. pneumoniae*. D1 represents the accessory domain and D2 represents the LytR domain, which are connected by an intradomain region. (Kawai, *et. al.* EMBO Journal. 2011. 4931-4941)

GBS CpsA is unique in that it is the only member of the protein family which possesses an extracellular accessory domain which has no homology to any other protein domains [17]. While no previous research has deleted the accessory domain, one study suggested that, in the absence of the LytR domain, the extracellular accessory domain decreases capsule production and cell wall integrity [17]. This mutation causes a dominant negative effect, where the mutation interferes with the wild type form of the protein [17]. A dominant negative effect occurs extracellularly when the accessory domain interferes with normal mechanisms of cell wall integrity and capsule production [17]. It has been hypothesized that specific conformations of CpsA might allow the accessory domain to negatively impact CpsA binding interactions, thus decreasing capsule production and cell wall synthesis [17]. Overall, this dominant negative effect

indicates that the accessory domain plays an important role in virulence, whether it is capsule dysregulation or cell wall instability remains unknown [17]. The production of a dominant negative effect is of clinical importance in the treatment of GBS infection. Much of the function and mechanisms of the accessory domain are not understood, suggesting the need for further research to determine its implications in capsule production and GBS virulence.

Previous research performed on the extracellular domains of CpsA focused on the LytR domain, leaving the function and mechanism of the accessory domain largely unknown. Much of this research was also studied in *Streptococcal* species other than GBS, as well, making the specific mechanisms of these domains in GBS unknown as well. Previous research on the LytR domain, however, suggests that it aids in the attachment of cell wall carbohydrates, capsule, and teichoic acids to the cell wall [17]. Carbohydrate precursors are first made on the inner leaflet of the bacterial membrane, then the outer membrane, where it is hypothesized that the LytR domain acts as a ligase, attaching the precursors on the cell wall [17].

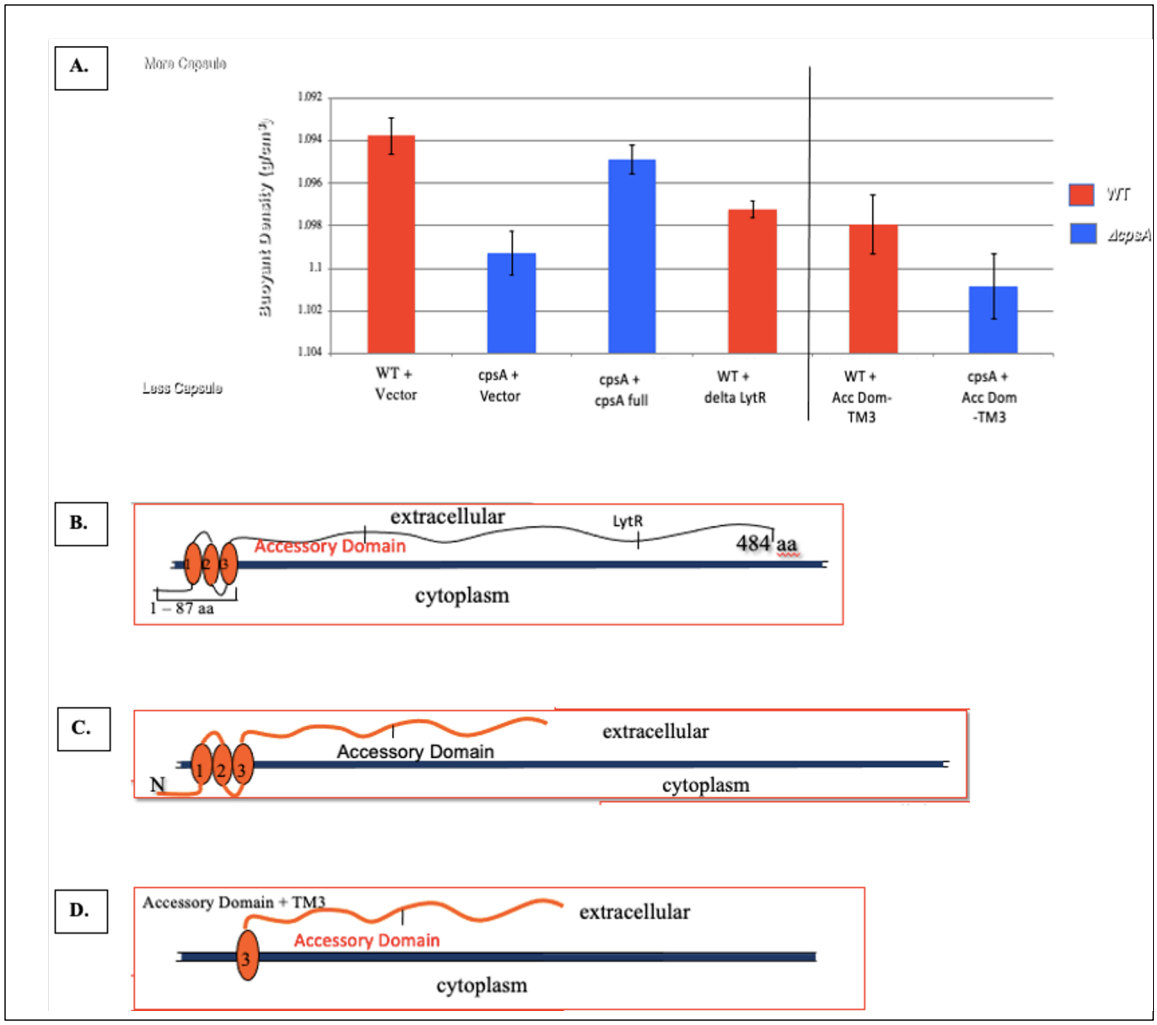


Figure 6. A) Buoyant density measurements of capsule expressed by full and truncated GBS CpsA proteins. B) Full length GBS CpsA protein. C) ΔLytR truncated GBS CpsA protein. D) ΔIntracellularDomains, ΔTransmembraneDomain, ΔLytR truncated GBS CpsA protein. (Neely lab)

Previous research performed in the Neely lab suggests that the accessory domain has some function independent of the LytR domain (Figure 6). In this study, a buoyant density assay was performed in order to determine the buoyancy of cells based on a sugar gradient which is directly correlated to capsule expression. Figure 6 displays the measurements of capsule expressed by both full and truncated versions of GBS CpsA. The first four bars were used as controls and the last two bars were the experimental samples. The first sample shows WT GBS and an empty vector, displaying the standard

amount of capsule expressed. The second bar shows the deletion of CpsA with an empty vector, showing how much less capsule is produced when CpsA is deleted from GBS. When compared to the WT + vector sample, approximately half as much capsule is expressed when CpsA is deleted. The third bar displays the deletion of CpsA with the full CpsA protein expressed on a plasmid, showing how much capsule production can be complemented back with CpsA from a plasmid. When compared to the WT + vector sample, almost all of the capsule is complemented back with CpsA from a plasmid. The fourth bar displays WT GBS and the deletion of the LytR domain expressed from a plasmid and shows the effect of capsule production if the extracellular domain is truncated. When compared to the WT + vector sample, less capsule is expressed, indicated the dominant negative effect of this mutation, because the WT CpsA is still present on the chromosome. Therefore, the presence of the truncated CpsA can override the function of the WT full length protein. The fifth bar displays the deletion of the first and second transmembrane domain, intracellular domains, and the LytR domain, leaving just the third transmembrane domain and the accessory domain remaining and expressed from a plasmid in the WT strain (WT CpsA still present). This data shows that when DNA binding domains were removed, capsule production was approximately the same as the WT + ΔLytR capsule production, supporting the hypothesis that inhibition of capsule production is due to the extracellular domain, not the intracellular domains. Finally, the sixth bar shows the CpsA deletion mutant GBS with the truncated CpsA (accessory domain and third transmembrane domain) expressed from a plasmid. When compared to the ΔCpsA + CpsA data (third bar), the data suggests that this truncated version of CpsA cannot complement back the amount of capsule production. All of this data taken

together suggests that the accessory domain functions independently of the LytR and intracellular domains, inhibiting capsule production in some way.

Research Goal

The polysaccharide capsule of GBS is suggested to be the most important virulence factor in GBS infection [8]. The sugar coating the outside of the cell wall is decorated in sialic acid residues which mimic host cells, aiding in immune evasion [21]. Because of this molecular mimicry, GBS is able to target and colonize different parts of the body, including the spleen, heart, and brain, making it the leading cause of neonatal meningitis and sepsis [20]. It is of clinical relevance to understand the mechanisms of dissemination used by GBS and other human pathogens to survive in various environments, such as the epithelium of the vaginal tract [7]. Because of the function of capsule, it is important to understand its interactions in the human body in order to further antimicrobial therapy [3].

The goal of this research study is to alter part of the CpsA protein of GBS in order to determine its function. The region of interest is the accessory domain of the CpsA protein. The CpsA protein belongs to the LytR-CpsA-Psr protein family, which is known to act as a ligase, attaching the capsule to the cell wall. The CpsA protein is the only protein in this family that has an extracellular accessory domain, but the specific function of the accessory domain remains unknown. This research study aims at determining the function of the accessory domain of the CpsA protein through its deletion and to determine the virulence of GBS with the ΔAccessoryDomain mutation.

Digestion

The plasmid pLZ12-rofA-pro (strain #12E34) was digested with *Bam*HI and incubated at 37ºC for 15 minutes. Then, the plasmid was digested again with *Pst*I and incubated at 37ºC for 30 minutes and was then moved to 80ºC for 5 minutes to inactivate the restriction enzymes. The digested plasmid was separated on a 0.8% agarose gel to check for a band at 4500 bp. GeneJet Gel Extraction Kit (ThermoFisher) was used to isolate the plasmid from the gel.

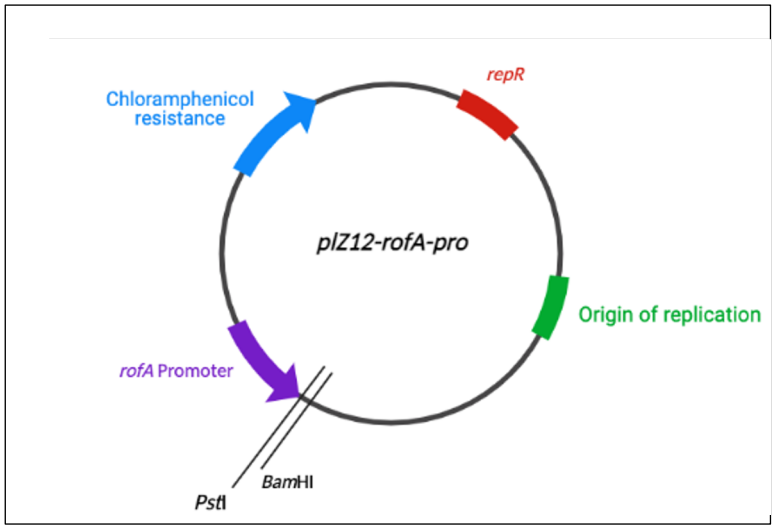


Figure 7. plZ12-rofA-pro plasmid map. Mutant strains of CpsA were inserted into the plasmid using *Bam*HI and *Pst*I restriction sites. Chloramphenicol was used as a selectable marker for positive transformants. *repR* is an initiator protein which allows the plasmid to be expressed at a low copy number. (Image created using BioRender)

Polymerase Chain Reaction (PCR)

Streptococcus agalactiae genomic DNA was used as a template to amplify the *cpsA* gene region. A high-fidelity DNA polymerase, Q5 (New England Biolabs), was

used and the primers were specific to each reaction. The long fragment (Reaction 1, Figure 8) used primers 5' GBS-CpsA-RBS-*Bam*HI and 3' GBS-CpsA-ΔAD (#1740, #1903). The short fragment (Reaction 2, Figure 8) used primers 3' GBS-CpsA-full-*Pst*I and 5' GBS-CpsA-ΔAD (#1672, #1902). PCR was performed using a Thermocycler at an annealing temperature of 68ºC. Both reactions were separated on 0.8% agarose gel to confirm fragments of ~300bp and ~1178bp. Both reactions were gel purified using the GeneJet Gel Extraction Kit (ThermoFisher).

PCR Splice Overlap Extension (SOE)

The two PCR fragments created were used as a template to SOE the fragments together, forming a full-sized fragment of ~1000 bp. Q5 DNA Polymerase (New England Biolabs) and primers #1672 and #1740 were used at an annealing temperature of 65ºC. The reaction was separated on a 0.8% agarose gel to confirm the final full-sized fragment. The full-sized fragment was gel purified using the GeneJet Gel Extraction Kit (ThermoFisher), followed by digestion with *Bam*HI and *Pst*I.

Ligation

The digested PCR SOEing fragment was inserted into the plasmid using T4 DNA ligase (New England Biolabs). The reaction was incubated at 37ºC for 30 minutes. Butanol and ethanol precipitations were performed to prior to electrotransformation.

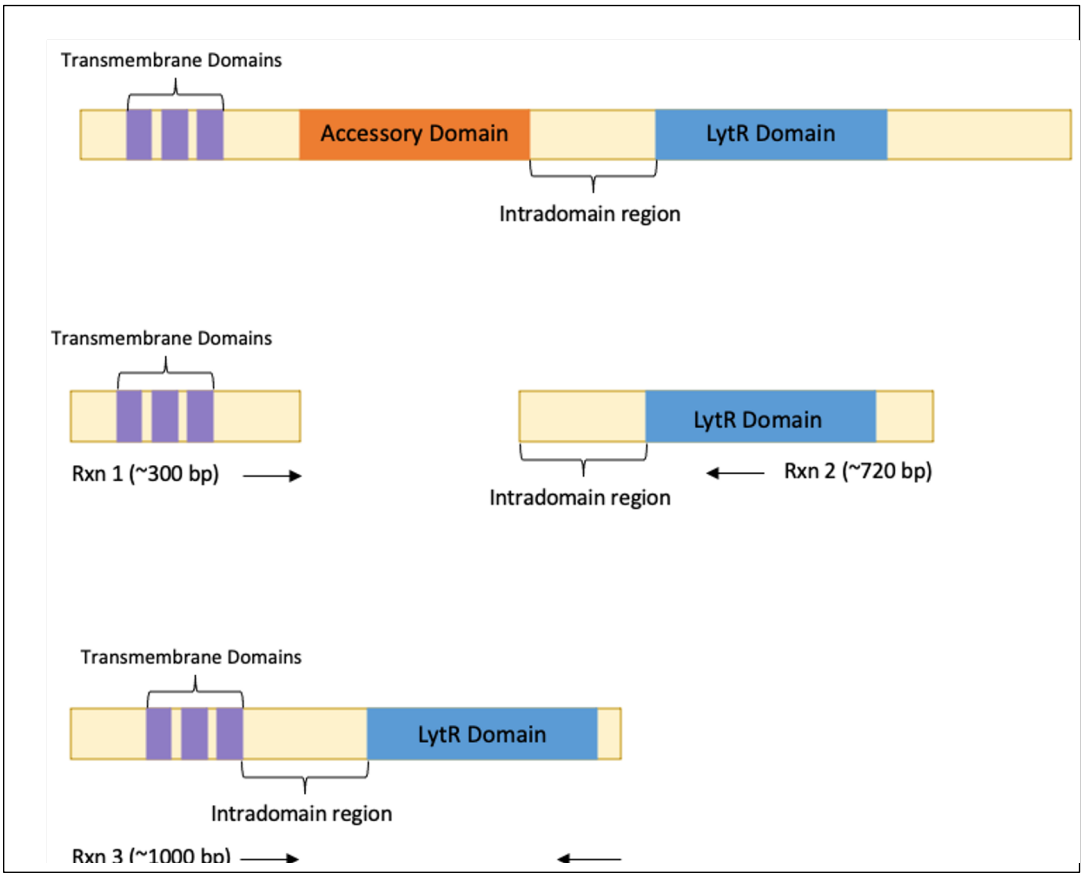


Figure 8. PCR SOEing model. The full protein contains the three transmembrane domains and both extracellular domains, the accessory and LytR domains. The truncated protein shows the deletion of the accessory domain, containing the three transmembrane domains and the LytR domain. Reactions 1 and 2 were used to delete the accessory domain of CpsA, and reaction 3 was used to SOE the fragments together to create the ΔAccessoryDomain CpsA fragment.

Transformation into *E. coli*

The ligation reaction was mixed with electro-competent cells and transferred to cold electroporation cuvettes (Figure 9). The cells were electroporated with a Gene Pulser (BioRad), with settings at 25 µF, 2.5 kV, and 200 Ω. Immediately after electroporation, the cells were transferred to SOC medium and incubated at 37ºC for 90 minutes with continual shaking. Cells were then plated onto Luria-Bertani (LB) agar plates supplemented with chloramphenicol (cam) at a final concentration of 15 µg/mL final

concentration. Plates were incubated at 37ºC overnight. Several transformants were purified on fresh LB-Cam plates. PCR of several purified transformants was performed to confirm the proper insertion of the PCR SOEing fragment.

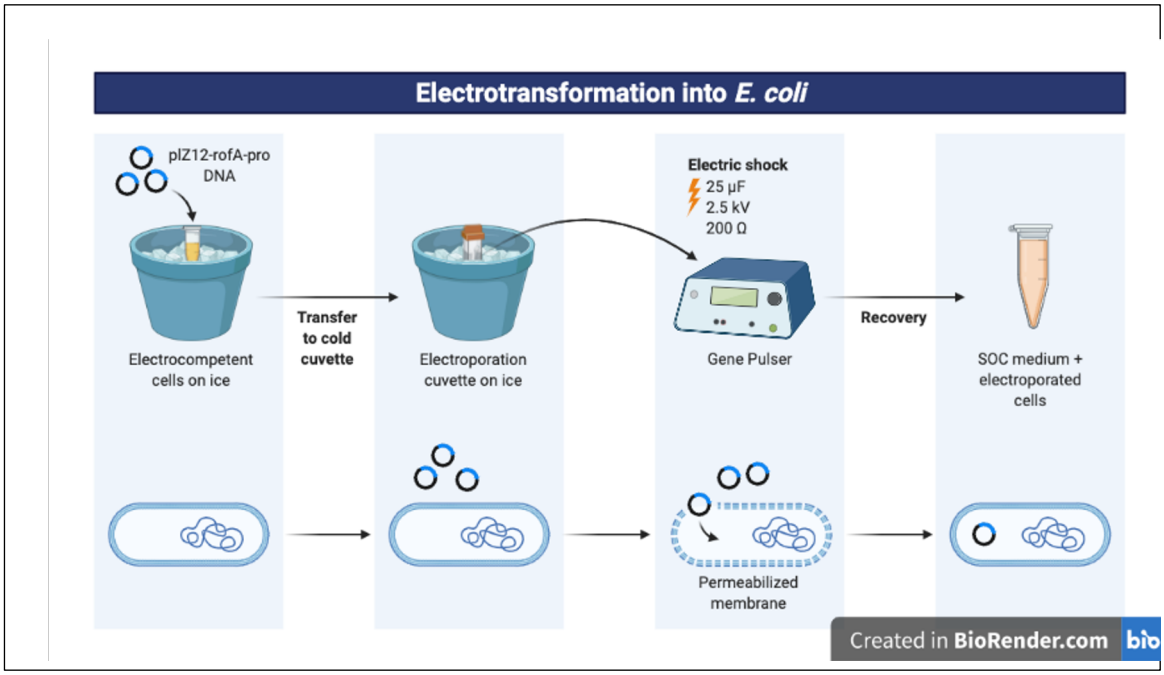


Figure 9. Electrotransformation of plZ12-rofA-pro plasmid DNA into *E. coli*. (Image created with BioRender.com)

Colony PCR

To confirm the proper insertion of the *cpsA* gene on the plasmid in *E. coli*, colony PCR was performed. The outside primers (#1672, #1740) were used to amplify the final fragment in the plasmid and then visualized by electrophoresis.

Plasmid Isolation

An overnight culture of a confirmed positive transformant was grown at 37ºC in a shaking incubator overnight in a 250 mL Erlenmeyer flask with 100 mL of LB broth supplemented with 15 µg/mL chloramphenicol. The next day, a PureLink HiPure Plasmid

Filter DNA Purification Kit (Invitrogen) was used to purify plasmid DNA. Isopropanol and ethanol precipitation were performed, and a Nanodrop (ThermoFisher) was used to determine the concentration of the isolated DNA. The plasmid insert was sequenced to confirm that the construct was correct and that no mutations were created by PCR.

Transformation into GBS

GBS515 cultures were grown statically in THY B (Todd-Hewitt-Yeast Broth) supplemented with 80 mM glycine overnight at 37ºC in a shaking incubator. A 1:20 dilution (1.25 mL) of the culture in 25 mL of THY B supplemented with 80 mM glycine the following day. The subculture was grown to an OD_{600} of 0.4 and harvested by centrifugation. The cells were washed and resuspended with 10% glycerol. Cells were then transferred to a cold electroporation cuvette. The isolated plasmid was added to the cold cuvette. The cells were electroporated with a Gene Pulser (BioRad), with settings at 25 μF, 2.0 kV, and 400 Ω (Figure 10). Immediately after electroporation, cells were transferred to THY B medium and incubated at 37ºC for 90 minutes with continual shaking. Cells were then plated on THY B supplemented with chloramphenicol at a final concentration of 3 μg/mL. Cells were transformed into both WT and ΔcpsA GBS strains. Plates were incubated overnight at 37ºC.

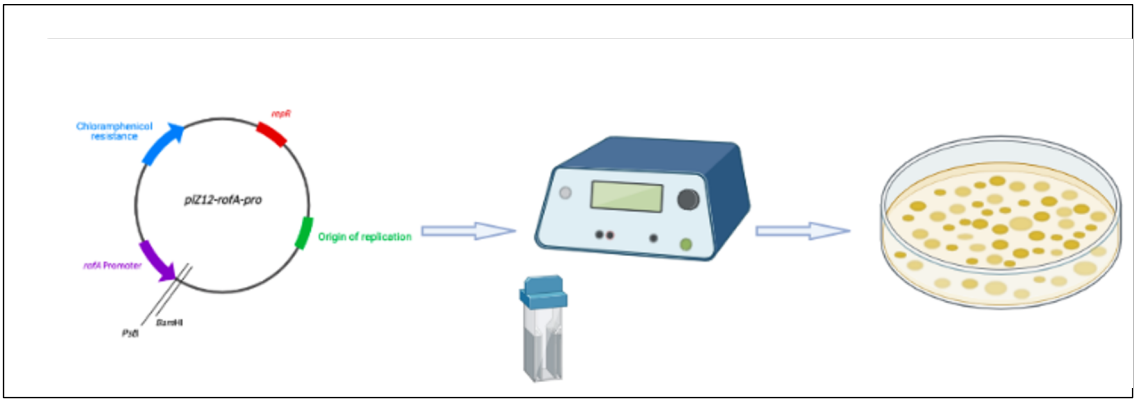


Figure 10. Electrotransformation into GBS. The plZ12-rofA-pro plasmid was isolated from *E. coli* and transformed into GBS using an electroporation protocol. (Image created with BioRender.com)

Enzyme-linked immunosorbent assay (ELISA)

Strains were grown in THY B-Cam3 broth at 37ºC overnight. Cultures were normalized to an OD_{600} of 0.75, pelleted, and washed three times with Tris-buffered saline + Tween (TBST). Primary antibody (rabbit anti-Serotype 1a GBS) was diluted to 1:20,000. Secondary antibody (secondary goat, anti-rabbit IgG conjugated to alkaline phosphatase [AP]) was diluted to 1:5,000. Secondary antibody was pre-absorbed to prevent nonspecific binding using both Δ*cpsA* and WT GBS strains. Cells were incubated with 1 µL primary antibody at 4ºC for 1 hour with rocking before being washed three more times with TBST. Cells were then incubated with secondary antibody for 1 hour with rocking at 4ºC before being washed three more times with TBST. Cells were resuspended in TBST and transferred to a 96-well plate. Alkaline phosphatase activity was measured after a 1 hour incubation in the dark at 37ºC after addition of p-nitrophenyl phosphate substrate (Sigma) (Figure 11). OD_{405} and OD_{600} were used to calculate AP activity and cell growth, respectively. Each sample was done in triplicate at a 1:1, 1:2,

and 1:4 dilution. Each experiment was repeated three times. Alkaline phosphatase (AP) units were calculated using the following equation:

$$AP\ Units = \frac{OD_{405}}{Vol * T * OD_{600}} * 1000$$

Vol = culture volume in well

T = time of incubation with substrate

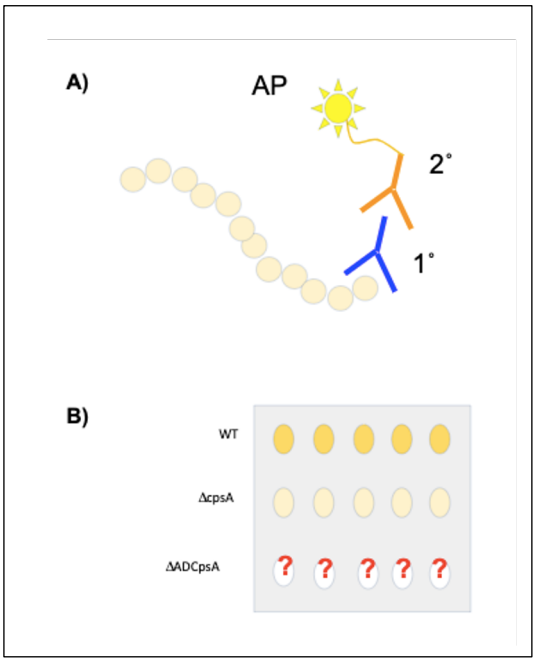


Figure 11. Enzyme-linked immunosorbent assay (ELISA) can be used to measure the amount of capsule on the cell. **A)** A primary antibody to the capsule is recognized by a secondary antibody, conjugated to alkaline phosphatase (AP). **B)** Plate of ELISA reactions. The wild type GBS will express a yellow color due to the presence of capsule. The ΔcpsA GBS will show a pale-yellow color due to the amount of capsule. The result of ΔAccessoryDomainCpsA GBS is unknown. (No citation, made by me)

Chain Length Analysis

Overnight cultures of all strains were grown statically at 37ºC in THY B medium. Using 7 µL of the overnight culture, wet mount slides were made and visualized with a Zeiss Axioskop compound microscope. Multiple images of each strain were documented, and the length of each chain was counted and analyzed.

Cell Aggregation Assay

Overnight cultures of all strains were grown statically in clear, round bottom tubes at 37ºC in THY B medium with antibiotics. Following 48 hours of growth, the suspension of the cultures in the tubes were documented.

RESULTS

Plasmid Construction

Plasmid construction was carried out as described in the materials and methods. Following plasmid construction, PCR was performed to create truncated versions of the fragment of sizes ~3000 bp and ~1178 bp. After gel purification, PCR SOEing was performed to form a truncated fragment of ~1000 bp. The full-length fragment was then digested and ligated before transforming into *E. coli*. Positive transformants were selected via colony PCR and DNA was isolated and used to transform into GBS after sequencing to confirm the correct mutation (Figure 12).

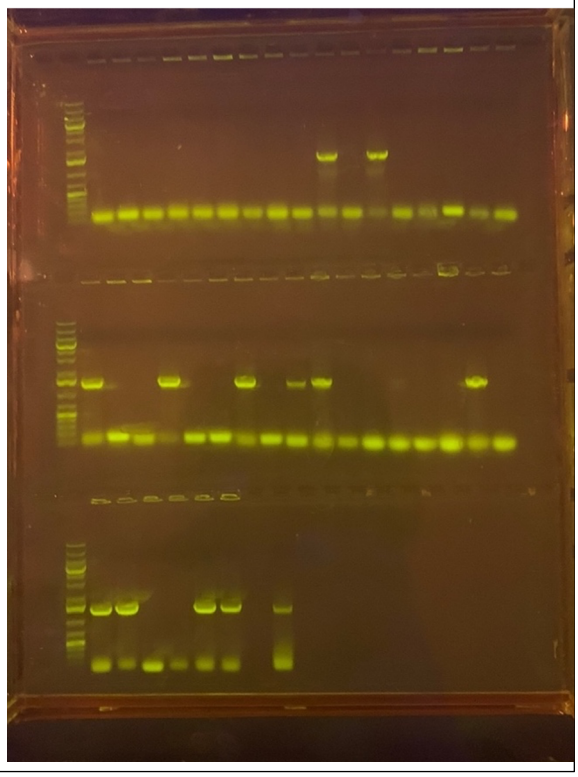


Figure 12. Gel electrophoresis of colony PCR shows positive colonies with a band at ~1470 bp. Positive colonies were those that included a successful deletion of the accessory domain, resulting in a fragment of ~1470 bp.

In this study, six different strains of GBS were constructed in order to compare the effects of a deletion of the accessory domain, both with and without the intradomain region remaining (Figures 13,14). In strains that have WT background, WT CpsA was on the chromosome and expressed in addition to what was expressed on the plasmid (Figure 14). In strains that have ΔCpsA background, the only CpsA expressed was from the

plasmid (Figure 14). Both the WT GBS and ΔCpsA GBS expressed either an empty vector, or a plasmid expressing WT CpsA, ΔAcc+ CpsA, or ΔAcc- CpsA (Figures 14-17). The ΔAcc+ CpsA included the intradomain region, while the ΔAcc- CpsA did not include the intradomain region (Figure 13).

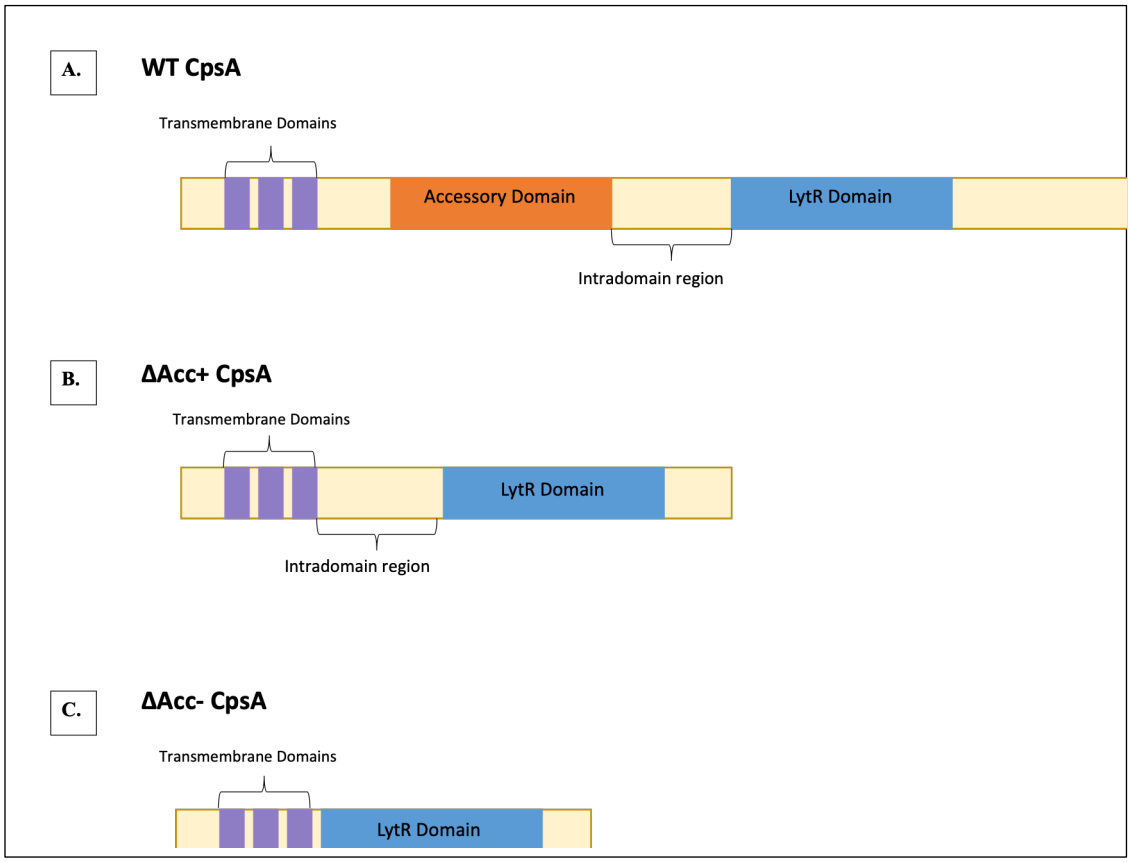


Figure 13. Comparison of WT CpsA to the truncated versions used in this study. A) Full length CpsA protein. B) ΔAcc+ CpsA, where the three transmembrane domains, intradomain region, and LytR domain remain, with a deletion of the accessory domain. C) ΔAcc- CpsA, where the three transmembrane domains and LytR domain remain, with the deletion of the accessory domain and intradomain region. (No citation, made by me)

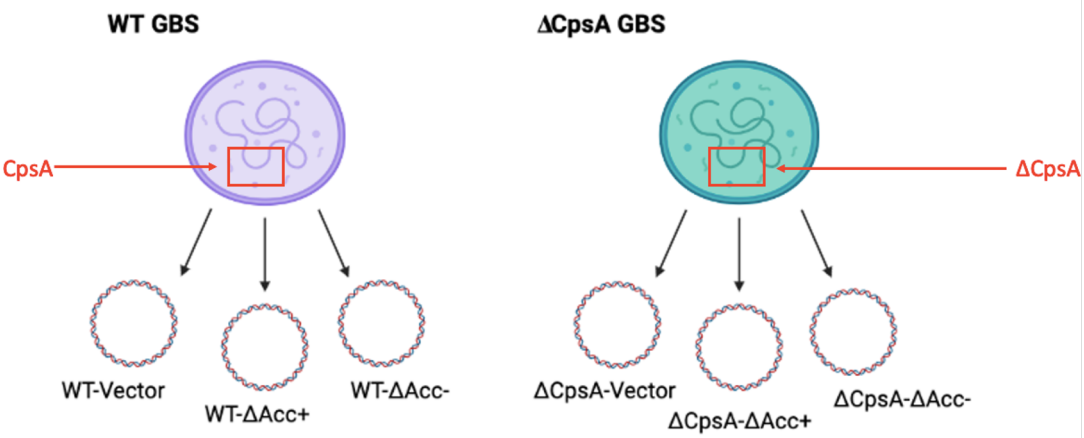


Figure 14. Construction of all 6 strains used in this study. WT GBS and ΔCpsA GBS expressing an empty vector, ΔAcc+ CpsA, or ΔAcc- CpsA on a plasmid. (Image created with BioRender.com)

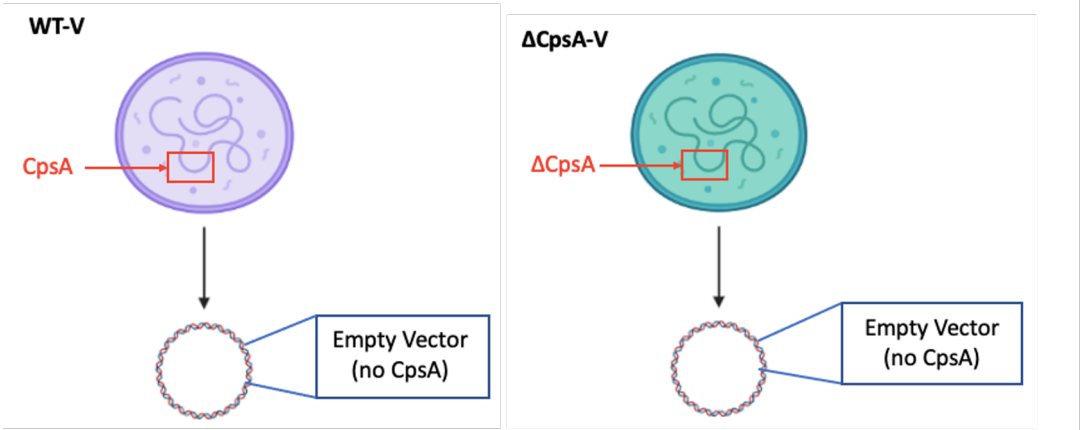


Figure 15. WT GBS and ΔCpsA GBS expressing the empty vector on a plasmid. (Image created with BioRender.com)

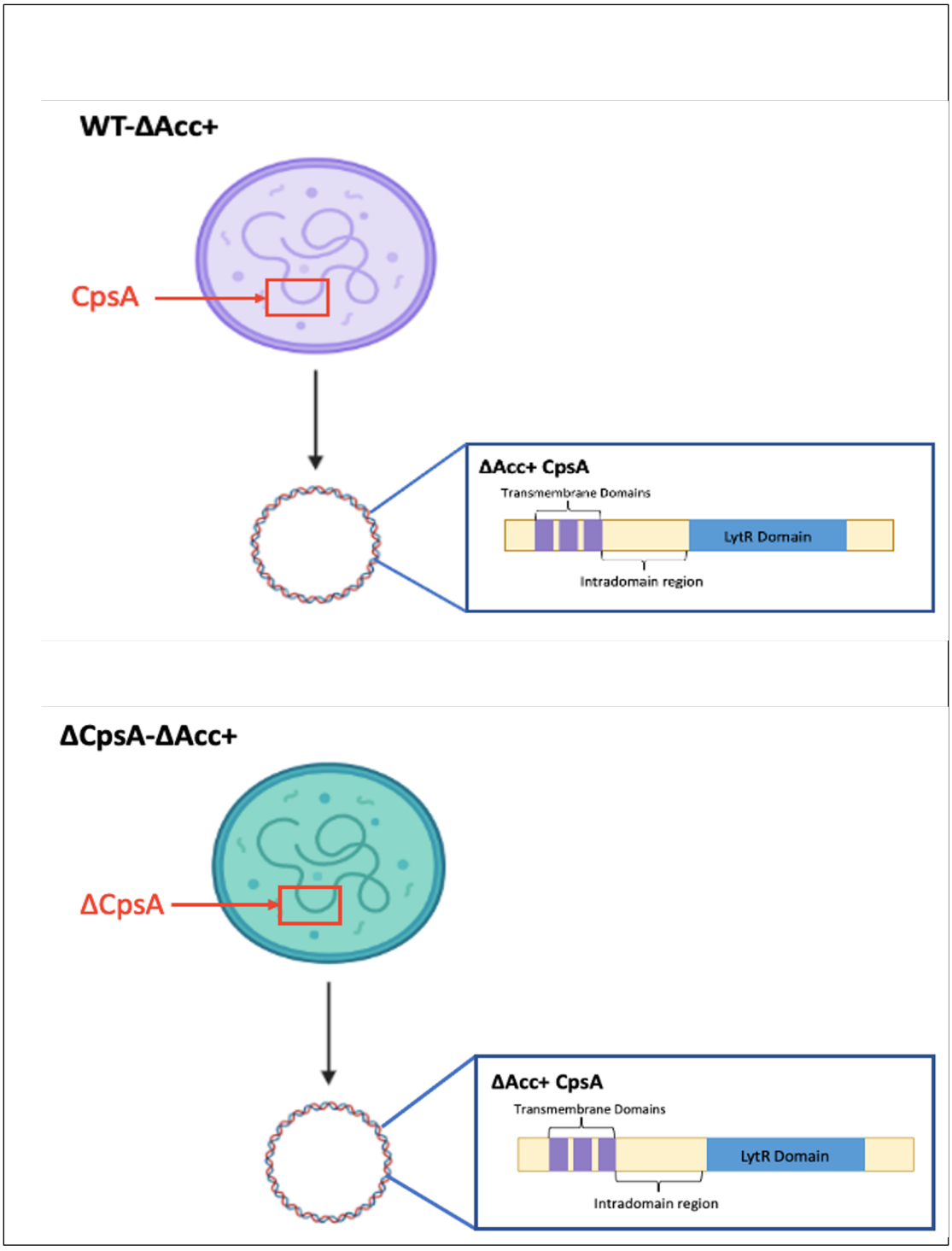


Figure 16. WT GBS and ΔCpsA GBS expressing ΔAcc+ CpsA on a plasmid. (Image created with BioRender.com)

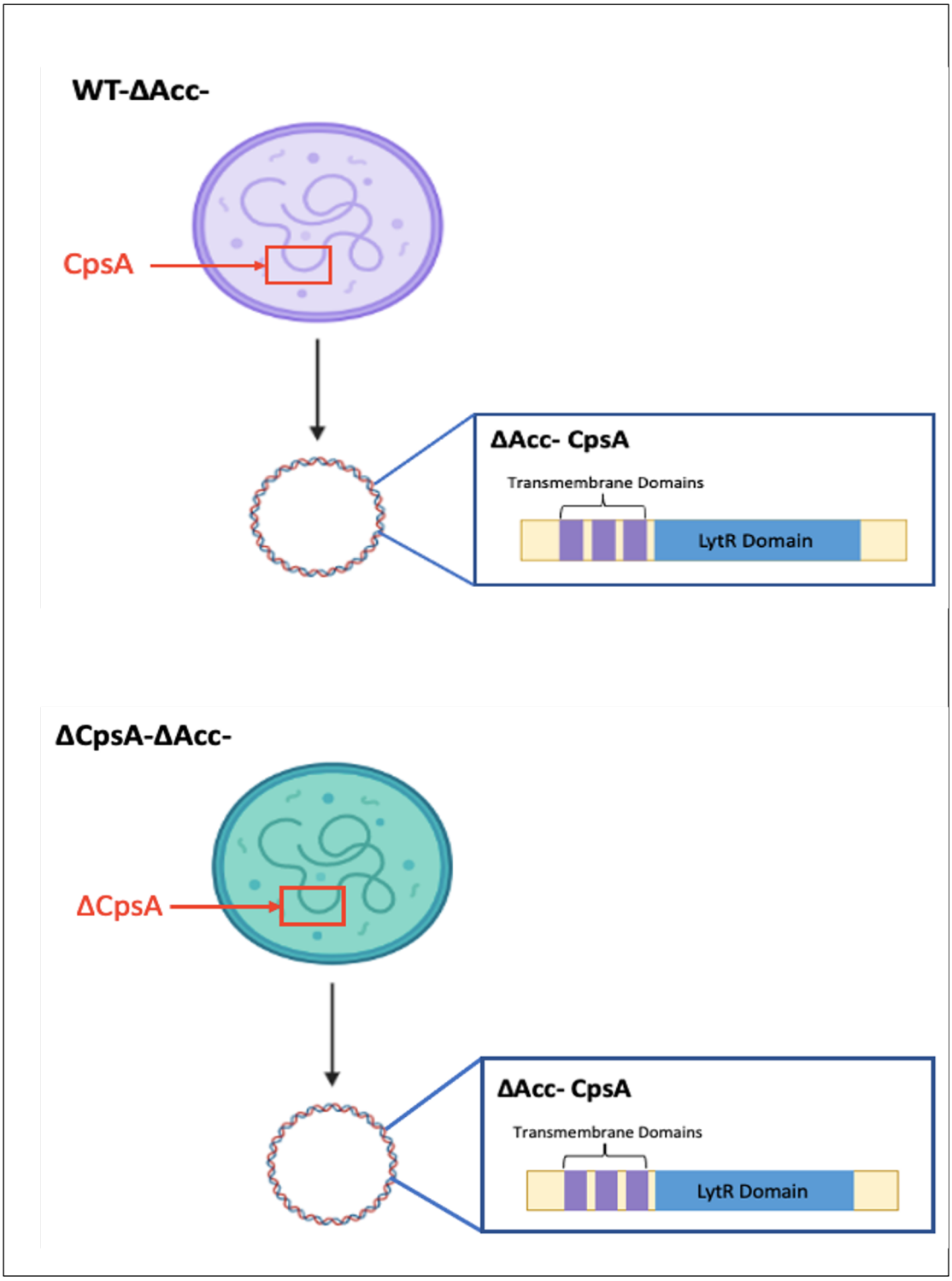


Figure 17. WT GBS and ΔCpsA GBS expressing ΔAcc- CpsA on a plasmid. (Image created with BioRender.com)

Chain Length Analysis

Previous research examined the relationship between chain length and cell wall integrity [7]. The data shows that WT strains express shorter chains of cocci, 2-6 cocci per chain, and ΔCpsA strains express much longer chains of cocci, up to 20 cocci per chain [7]. Long chain morphologies have been associated with less capsule expression [7]. In this study, researchers treated all GBS strains with a subinhibitory amount of lysozyme and compared the chain lengths between the treated and untreated strains [7]. Lysozyme has enzymatic activity that cleaves specific residues of the peptidoglycan cell wall. Specifically, it has muramidase activity which cleaves the *N*-acetyl-D-glucosamine residues of the peptidoglycan cell wall [7]. After growing in the presence of lysozyme, all cells existed as either diplococci or single cocci in both the WT and ΔCpsA strains [7]. This data suggests that CpsA dependent changes to the cell wall may be responsible for the observed chain length variances [7].

To determine the differences in morphology between the different GBS strains examined in this study, 10 images of each strain were visualized and documented using light microscopy at 1000X magnification using a Zeiss Axioskop compound microscope (Figure 18). The number of cocci in each GBS chain was counted and recorded. The average cocci per chain was determined for each GBS strain (Table 1). Overall, the ΔCpsA mutant strains had longer chain lengths relative to the WT strains. When compared to the WT-vector, ΔCpsA-vector expressed slightly longer chains. The average chain length in the WT-vector strain was 1.784 cocci, while the average chain length in the ΔCpsA-vector strain was 3.26 cocci (Table 1). The ΔCpsA-vector strain also showed slightly more distribution in chain length than the WT-vector strain (Figures 19, 20).

When compared to the WT-ΔAcc+ strain, the ΔCpsA-ΔAcc+ strain expressed much greater variation in chain length (Figures 21, 22). The average chain length of the WT-ΔAcc+ strain was 1.897 cocci per chain, while the average length of the ΔCpsA-ΔAcc+ strain was 6.35 cocci (Table 1). The majority of the chains in the WT-ΔAcc+ strain were between 1-4 cocci per chain, but the ΔCpsA-ΔAcc+ strain expressed much greater variation in chain length, with a range from 1 cocci per chain to 23 cocci per chain (Figures 21, 22, Table 1). When compared to the WT-ΔAcc- strain, the ΔCpsA-ΔAcc- strain expressed longer chain lengths (Figures 23, 24). The average chain length for the WT-ΔAcc- strain was 1.843 cocci, while the average chain length for the ΔCpsA-ΔAcc- strain was 4.059 cocci (Table 1). The ΔCpsA-ΔAcc- strain also expressed more distribution in chain length, with a range from one cocci per chain to 23 cocci per chain. The majority of the chains in the WT-ΔAcc- strain were between 1-4 cocci per chain (Figure 23). When each strain was compared to one another, the WT-V strain showed the least variation, while the ΔCpsA-ΔAcc+ strain expressed the most variation in chain length and the longest chains (Figure 25).

Table 1. Maximum, minimum, and average chain length of each sample documented in the chain length analysis.

	WT-V	**Δ-V**	**WT-ΔAcc-**	**Δ-ΔAcc-**	**WT-ΔAcc+**	**Δ-ΔAcc+**
Maximum	10	20	13	23	23	23
Minimum	1	1	1	1	1	1
Average	1.784	3.26	1.843	4.059	1.897	6.35

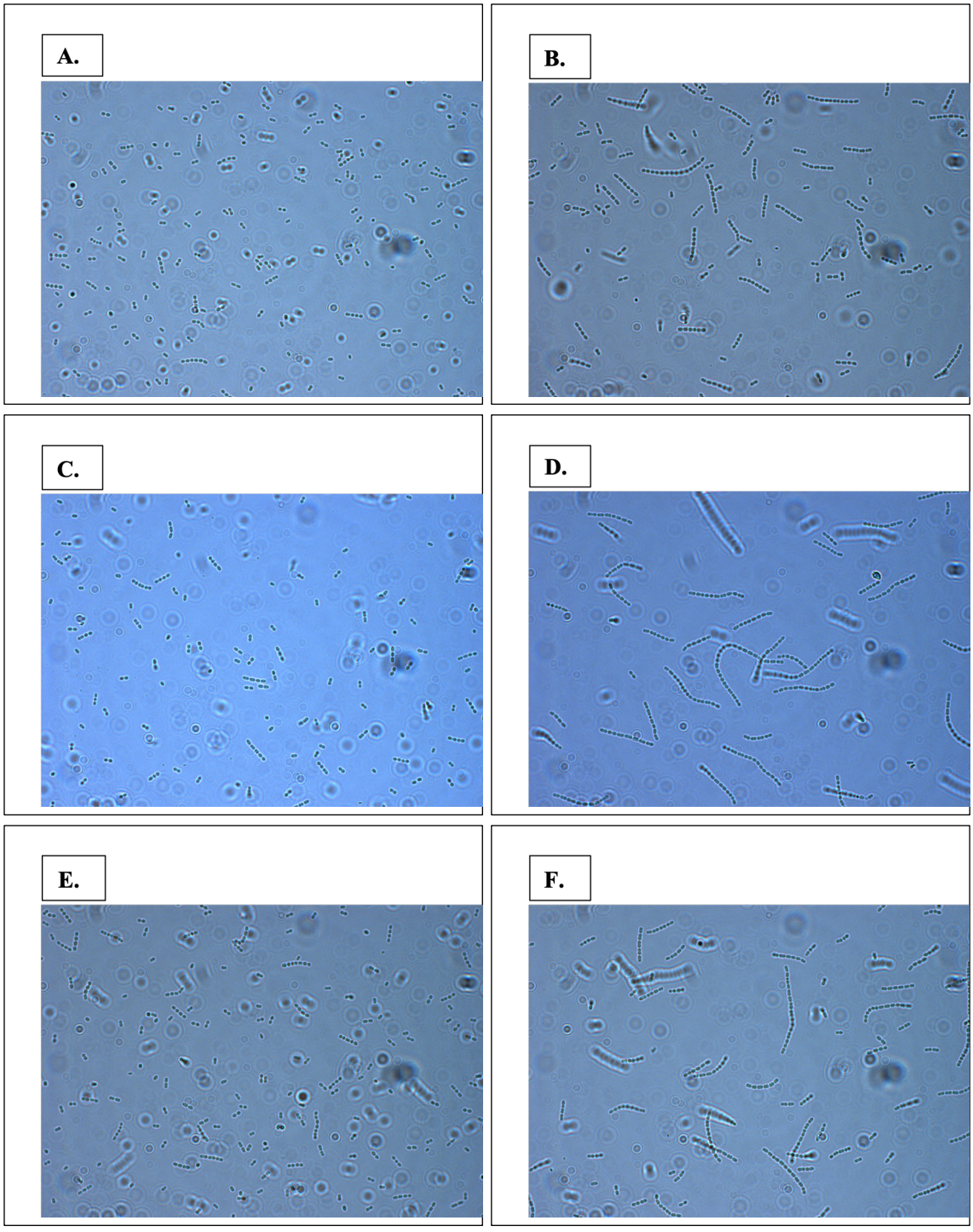


Figure 18. Images taken for chain length analysis using compound microscopy. A) WT-Vector. B) ΔCpsA-Vector. C) WT-ΔAcc+. D) ΔCpsA-ΔAcc+. E) WT-ΔAcc-. F) ΔCpsA-ΔAcc-.

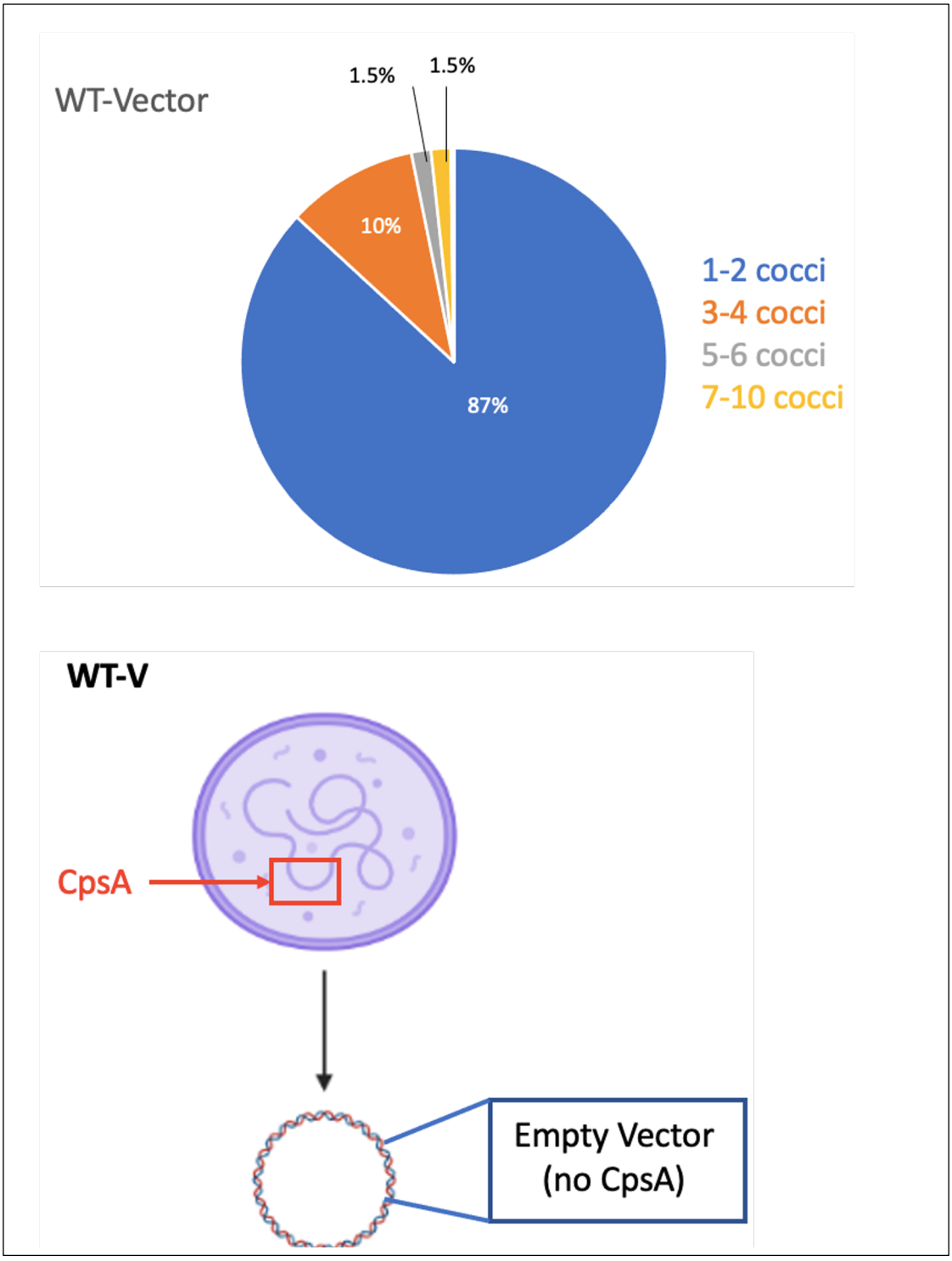


Figure 19. Chain length analysis of WT-Vector strain. (Image created with BioRender.com)

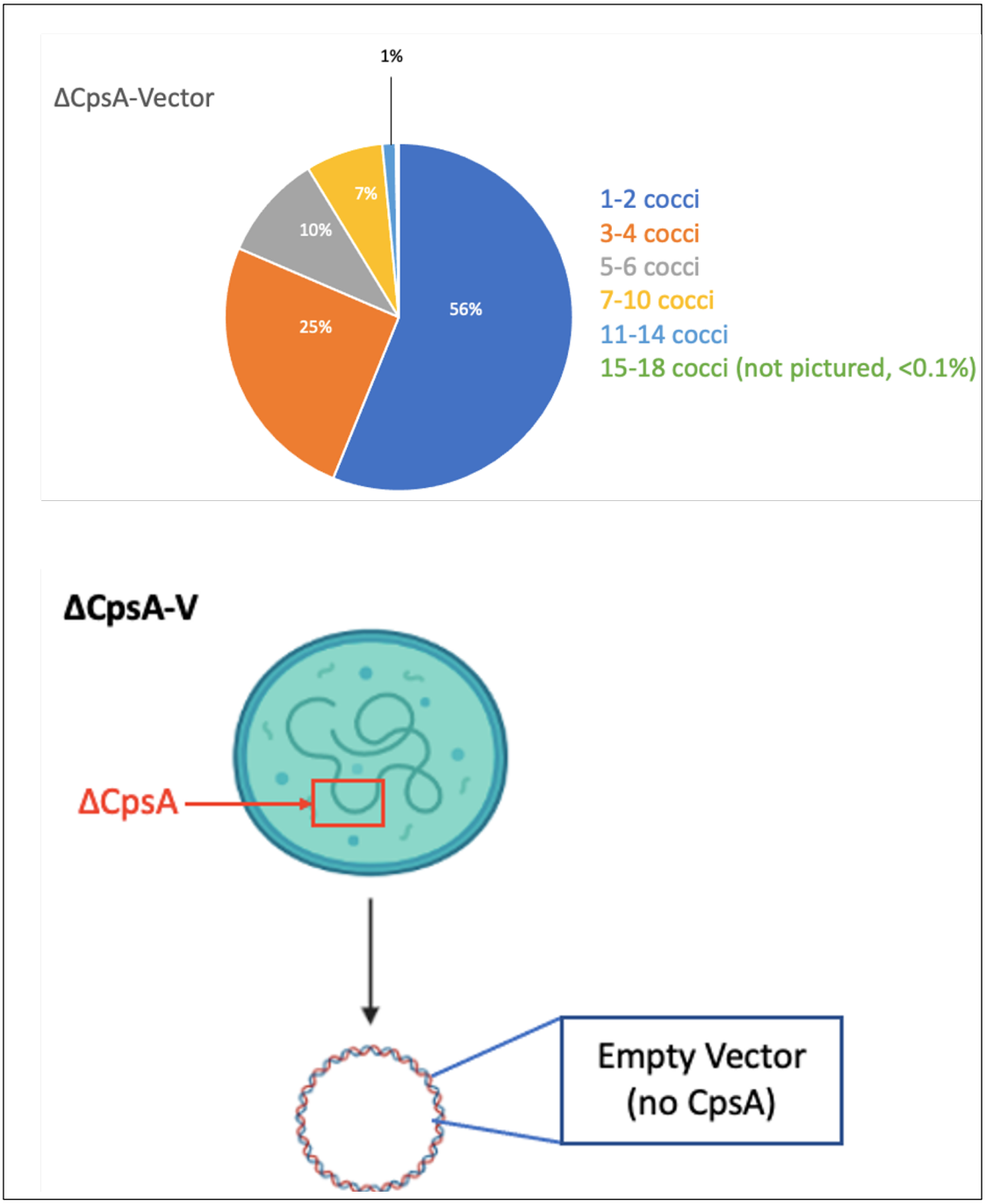


Figure 20. Chain length analysis of ΔCpsA-Vector strain. (Image created with BioRender.com)

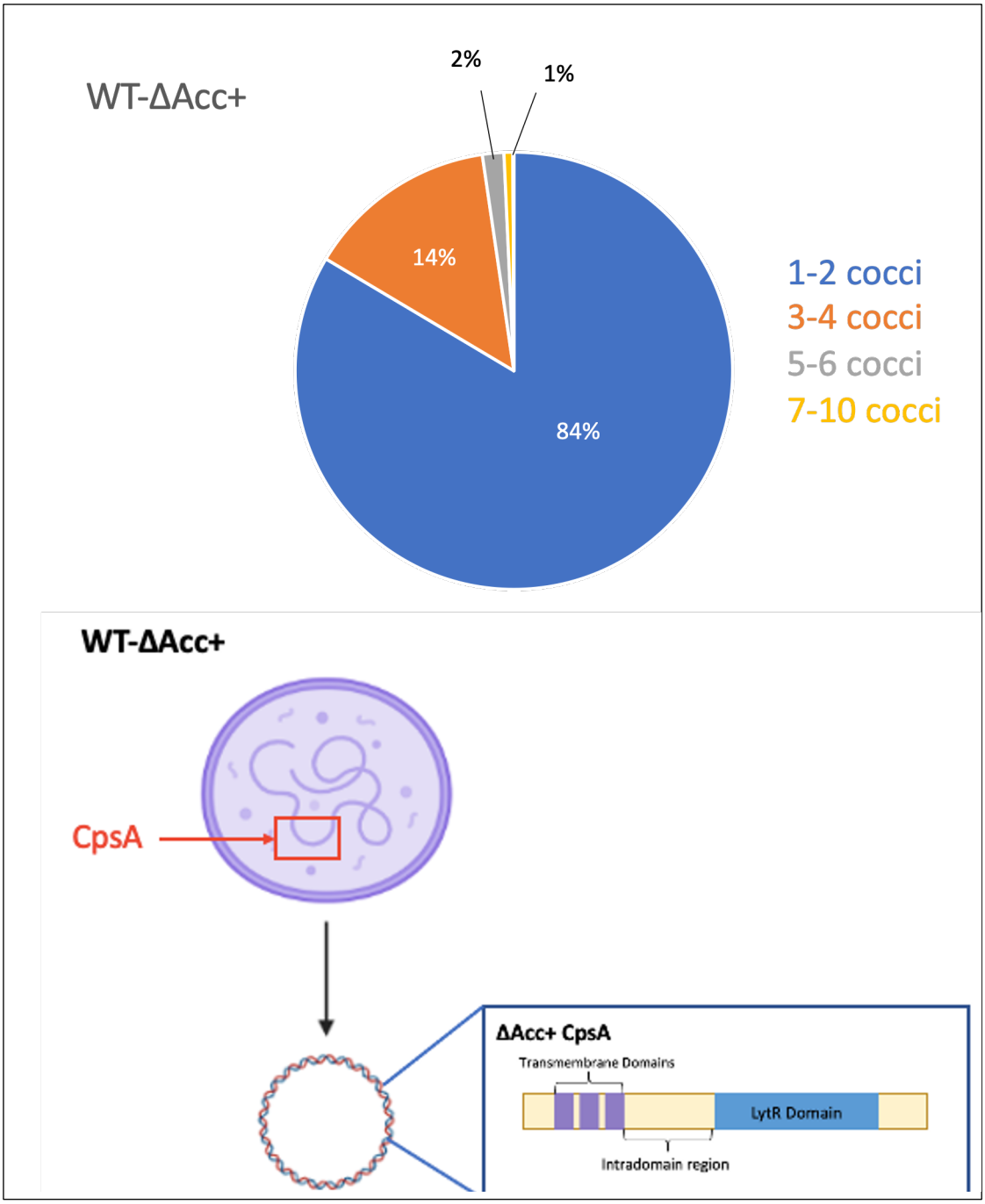


Figure 21. Chain length analysis of WT-ΔAcc+ strain. (Image created with BioRender.com)

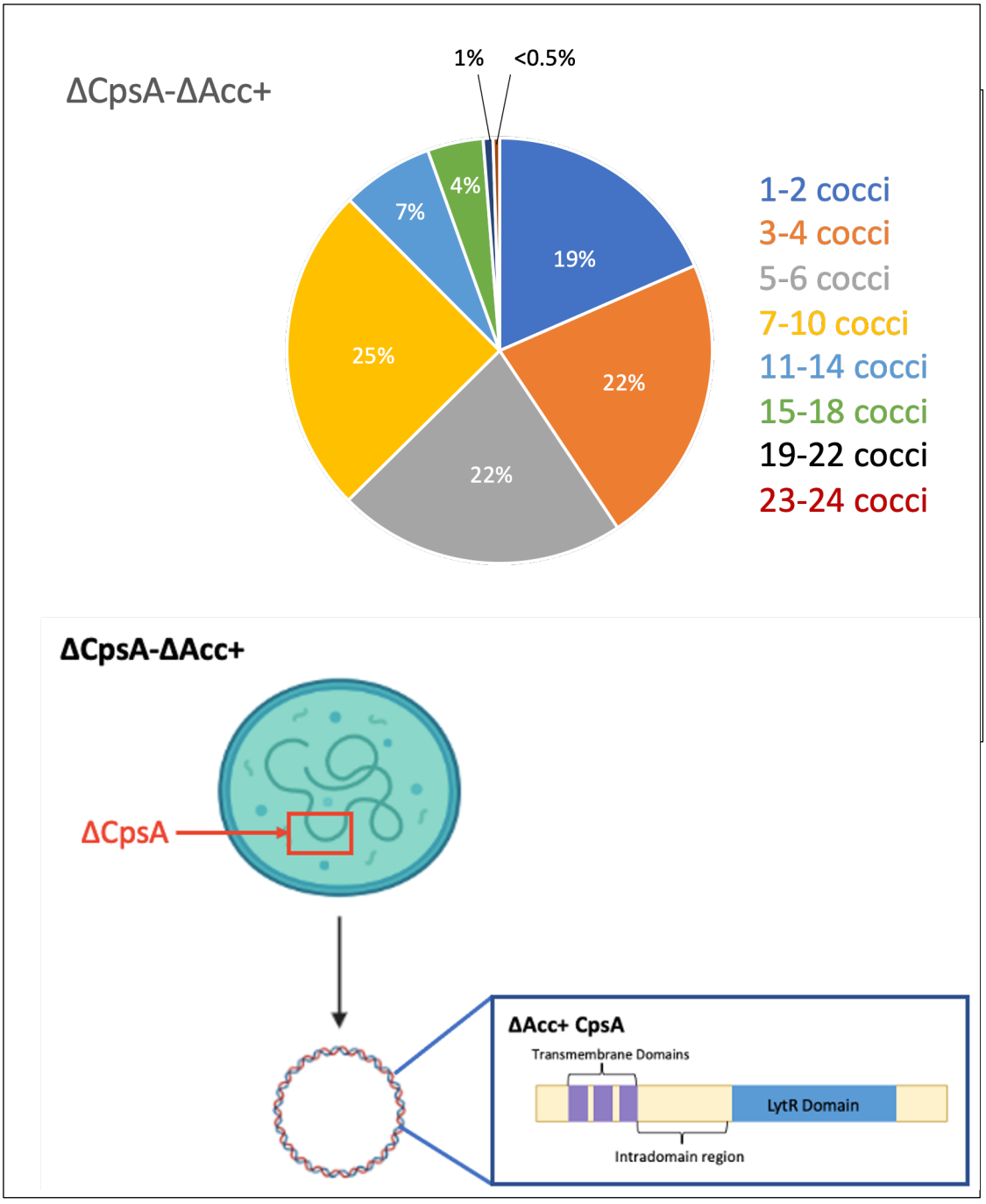


Figure 22. Chain length analysis of ΔCpsA-ΔAcc+ strain. (Image created with BioRender.com)

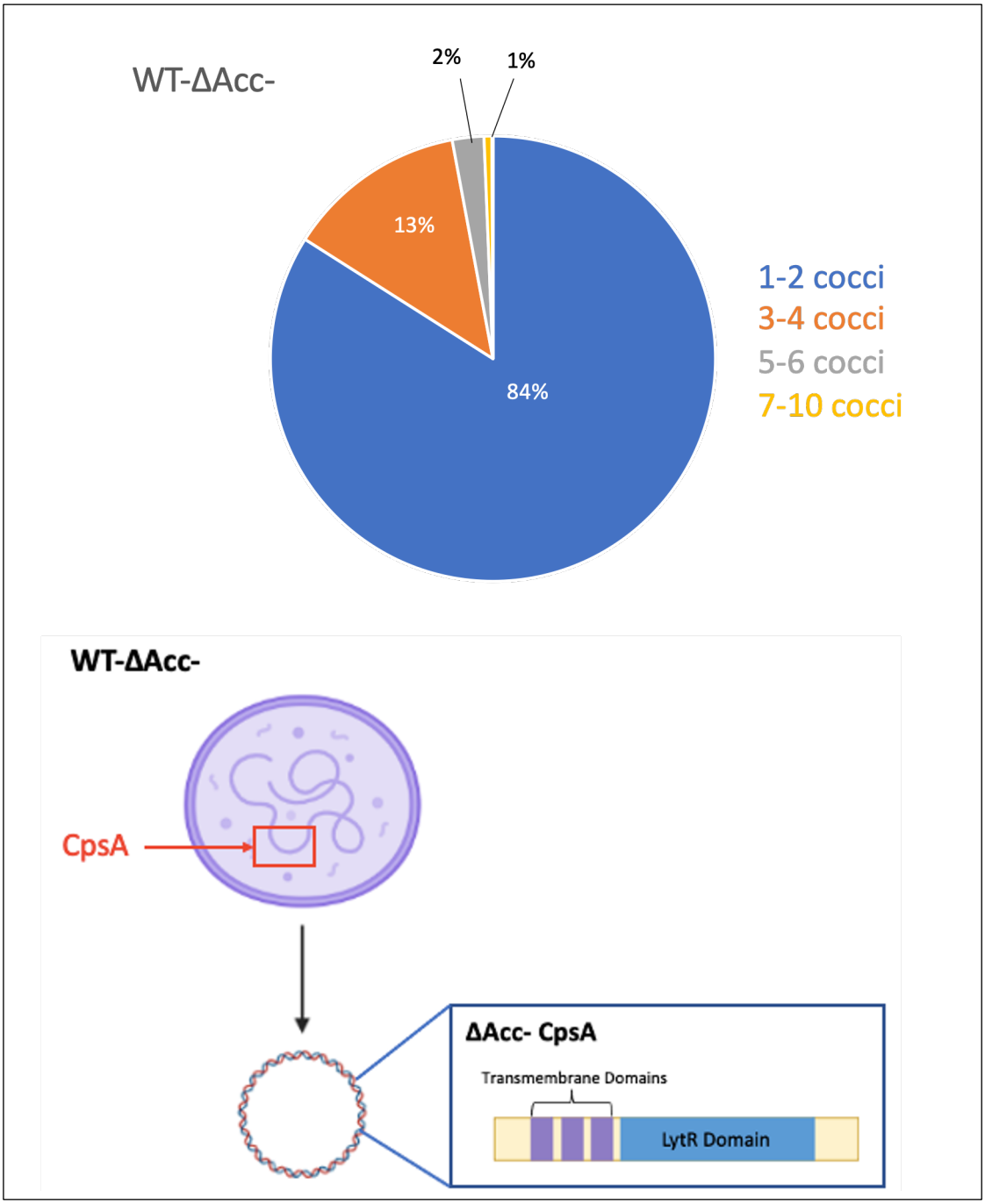


Figure 23. Chain length analysis of WT-ΔAcc- strain. (Image created with BioRender.com)

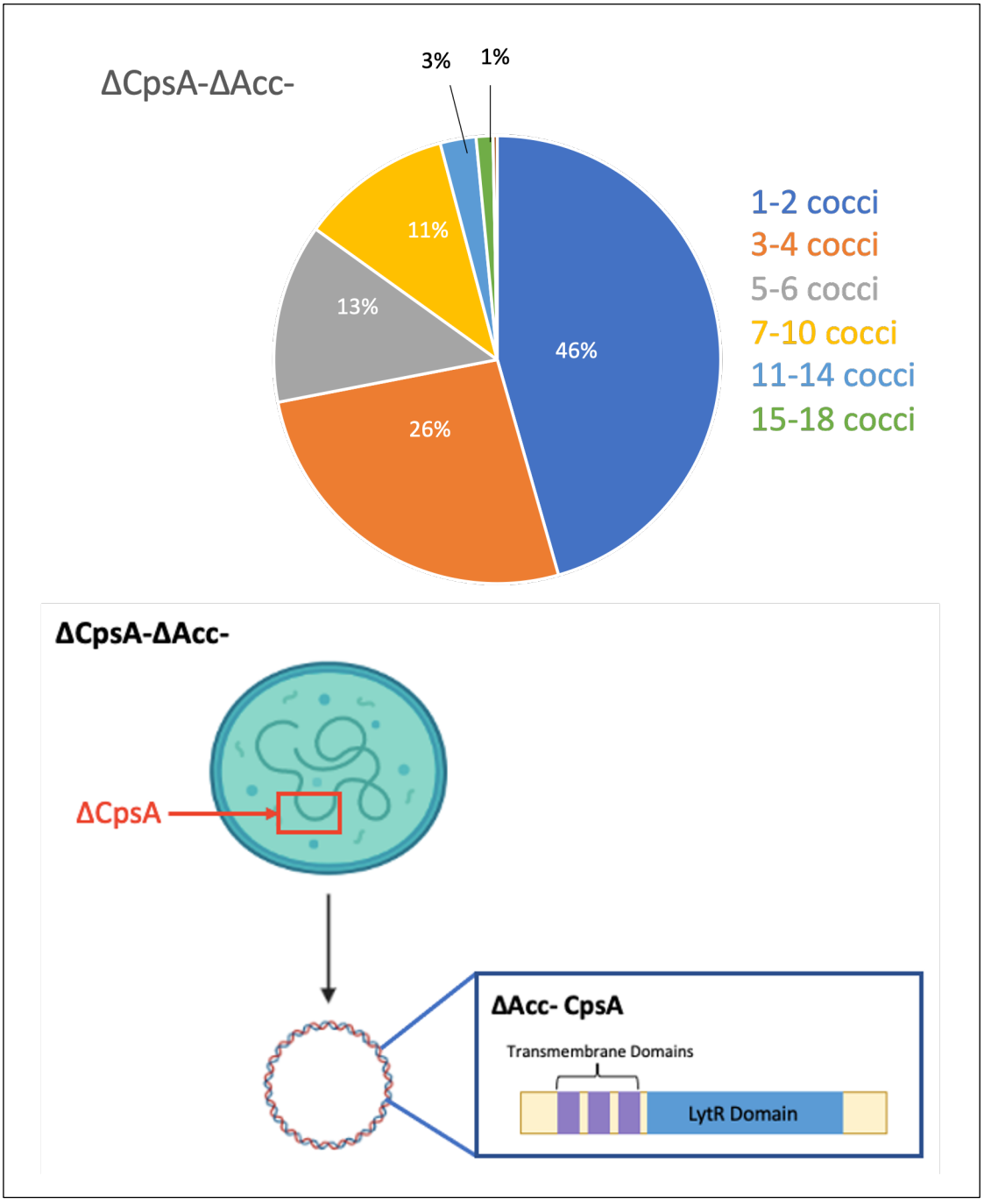


Figure 24. Chain length analysis of ΔCpsA-ΔAcc- strain. (Image created with BioRender.com)

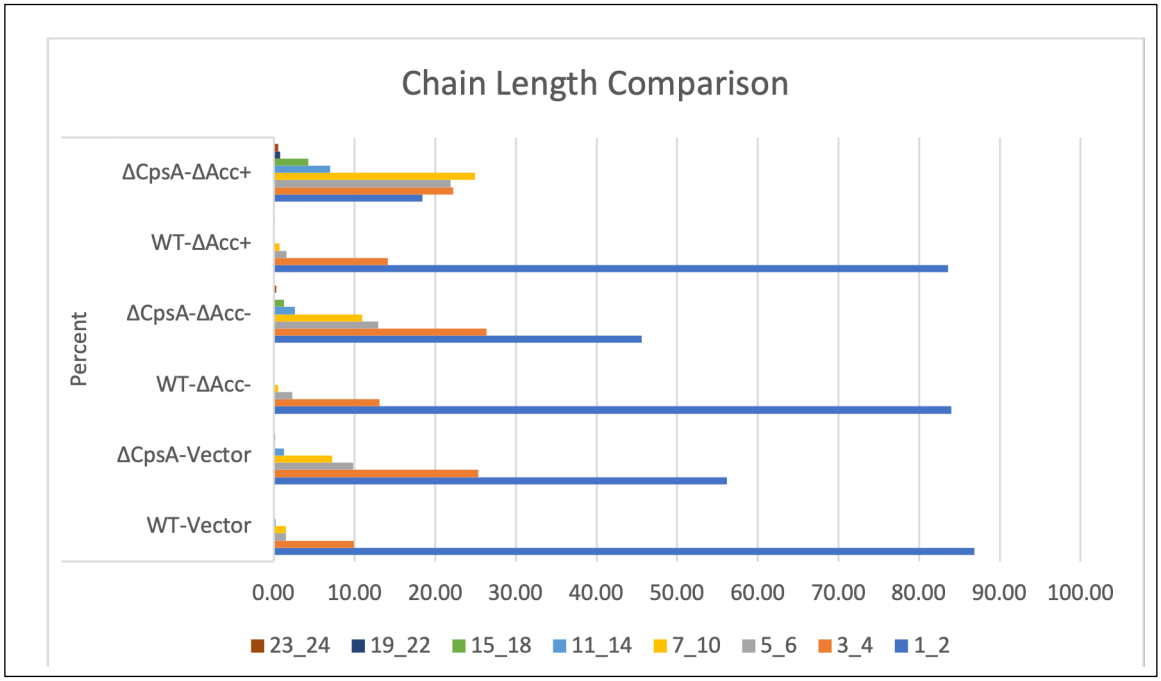


Figure 25. Chain length comparison between each strain of GBS.

Cell Aggregation Assay

Cell aggregation assays were performed according to materials and methods to examine the difference in culture growth in WT and ΔCpsA GBS strains. Upon examination of culture tubes, WT strains exhibited more turbidity than ΔCpsA strains, which exhibited more clearing (Figure 26). Each strain of WT and ΔCpsA GBS were compared against one another. In each strain, the ΔCpsA GBS exhibited less turbidity than its WT GBS counterpart. One strain, ΔCpsA-ΔAcc-, looked similar to WT-ΔAcc-, which could have likely been due to a disruption of the cell pellet. Although this could be an error, the ΔCpsA-ΔAcc- strain still exhibited slightly more clearing than the WT-ΔAcc- strain.

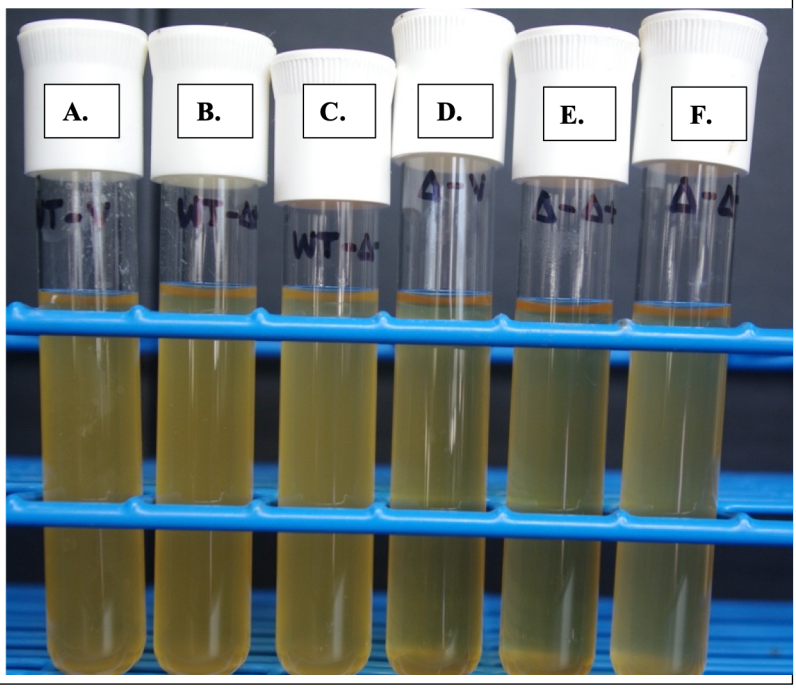


Figure 26. Comparison of cell aggregation of all six strains created in this study. A) WT-Vector. B) WT-ΔAcc+. C) WT-ΔAcc-. D) ΔCpsA-Vector. E) ΔCpsA-ΔAcc+. F) ΔCpsA-ΔAcc-.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to determine capsule expression. The amount of alkaline phosphatase (AP) units in each sample is directly correlated to the amount of capsule expressed by the cell. The AP units were calculated using the following equation:

$$AP\ Units = \frac{OD_{405}}{Vol * T * OD_{600}} * 1000$$

Vol = culture volume in well

T = time of incubation with substrate

ELISA was performed in triplicate in order to determine reproducibility of results. After three trials, AP units of each trial were averaged together (Figure 27). Standard error and significance values were determined (Figure 27).

When comparing WT-Vector and ΔCpsA-Vector strains, ΔCpsA-Vector is not able to complement back the same amount of capsule as WT-Vector (Figure 27). Calculated p-values suggest that there is significant difference between these strains.

When comparing the WT-mutant CpsA strains to the WT-Vector, the WT-ΔAcc+ strain was able to complement back approximately the same amount of capsule as the WT-Vector strain, but WT-ΔAcc- could not do the same (Figure 27). Calculated p-values suggest that there is a significant difference between the WT-Vector and WT-ΔAcc- strains, but there was no significant difference between WT-Vector and WT-ΔAcc+ strains (Figure 27). These p-values are consistent with the data shown in Figure 27.

When comparing the ΔCpsA-mutant to the ΔCpsA-Vector, both the ΔCpsA-ΔAcc+ and ΔCpsA-ΔAcc- strains were able to complement back approximately the same amount of capsule as the ΔCpsA-Vector (Figure 27). Calculated p-values suggest that there is no significant difference between these strains, and this is consistent with the data shown in Figure 27.

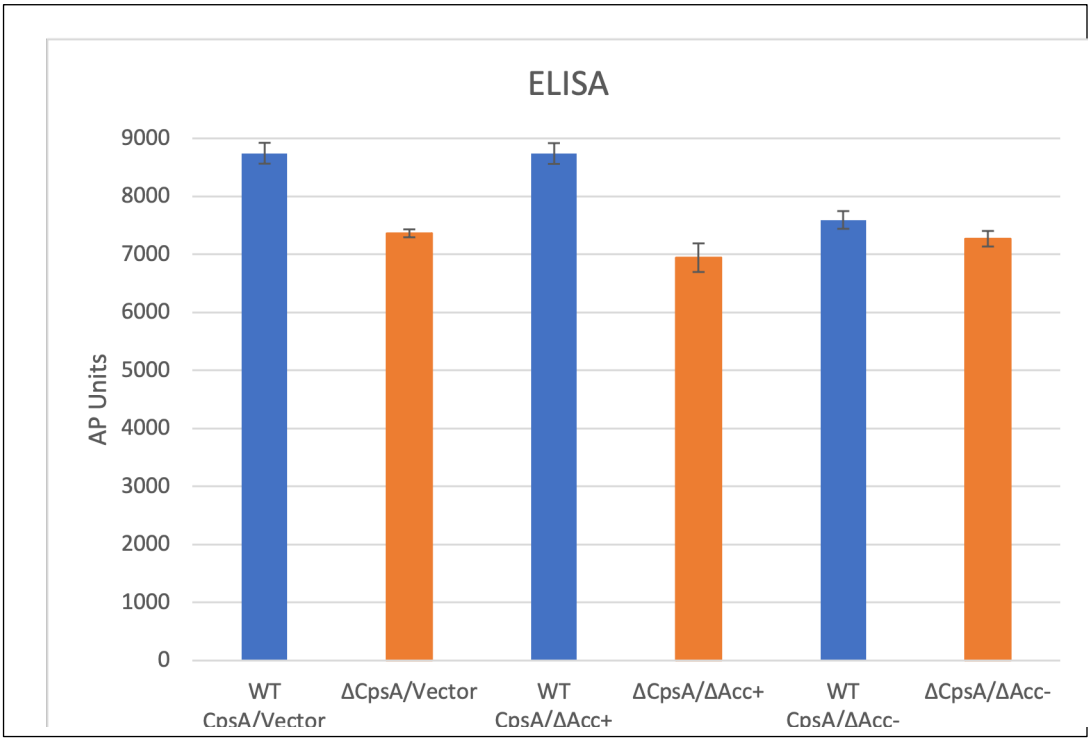


Figure 27. Average ELISA data to determine capsule expression. There is a significant difference between WT-Vector and WT-ΔAcc- strains (p=0.03522) and between WT-ΔAcc+ and WT-ΔAcc- strains (p=0.03607). No significant difference was found between WT-Vector and WT-ΔAcc+ strains (p=0.9). There is a significant difference between WT-Vector and ΔCpsA-Vector strains (p=0.0250), but no significant difference between any of the ΔCpsA strains. P-values were determine using a one-way ANOVA procedure including a Tukey Test.

DISCUSSION

S. agalactiae, also known as Group B *Streptococcus* (GBS), is a commensal organism that colonizes the vaginal and genitourinary tract of females, where it can act as an opportunistic pathogen in the immunocompromised, such as neonates and fetuses [17]. GBS is a widespread pathogen that affects over 22 million pregnant women worldwide, with approximately 320 million infant GBS cases as of 2015 [4]. In the United States alone, GBS is responsible for approximately 900,000 infections, with a mortality rate of 6% in neonates and infants [17]. GBS infection is known to be the leading cause of neonatal meningitis, and it can also cause other implications, such as septicemia, premature birth, and stillbirth [20]. This pathogen can be passed from mother to baby, either in utero or during birth, posing a serious threat to neonates and fetuses [17].

Because of this threat, pregnant women undergo late gestational screening in order to prevent the spread of infection. If the pregnant woman is a colonizer of GBS, intrapartum antibiotic prophylaxis (IAP) is administered intravenously during delivery [20]. While this treatment is effective at reducing the incidence of GBS infection within the first few weeks of life, the long-term effects of antibiotic use in newborns can be devastating. A recent study linked antibiotic use in the first two years of life with a higher risk of celiac disease, childhood onset asthma, allergic rhinitis, atopic dermatitis, overweigh, obesity, and ADHD [2]. Therefore, the need for improved, specific treatment for GBS infection in neonates is of the utmost importance.

The most important virulence factor of GBS infection is known to be the capsular polysaccharide [8]. The capsule has been a novel target for the treatment of GBS

infection. In GBS, capsule is dependent upon the CpsA protein, which is encoded by the *cpsA* gene. This protein acts as one of the transcriptional regulators of the capsule operon and is a multifunctional protein containing an intracellular DNA-binding domain, 3 transmembrane domains, and two extracellular domains, the LytR and accessory domains [6]. Crystal structure of the extracellular domains suggests that they fold separately and have separate functions [12]. The CpsA protein is highly homologous among *Streptococcal* species, such as *S. iniae*, *S. pneumoniae*, and *S. suis* [6,8]. Previous research suggests that the CpsA protein directly aids in the production of capsule and thus, the virulence of GBS [7]. Since capsule is required for GBS survival in the bloodstream, understanding the mechanisms of dissemination used by GBS to survive in tissue environments, such as the vaginal epithelium, is of crucial importance.

Prior research performed in the Neely lab compared percent zebrafish survival over days post injection of WT and mutant GBS and *S. iniae* (Figure 3). This data demonstrated that the mutant strains were less virulent, reducing the lethality of infection by approximately 60%, compared to the WT strains where 100% of zebrafish died after 7 days post injection. This data suggested that CpsA plays an unknown, but important role in virulence of GBS.

Much of the previous CpsA research has focused on the LytR domain, however no previous research has analyzed the effect of a deletion of the accessory domain. The goal of this study was to determine the role of the accessory domain on CpsA function and capsule production in GBS by deletion of the accessory domain and then analysis of the mutated CpsA using a series of virulence assays. The accessory domain was successfully deleted through plasmid construction and electrotransformation, first into *E.*

coli, and then into GBS. Once the mutant strain was transformed, chain length analysis was performed to determine the effect of mutant CpsA on chain length morphology.

Prior studies have suggested that WT GBS strains express shorter chains of cocci compared to ΔCpsA GBS strains, which are known to express longer chains of cocci due to decreased capsule expression. In this study, when WT CpsA was expressed on the plasmid, chains of cocci were shorter than compared to that of the ΔCpsA strains, on average (Table 1). This data is consistent with the findings of the chain length analysis performed in this study, however, these results provide new information about chain length. Interestingly, there was a difference in chain length between the ΔAcc+ and ΔAcc- mutant strains expressed on both WT and ΔCpsA plasmids, suggesting that the intradomain region has some effect on chain length. No previous research has deleted both the accessory domain and intradomain region from CpsA. The intradomain region connects the accessory domain and LytR domain and is 180 bp in length (60 amino acids). While this is a small fragment of CpsA, the results of the chain length analysis suggest that this region could play an important role in GBS virulence. Future studies should perform the same chain length analysis on WT-WT and ΔCpsA-WT strains as an additional control in order to fully understand the impact of mutant CpsA on chain length phenotypes.

An ELISA was used to determine the difference in capsule presence on the cell between each strain in this study. In previous analyses, long chain phenotypes have correlated to less capsule expression [7]. This is consistent with the data collected in this study, where the ΔCpsA-ΔAcc+ and ΔCpsA-ΔAcc- strains expressed the longest chains of cocci and expressed the lowest amount of capsule (Figures 22, 24, 27). Interestingly,

the WT-Vector strain looks phenotypically similar to the WT-ΔAcc+ and WT-ΔAcc- strains, even though the capsule expression between the three strains varied significantly. All three WT strains expressed similar chain lengths, with approximately 85% of those samples being 1-2 cocci per chain. However, the WT strains expressed different capsule levels, with WT-V and WT-ΔAcc- being statistically significant from one another.

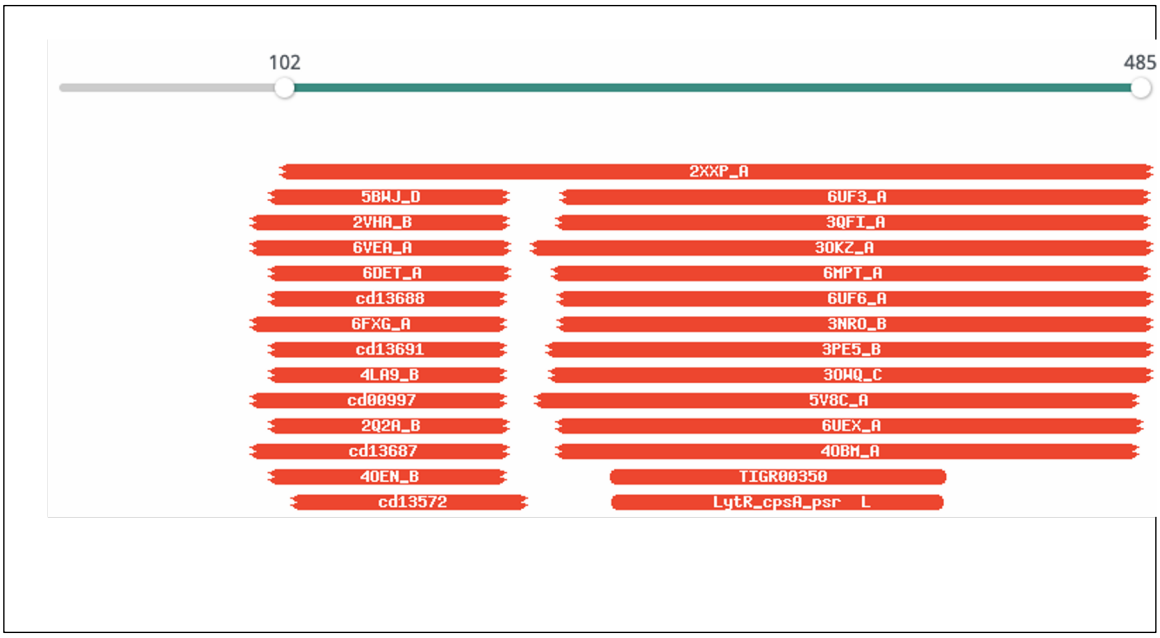
Based on the ELISA data, the WT-ΔAcc- strain showed less capsule than the WT-Vector strain, even though WT CpsA was expressed from the chromosome. This suggests that the WT-ΔAcc- strain causes a dominant negative effect because this mutation is interfering with the normal function of CpsA (Figure 27). The expression of only the LytR domain on both the vector and plasmid in the WT-ΔAcc- strain is inhibiting capsule expression in some way. The WT-ΔAcc+ strain does not cause a dominant negative effect, however, because this strain is able to complement back approximately the same amount of capsule expressed by the WT-Vector (Figure 27). Furthermore, the difference in capsule expression between the WT-ΔAcc- and WT-ΔAcc+ strains suggests that the intradomain region plays an important, but unknown role. This data is interesting, since no previous work has studied the effect of the intradomain region on capsule expression in GBS CpsA.

A cell aggregation assay was performed in order to determine the difference in bacterial culture suspension between each strain. When comparing all six strains overall, the WT GBS strains exhibited more turbidity than the ΔCpsA GBS strains (Figure 26). Each WT GBS strain was also compared against its ΔCpsA GBS counterpart (Figure 26). In each strain, the WT GBS expressed more turbidity than the ΔCpsA GBS strain. In addition, the ΔCpsA GBS strains showed a slightly larger cell pellet than the WT GBS

strains. This difference in turbidity and cell pellet size is likely due to the decrease in capsule that is caused by the deletion of CpsA. When CpsA is not present, it is understood that there is a decrease in capsule and an increase in chain length, which causes the cells to be less buoyant and, thus, sink more towards the bottom of the tube, causing both a larger cell pellet and less turbidity.

HHpred is a bioinformatics toolkit that aligns amino acid sequences to determine protein homology and predict structure. Previous HHpred data analysis showed no protein homologies to the accessory domain of the CpsA protein, until April 2021. The most recent data observed via HHpred suggests that the accessory domain has homology with substrate binding proteins (Figure 28). These proteins are mostly amino acid binding transport proteins, including sensory transduction histidine kinase, periplasmic binding transport proteins, glutamate receptors, and ABC transporters (Figure 29) [HHpred]. This data suggests that CpsA is homologous to a periplasmic L-aspartate/L-glutamate binding protein (DEBP) from *Shigella flexneri* [HHpred]. Previous data suggests that this protein acts as a periplasmic binding protein (PBP) of the ATP-binding cassette (ABC) transport system [10]. The PBP aids in transport by trapping the ligand that diffuses through the outer membrane into the periplasmic space of bacteria [10]. Further characterization of both proteins needs to be performed in order to determine the structural and functional homology between the two.

A.



B.

2VHA_B PERIPLASMIC BINDING TRANSPORT PROTEIN; TRANSPORT PROTEIN, PERIPLASMIC BINDING PROTEIN, LIGAND BINDING, ULTRAHIGH RESOLUTION, STRUCTURAL BASIS OF STRUCTURAL BASIS OF SPECIFICITY; HET: TRS, GLU; 1.0A {SHIGELLA FLEXNERI}

Probability: 97.69%, E-value: 0.0002, Score: 70.49, Aligned cols: 109, Identities: 8%, Similarity: 0.145, Template Neff: 12.3

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Q Q_5032959     89  VATTLYGFKSTIDLTNNLNKTASYSEIEMSVIVPKDSKITNIEAVSK-LAAPVKNDTSNITDLIEHIKSEKGISITPQKT   167 (485)
Q Consensus     89  ~~~~~~~it~~r~~~~~s~~~~~~~~~~vvv~kds~i~si~DL~g~~igv~~~~~~~~~~l~~l~~~~~~~~~~~~y   167 (485)
                    ++++.+..+..+.....++.++..  ....++++++++++||+| ++|+.  .++.....+.+...+.+...+++.+
T Consensus    86  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~i~~~~dl~g~~i~~~~~~~~~~~~~~~~~~~~   161 (287)
T 2VHA_B       86  FECGSTTNNVERQKQAAFSDTIFV--VGTRLLTKKGGDIKDFADLKGKAVVVT--SGTTSEVLLNKLNEEQKMNMRIISA   161 (287)
T ss_dssp           EECSSCBCCHHHHTTCEEEEEEEE--EEEEEEEETTSSCSSGGGGTTCEEEEE--TTSHHHHHHHHHHHTTCCCEEEEE
T ss_pred           EEecccCChhHhccceeccceeE--eceEEEEecCCCCCCHHHhCCCEEEEc--CCChHHHHHHHHHHcCCCcEEEEe


Q ss_pred           CCHHHHHHHHHcCCCcEEEEchHHHHHHHHhCccc
Q Q_5032959    168  DSYQDAYNRIKNGDSQAMVLNNAYVSLIELSTPD   201 (485)
Q Consensus    168  ~s~~~~~~aL~~g~vDAii~~~~~~~~~l~~~~~   201 (485)
                    .+..+++.+|.+|++|+++++.....+.+..+.
T Consensus    162  ~~~~~~~~~l~~g~vd~~~~~~~~~~~~~~~~~   195 (287)
T 2VHA_B       162  KDHGDSFRTLESGRAVAFMMDDAALLAGERAKAKK   195 (287)
T ss_dssp           SSHHHHHHHHHTTSCSEEEEEHHHHHHHHTTSSS
T ss_pred           CChHHHHHHHHhCCCCEEEeCHHHHHHHHHcCC
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Figure 28. A) Alignment of WT CpsA showing homology to other proteins. B) Amino acid alignment of WT CpsA with a periplasmic binding transport protein, showing 97.69% probability. (Images from HHpred)

CONCLUSIONS

While previous studies have deleted the LytR domain of CpsA, none have deleted the accessory domain or intradomain region. These studies have suggested that when there is a deletion of the LytR domain, significantly less capsule is expressed. This data led to the conclusion that the LytR domain is the important in the attachment of capsule to the cell wall, acting as a ligase. The accessory domain does, in fact, play a role in capsule expression and chain length. The data collected in this study from ELISA data suggests that the LytR domain cannot do this alone, and that the accessory domain is also required. The accessory domain might be involved in this process initially, by picking up the polysaccharide and handing it over to the LytR domain for further attachment. Furthermore, the intradomain region might also have a role in capsule attachment to the cell wall. The intradomain is important because there are differences between strains that express and do not express this region.

These conclusions come with some caveats, however. This data has not shown that when the protein is expressed from a plasmid that it is actually placed in the membrane. Since this study has suggested a dominant negative effect, CpsA most likely does make it to the membrane, but further experiments would need to be designed and analyzed to monitor the placement of the protein in the membrane. In addition, the ΔAcc- strains are missing the 60 amino acid intradomain region which allows the LytR domain to be closer to the membrane itself. The WT-ΔAcc- strain caused a dominant negative effect, suggesting that the LytR domain might not be able to function correctly, without the intradomain region being present.

FUTURE DIRECTIONS

There are still many more assays that need to be performed in order to determine the role of the accessory domain on CpsA function and capsule production in GBS. These experiments include fluorescent vancomycin assays, Western blots, and virulence studies using zebrafish as a model organism.

Fluorescent vancomycin assays can be used to determine the cell wall integrity of modified CpsA. In this experiment, newly modified cell walls in the samples are tagged with fluorescent vancomycin stain and observed with confocal microscopy. This allows for the visualization of defects in the cell wall which could be attributed to mutated CpsA. Previous studies have shown that WT CpsA fluoresces just around the outside of the cell wall, while ΔCpsA strains can be fully fluorescent. This difference is likely due to lower cell wall integrity of ΔCpsA strains, which allows the cell walls to be leaky and the stain to enter the entire cell. It is hypothesized that mutations in CpsA will cause a decrease in cell wall integrity due to the proposed interactions of CpsA with protein components associated with the cell wall.

A Western blot can be used to confirm if the protein is being displayed on the outside of the cell. This allows for the confirmation of whether or not the protein is actually making it to the membrane or not. In this experiment, the cell wall and cell membrane would be separated from the cytoplasm, resulting in two fractions. An antibody to the MBP protein, which is used to tag CpsA, would be used. If the CpsA protein is located properly, it would appear in the membrane fraction, but not in the cytoplasm fraction.

Microinjection of zebrafish can be used to visualize the path of infection via fluorescently tagged immune cells. This allows for the understanding of the function of the accessory domain as a virulence factor of GBS. In these experiments, two types of injections would occur. The first is a yolk sac injection, which would cause a systemic infection and could be used to measure overall virulence of the strain. The second type of injection that would occur is an otic vesicle injection, which would cause a localized infection and can be used to determine the chemotaxis of neutrophils to the site of infection. Previous research performed in the Neely lab suggests that 100% zebrafish succumb to WT GBS infection by 72 hours post injection. The effect of mutant CpsA injection can be compared to this data to determine if the mutant strain is more or less virulent. It is hypothesized that mutated CpsA will decrease the amount of capsule on the cell and will allow the immune system to recognize and clear the infection.

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Gina DiFederico was born and raised in Milford, Connecticut, attending Jonathan Law High School and Bridgeport Regional Aquaculture Science and Technology Education Center. While pursuing a Bachelor of Science in microbiology, Gina served as the Vice President and Personnel Chair of Chi Omega, as well as the President and Secretary of Operation H.E.A.R.T.S. As a member of the Neely Laboratory and the Honors College, she received the E. Reeve Hitchner Scholarship given to outstanding students in Microbiology in both 2020 and 2021. Gina was also a member of Sophomore Eagles and Alpha Lambda Delta Honors Societies and was nominated as Outstanding Student Leader of the Year in 2021. She also worked as a Campus Aide at the Hope House Health and Living Center, located in Bangor, Maine through her senior year. Upon graduation, Gina plans to pursue a career as a Physician Assistant.