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Analysis of the streptococcal cpsa protein in dna-binding and regulation of capsule and cell wall maintenance

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ANALYSIS OF THE STREPTOCOCCAL CPSA PROTEIN IN DNA-BINDING AND REGULATION OF CAPSULE AND CELL WALL MAINTENANCE

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GENERAL INTRODUCTION

Gram-positive streptococcal pathogens are the causative agent of a large number of infections that result in morbidity and mortality, representing a significant burden to the health care system (105, 134). Among these pathogens are streptococci that typically reside as commensal colonizers of the human body, but upon introduction to certain host sites cause opportunistic infections. *Streptococcus agalactiae* and *Streptococcus pneumoniae* represent two such streptococcal species. A variety of diseases can result from bacterial displacement to other host sites, with systemic infection the cause for greatest concern. Systemic infection entails entry of streptococci into the host bloodstream where dissemination to a number of host organs as well as the cerebrospinal fluid can occur. Ultimately, systemic infection can result in endocarditis (60) or meningitis (25, 64) which can both proceed rapidly to a fatal disease.

Streptococcus agalactiae, also known as Group B Streptococcus (GBS), is a commensal colonizer of the human gut and genitourinary tract (15), with up to 30% of women experiencing vaginal colonization (7). GBS is a leading cause of sepsis and meningitis in newborns (144), with both vertical and horizontal transmission of GBS to neonates occurring (146). Recent reports have highlighted a decline in incidence of neonatal disease due to GBS (117), but have also documented an increase in invasive disease in elderly patients with at least one underlying medical condition (117, 141), illustrating that GBS remains a systemic pathogen of medical importance.

Streptococcus pneumoniae is a commensal colonizer of the human nasopharynx (86) and is a significant cause of pneumonia, otitis media, sepsis and meningitis in the young and immunocompromised (71). Current vaccines have shown efficient coverage of the most prevalent disease-causing serotypes (166), but an increase in infections caused by serotypes not

covered by vaccination has been observed (61). Additionally, antibiotic resistance of non-vaccine serotypes appears to be increasing over time (41), highlighting the challenges faced in overcoming systemic streptococcal pathogens.

In the laboratory setting, the aquatic systemic pathogen *Streptococcus iniae* (45), is often used to model systemic disease in a natural host, the zebrafish (*Danio rerio*). Many of the virulence factors that contribute to systemic disease caused by GBS and *S. pneumoniae* are conserved in *S. iniae*, allowing investigation of virulence in an *in vivo* setting. While *S. iniae* only causes a relatively mild opportunistic sepsis or cellulitis in humans (45), disease within an aquatic host mirrors what is seen for human specific pathogens, providing a powerful tool for analysis of pathogenesis.

The ability of GBS, *S. pneumoniae*, and *S. iniae* to cause systemic disease is dependent on the production of a polysaccharide capsule that shields the bacteria from clearance by host immune components such as complement deposition (88) and phagocytosis (67). Alteration of the capsular polysaccharide composition within a single species results in different serotypes that are able to evade an immune response generated by other serotypes, making it difficult to generate a comprehensive vaccine that covers all serotypes of a given species.

Streptococcus iniae

The β-hemolytic aquatic pathogen *Streptococcus iniae* was initially isolated from the abscess foci of an Amazon freshwater dolphin, *Inia geoffrensis*, in 1976 (118). Antisera to antigens from *Streptococcus* groups A to U did not crossreact with *S. iniae* and therefore it was designated as a new non-Lancefield *Streptococcus* species (118). Subsequent to its identification, *S. iniae* was later observed to spread from diseased wild fish to cultured marine

fish, representing a threat to aquaculture (174). A wide number of fish species have demonstrated infection with *S. iniae* (2), including tilapia, trout (39), and hybrid striped bass (140), with an estimated annual financial impact on aquaculture of $10 million in the US and $100 million globally (140).

In 1995 an *S. iniae* specific vaccine was introduced for farmed rainbow trout, which reduced mortality from greater than 50% to less than 5% (40). However, by 1997 massive outbreaks of *S. iniae* infection in immunized fish were reported, with the eventual determination that a new serotype able to evade the vaccine triggered immune response was responsible (5). The vaccine escape observed with varying serotype of *S. iniae* is reminiscent of what has been observed for *S. pneumoniae*, with non-vaccine serotypes filling the void left by vaccination to more prevalent serotypes (61).

Recent work has characterized a live-attenuated strain of *S. iniae* lacking the phospoglucomutase gene, which provokes a robust immune response and has promise as a vaccine candidate (16). However, it is not apparent how this strain would overcome the serological diversity that has provided vaccine escape previously (5). Other vaccine strategies are currently being employed (2), but a better understanding of what regulates serological diversity in *S. iniae*, or the generation of a serotype-independent vaccine, is necessary to comprehensively protect aquaculture from *S. iniae* infection.

Fish infected with *S. iniae* exhibit multisystem organ involvement and diffuse hemorrhaging (5), and dissemination to the heart, brain, and spleen is observed as early as 15 minutes after intramuscular injection during experimental infection of zebrafish (79). Infection with *S. iniae* is overwhelmingly fatal in zebrafish, with 92% of fish succumbing to disease 4 days post infection (dpi) at an infectious dose of 1 x 10^5 CFU (79). Conversely, human infections

caused by *S. iniae* are limited in severity, and typically consist of bacteraemic cellulitis from patients who suffered a puncture wound while preparing contaminated fish (2). Many patients presenting with *S. iniae* infection were elderly and had one or more underlying medical condition concurrent with infection, indicating that zoonotic infection with *S. iniae* is primarily opportunistic and limited to immunocompromised individuals (2).

Phylogenetically, *S. iniae* is closely related to GBS when comparing the 16S ribosomal RNA sequence (68). A number of virulence determinants are also shared between *S. iniae* and GBS, including homologs of a C5a peptidase, enolase, and CAMP factor (6). Additionally, *S. iniae* also contains virulence factors homologous to those utilized by *Streptococcus pyogenes*, including an M-like protein and the cytolysin streptolysin S (6). Perhaps most important, the production of a polysaccharide capsule is highly conserved between *S. iniae* and GBS, with an alignment of the first four genes giving greater than 70% similarity at the translated amino acid sequence level. These first four genes are associated with regulation of capsule synthesis, and the high degree of homology between *S. iniae* and GBS is suggestive of a conserved regulatory mechanism. The importance of capsule in virulence was recently highlighted in a large scale signature-tagged random transposon mutagenesis (STM) study that identified *S. iniae* mutants incapable of surviving within the zebrafish host, with a large percentage of mutants containing transposon insertions in genes of the capsule operon (94).

Streptococcus agalactiae or GBS

Streptococcus agalactiae are β-hemolytic cocci that contain the Group-B Lancefield carbohydrate and are comprised of nine distinct serotypes (Ia, Ib, and II-VIII), with serotypes Ia, Ib, II, III, and V primarily responsible for invasive disease (63). GBS was originally associated

with bovine mastitis (12), and only recently has it become associated with invasive neonatal disease. Beginning in the 1960s GBS was identified as a causative agent of neonatal sepsis and meningitis, as well as adult bacteraemia (19, 38), and by the 1970s became the leading cause of neonatal infection in the developed world (30, 56, 91). At this time it is unclear whether GBS had a bovine origin and crossed the species barrier to become a colonizer and pathogen of humans (12), or rather that GBS was originally associated with humans and crossed in the other direction (15).

In the context of invasive neonatal disease, two distinct clinical manifestations are routinely observed. Early onset disease is due to intrapartum transmission of GBS from the genitourinary tract and occurs prior to 7 days of age (117). Late-onset disease occurs on or after day 7 and may be caused by horizontal transmission to the newborn (117). Developed countries routinely use a combination of antenatal culture screening of pregnant women and intrapartum antibiotic prophylaxis to prevent invasive GBS neonatal disease (133). However, this represents a significant cost to the health care system, and is economically untenable in developing countries (134). In this age of antibiotic resistance, the constant use of antibiotic prophylaxis is also a concern, and the need for an efficient comprehensive solution to GBS infection of neonates is great.

Humans are typically asymptomatically colonized by GBS (7, 15), and it is only introduction of bacteria to the bloodstream in immunocompromised or immune-incompetent individuals that results in disease. Transmission of GBS to the normally sterile circulatory system requires a dynamic shift in functional goals for the bacterium, as they move from a site associated with an acidic environment (genitourinary tract) to one with a more neutral pH (blood), encounter differing levels of nutrients, and enter into an environment under intense

immunological scrutiny. This requires an efficient switch from a form that promotes colonization to one that enhances survival, and while not mutually exclusive, these two states entail a number of differentially regulated genes. This has recently been demonstrated in two separate studies in which GBS was incubated *ex-vivo* in human whole blood, global gene expression analyzed, and demonstrated extensive changes to the GBS transcriptome when introduced to human blood (92, 93). A significant drawback to these studies was that they used a single clinical isolate of GBS for transcriptome studies, and they observed that expression data from the bacteria as well as cytokine levels in response to bacteria differed greatly for blood taken from different individuals.

Once in the host bloodstream, GBS utilizes a variety of virulence factors to survive and propagate. A number of these factors were identified by using STM in conjunction with a neonatal rat sepsis model to identify mutants unable to survive *in vivo* (65). The authors report the identification of gene classes associated with virulence, including binding and transport of small molecules, two-component signaling systems (TCSSs), metabolism, cell envelope regulation, adherence, protein secretion, and a number of genes of unknown function (65). Taken together with transcriptomic studies in human blood, it is evident that GBS pathogenesis is a multifaceted process that currently is not well understood.

Current efforts at controlling GBS infection are focused on vaccine design, with vaccines to surface proteins (111, 127, 145) and pilus (87, 104) currently being investigated. Promising results have been obtained thus far using a combination of pan-genome reverse vaccinology and structural vaccinology. Pan-genome reverse vaccinology entails the collation of whole genome sequences for a large number of strains of a given organism, allowing identification of vaccine targets that would provide protective immunity to a broad contingent of the organism (137).

Structural vaccinology pertains to the identification of antigenically unique structures, and the synthetic combination of these structures into a single molecule for multivalent protection for a given organism (104, 131). Both of these strategies have been utilized relatively successfully for GBS, by showing that all GBS strains sequenced so far contain at least one of three different pilus islands encoding the genes necessary for pilus assembly (87). While two of the pili are highly conserved amongst different strains of GBS, antigenic variation exists for one of the pilus islands (104). However, using pan-genome reverse vaccinology, variable regions corresponding to all sequenced strains were identified, and using structural vaccinology were combined into a chimeric protein that elicited protection for all strains carrying the variable pilus subset in a mouse-challenge model (104).

Despite these promising advances in vaccine design for GBS, more work is needed to characterize the immune responses elicited by pili-based vaccines as well as the efficacy of the immune responses. Further characterization of the pilus encoding islands is necessary to determine contributions to variability that may arise under selective pressure, and any implications this may have on vaccine escape. There are a number of lessons to be learned from the relatively successful adoption of a multi-valent *S. pneumoniae* vaccine (168), and subsequent escape by various strains (61).

Capsule and its regulation

The importance of the polysaccharide capsule of streptococcal pathogens capable of causing systemic disease is well established (23, 69, 79, 80, 82, 94, 107, 143, 165, 173). The capsular polysaccharide (CPS) consists of repeat polysaccharide units that are typically species and strain specific, with most of the enzymes required for generating CPS encoded within a

single genetic locus (173) (Fig. 1). All nine serotypes of GBS utilize differing linkages of glucose, galactose, and *N*-acetylneuraminic acid in the CPS repeat unit (26). *N*-acetylglucosamine is also used in all serotypes but type VI and type VIII, and type VIII also uses rhamnose in the CPS repeat unit (26). The structural variability observed for CPS in different serotypes of GBS has been attributed to genetic differences within the CPS synthesis locus, with en bloc replacement of glycosyltransferase genes putatively occurring through horizontal gene transfer, leading to different enzyme linkage specificities (26). As protective antibodies can be made to GBS CPS, these events were likely influenced by selective pressures exerted by the host immune system, necessitating the adaptation of alternative CPS structures for immune evasion. Despite the genetic diversity in the CPS synthesis locus, a subset of highly conserved genes is present in all serotypes, including *cpsA*, *cpsB*, *cpsC*, *cpsD*, *cpsE*, *cpsL*, *neuB*, *neuD*, *neuA*, and *neuC* (26). This conservation suggests that these genes are integral to success of GBS in the face of selective pressure by the host immune system.

In contrast to the nine serotypes currently identified for GBS, *S. pneumoniae* demonstrates a much larger repertoire of polysaccharide repeat units, with 93 individual serotypes identified thus far (173). This is primarily attributed to the natural competence exhibited by *S. pneumoniae*, allowing for increased acquisition of foreign DNA contributing to genetic variability within the CPS synthesis locus. Despite the large degree of serotype variability observed for *S. pneumoniae*, the first four genes of the CPS synthesis locus are conserved amongst serotypes, with *cpsB*, *cpsC*, and *cpsD* divided into two major clusters, and *cpsA* highly conserved amongst all serotypes (173).
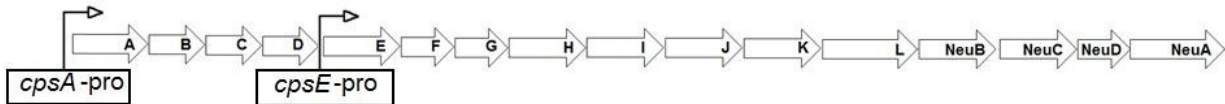
Gene	Function
cpsA..........	Putative transcriptional activator of capsule locus
cpsB..........	Phosphotyrosine-protein phosphatase, regulation of polysaccharide polymerization
cpsC..........	Accessory protein for CpsD, regulation of polysaccharide polymerization
cpsD..........	Tyrosine-protein kinase, regulation of polysaccharide polymerization
cpsE..........	Glycosyltransferase, initiates repeat unit synthesis
*cpsF..........	Putative polysaccharide biosynthesis protein
*cpsG..........	Putative galactosyltransferase
*cpsH..........	Polysaccharide repeat-unit polymerase
*cpsI..........	Glucosaminyltransferase
*cpsJ..........	Putative galactosyltransferase
*cpsK..........	Sialyltransferase
cpsL..........	Putative polysaccharide biosynthesis protein
neuB..........	Neuraminic acid synthesis
neuC..........	Neuraminic acid synthesis
neuD..........	Neuraminic acid synthesis
neuA..........	Neuraminic acid synthesis

Figure 1: Organization of genes in the polysaccharide capsule synthesis operon of GBS serotype Ia (26) with putative promoter elements (171) indicated with directed arrows. Functional assignments of capsule operon genes are shown below. Asterisks indicate genes with significant sequence variance between serotypes.

Currently, *S. iniae* has only two known serotypes, I and II, which are biochemically identified by arginine dihydrolase activity, with serotype I positive for enzyme activity and serotype II negative (10). Additionally, *S. iniae* of serotype II appear to produce more CPS than serotype I (10), which may explain the heightened severity of disease manifested by infection with serotype II in fish (5). This observation is also consistent with the determination that the avirulent commensal *S. iniae* strain 9066 is missing genes *cpsF* through *cpsL* of the capsule operon, supporting the notion that capsule is required to initiate disease (79). As with GBS, it appears that horizontal gene transfer has contributed to the presence and diversity of the *S. iniae* CPS locus (79).

The production of CPS is canonically associated with protection from immune clearance, but an indirect role for CPS in controlling adhesion and invasion of epithelium has also been observed. The acapsular commensal *S. iniae* strain 9066 demonstrated increased adherence to, and invasion of, human brain microvascular endothelial cell (BMEC) monolayers when compared to the encapsulated virulent 9117 strain (45). Additionally, an acapsular isogenic mutant of the type III GBS strain COH-1 demonstrated increased invasion of human umbilical vein endothelial (HUVE) cell monolayers compared to the encapsulated parent strain (47). Consistent with these results is the observation that *S. pneumoniae* strains with reduced levels of CPS or no CPS were better able to adhere to and invade the human lung alveolar carcinoma epithelial cell line A549 (51). Subsequently, it was determined that encapsulated strains of *S. pneumoniae* actively reduce levels of CPS when coming into contact with epithelial cells (51), which may represent a physiological adaptation prior to colonization. Taken together, these observations suggest that these bacterial pathogens tend to exist in one of two dynamically regulated states, with production of CPS enhancing survival during dissemination from a site of

commensal colonization, and reduction of CPS to enhance colonization when a suitable host site is encountered. Supporting this hypothesis is the recent determination that the GBS transcriptional regulator RogB coordinately regulates the CPS synthesis locus and genes that facilitate adherence. The authors demonstrated that a *rogB* null mutant had increased levels of transcript from the CPS locus and decreased levels of *fbsA* (50), which encodes a fibrinogen-binding protein that contributes to GBS adherence (135). RogB, or upstream effectors, may act to manage these opposing functions in response to environmental signals at specific sites within the host to promote protection from immune clearance or colonization appropriately.

Regulation of CPS production by systemic streptococcal pathogens is not well understood, especially in the context of host interactions that can be either commensal or pathogenic. Current understanding of CPS regulation is predicated on its polymerization, export, and ligation to the cell wall, and involves the genes *cpsB*, *cpsC*, and *cpsD*, which are highly conserved amongst *S. iniae*, GBS, and *S. pneumoniae*. In this regulatory scheme, CpsD acts as an autophosphorylating tyrosine-protein kinase (100), and is tightly associated with CpsC which acts to anchor CpsD to the cell membrane (22). Structural studies of CpsC and CpsD homologues in *Staphylococcus aureus* indicate that unphosphorylated CpsD forms an octameric ring structure in conjunction with CpsC, and upon autophosphorylation CpsD dissociates into a monomeric form while remaining associated with the intact CpsC octamer (109). In this system, CpsB is a manganese-dependent protein-phosphotyrosine phosphatase (98), and acts to dephosphorylate CpsD to recycle the system to its original state. The conformational changes induced by phosphorylation appear to regulate the length of CPS polymer exported to the cell surface as well as the amount of CPS that is exported (11). Deletion of *cpsB* is accompanied by an increase in phosphorylated CpsD with a concomitant increase in capsule, demonstrating a

positive correlation between phosphorylated CpsD and levels of CPS (11). Deletion of either *cpsC* or *cpsD* results in a severe reduction in CPS polymer length and export (11), consistent with their predicted role as regulators of this process. Taken together, the data suggests a model in which the CpsC and CpsD complex exists in either an "open" or "closed" state, with the open conformational state induced by CpsD phosphorylation leading to increased CPS chain length and export, and the closed conformational state induced by CpsB dephosphorylation of CpsD leading to decreased CPS chain length and export. The signals or upstream effectors that contribute to promoting either an open or closed conformation are unclear, but may involve environmental signals encountered within the host. This hypothesis is supported by the observation that the decrease in CPS observed for *S. pneumoniae* interacting with epithelial cells was not transcription dependent (51), and may have been coordinated through the CpsB, CpsC, and CpsD regulatory network.

Transcriptional regulation of the CPS synthesis locus has not been studied as extensively as the CpsB, -C, -D phosphorelay system, and it is still unclear what transcriptional control mechanisms are utilized by streptococcal pathogens to determine expression of CPS genes. As mentioned previously, RogB appears to contribute to this regulation in GBS, likely as a repressor (50), but the exact way in which this occurs has not been described. In *S. iniae*, the TCSS SivS/R regulates a number of virulence genes (14), and appears to contribute to transcriptional regulation of the CPS synthesis locus, with deletion of *sivS/R* leading to reduced levels of CPS and a decrease in transcript from the CPS synthesis locus (13). However, upon testing this same strain in our lab we were unable to confirm a decrease in CPS level (B.H. and M.N., unpublished data), leaving these findings contentious in our view. In *S. pneumoniae*, a homologue of catabolite control protein A (CcpA) called RegM was shown to directly influence transcriptional

regulation of the CPS synthesis operon, with deletion of *regM* leading to reduced levels of CPS expression when grown in the presence of glucose or sucrose, and thereby indicating *regM* is normally an activator of CPS expression (46). These findings are further supported by recent work in *S. suis*, with deletion of *ccpA* resulting in reduced levels of CPS, and again implicating CcpA related proteins as transcriptional activators of the CPS synthesis locus (167). CcpA proteins act to control sugar metabolism networks such that polysaccharides are utilized most efficiently under specific nutrient availabilities, and the involvement of a CcpA homologue in regulation of capsule is consistent with the presence of CPS synthesis substrates within interrelated sugar regulatory networks.

The first gene of the capsule operon, *cpsA*, has been described as a putative transcriptional regulator of the CPS synthesis operon in *S. iniae* (54), GBS (27), and *S. pneumoniae* (49, 98, 100). This was initially based on protein sequence homology of a C-terminal portion of CpsA with the LytR family of proteins, which are associated with transcriptional attenuation of autolysin genes (24, 58, 62, 74, 110). However, deletion of *cpsA* in GBS results in a decrease in transcription from the CPS synthesis locus and CPS levels, suggesting that CpsA is a transcriptional activator of the CPS synthesis locus (27). CpsA is an integral membrane protein, and was recently shown to have three transmembrane domains conferring a distinct membrane topology to the protein in which the majority of the protein resides extracellularly and only a small N-terminal portion of the protein is within the cytoplasm (Fig. 2) (54). Unlike CpsA, LytR proteins have only a single transmembrane domain, but it was recently established that similar to CpsA, the majority of the LytR protein is extracellular with a small N-terminal cytoplasmic domain (58). CpsA also differs from LytR proteins with the presence of a second protein domain, termed the DNA Polymerase Processivity Factor
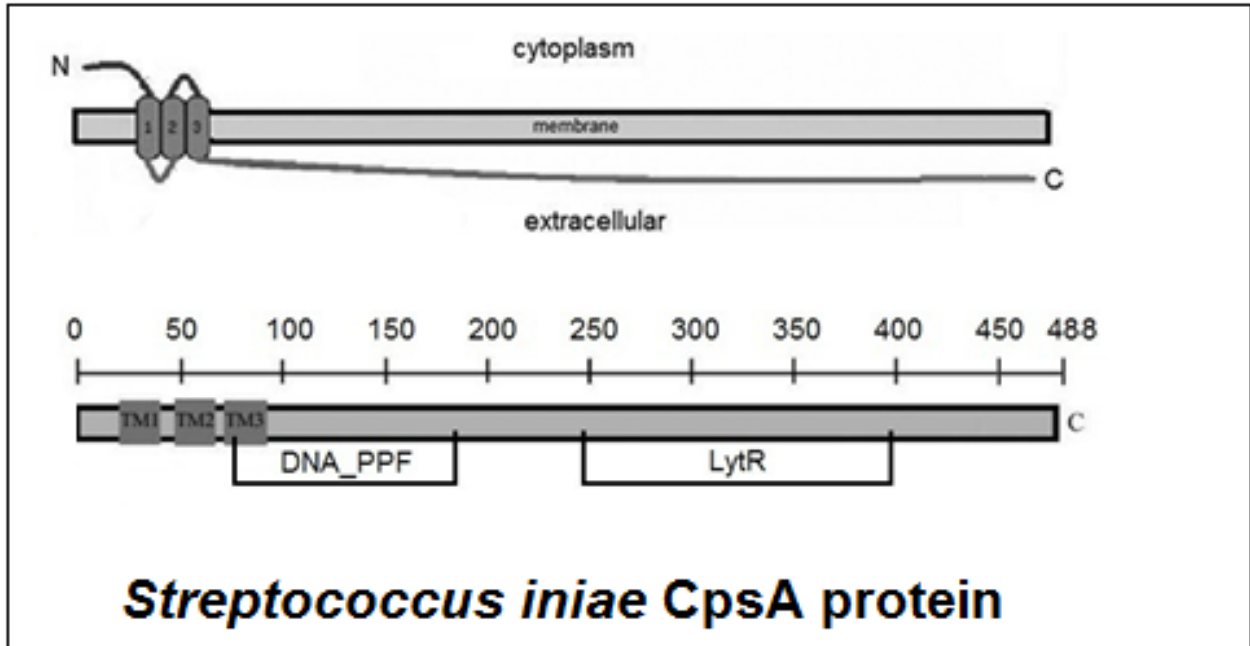
Figure 2: Membrane topology and conserved domains of the Streptococcal CpsA protein, with the CpsA protein of *Streptococcus iniae* shown as an example.

(DNA_PPF) domain, which is typically associated with sliding clamp function that enhances processivity of DNA replication and repair (97, 138). However, extensive protein sequence divergence from traditional sliding clamp structures, as well as the determination that the DNA_PPF domain resides extracellularly (54), indicates that the DNA_PPF domain of CpsA likely contributes to some other unrelated function. Although CpsA has been suggested as a transcriptional regulator of the CPS synthesis locus, many considerations are unclear, including how CpsA contributes to regulation, whether the conserved DNA_PPF and LytR domains play a role in this regulation, and the overall contribution of CpsA to virulence during systemic infection with streptococcal pathogens.

Pleiotropism and constituents of the cell surface

Gram-positive pathogens utilize complex defense mechanisms in response to extracellular stress, be it environmental or host-derived. One such defense mechanism involves modification of individual molecules of the cell surface. Unlike Gram-negative bacteria, Gram-positive species have only a single cell membrane composed of the well-characterized phospholipid bilayer (Fig. 3). Protection for the membrane is provided by the adjacent thick peptidoglycan layer, which also acts as a scaffold for other components of the cell surface. Interspersed throughout the multilayered peptidoglycan are the anionic teichoic acid molecules that provide cell wall integrity as one of their many functions, as well as supplying the major portion of the overall negative charge of the cell surface. Cell surface-exposed proteins are attached to the cell wall and function in multiple interactions with the extracellular environment. Lastly, a polysaccharide capsule serves as the outer most layer of the cell surface, providing additional protection from extracellular assaults. This highly structured consortium functions

cooperatively to provide stability and protection from the external environment. Recent work on individual structural components of the cell surface has demonstrated that disturbance of a single factor can result in pleiotropic effects, underscoring the interdependence of each component to the overall function of the cell surface. Consequently, loss or disturbance of any one of these closely associated factors may affect the regulation or ultimate function of another factor, resulting in a change in the equilibrium in which the cell surface typically exists.

Although a complete understanding of the cell surface network is currently unrealized, recent work has provided important new insights into the complex associations occurring between multiple elements that contribute to overall pathogen survival. Consideration of these distinctions is important to our understanding of bacterial pathogen virulence, as the cell surface remains an extremely important target for future antimicrobial therapy (17, 77, 132).
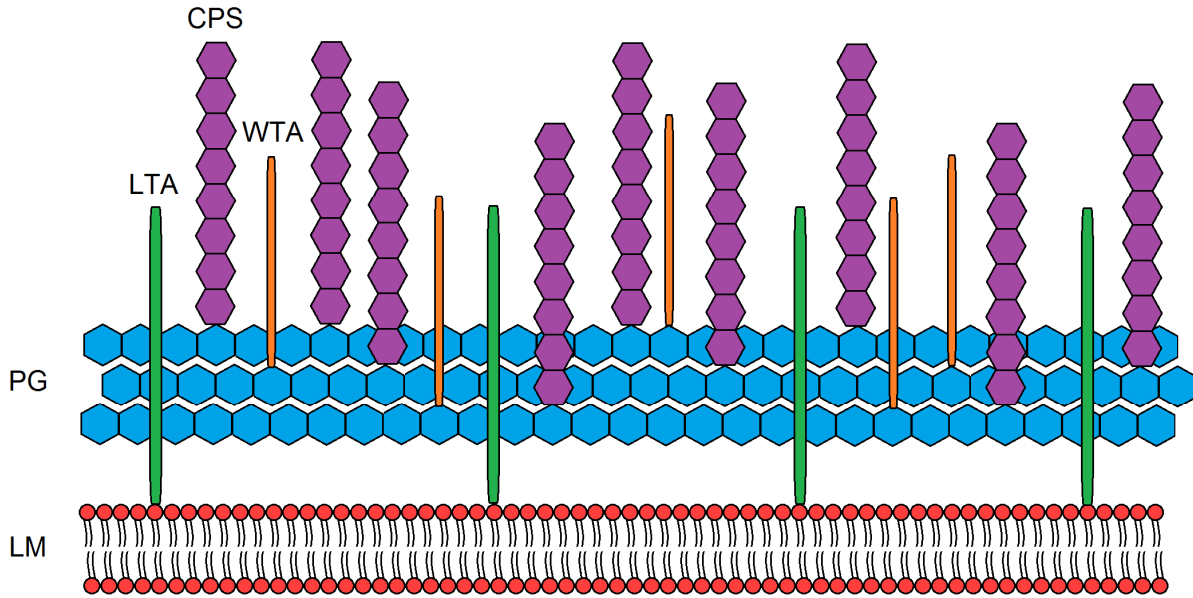
Figure 3: Simplified overview of the cell surface of Gram-positive bacteria, excluding proteins, with the lipid membrane (LM), peptidoglycan (PG), lipoteichoic acid (LTA), capsular polysaccharide (CPS), and wall teichoic acid (WTA).

Peptidoglycan

The cell surface of Gram-positive pathogens is a highly complex assembly consisting of a lipid bilayer, cell wall peptidoglycan (PG) (155), cell wall associated teichoic acids (WTA) (148), membrane associated lipoteichoic acids (LTA) (130), capsular polysaccharide (CPS) (173), and a variety of proteins associated with the cell membrane (72, 123, 147) or covalently attached to the PG of the cell wall (89, 114, 151).   Peptidoglycan of the cell surface exists as alternating repeats of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) with peptide side chains attached to the NAM residue. Although made up of simple repeating subunits, a complex association network of PG with multiple cell surface components occurs through cross-linking and covalent and non-covalent interactions. Although a number of methods have been employed to arrive at current models of peptidoglycan macromolecular structure, including atomic force microscopy (AFM), nuclear magnetic resonance (NMR), and cryo-transmission electron microscopy (TEM) (156), major obstacles still exist, including the structural diversity of PG between different organisms. Future strategies will likely focus on not just PG structures, but the full complement of interactions between PG, WTAs, LTAs, and CPS, with subsequent comparisons to surface structures in which one or more components are missing or altered by targeted mutations.

The importance of elucidating the actual structure of PG is highlighted by recent studies involving the deletion of genes with homology to *lytR*, a member of the *lytR_cpsA_psr* family, which is associated with transcriptional attenuation of PG hydrolases, their processing and transport. The interruption of LytR function results in pleiotropic effects, such as defective cell division, asymmetric septation, and altered antimicrobial sensitivity (24, 62, 110). Furthermore, the production of multiple phenotypes can be difficult to reconcile, as shown in *Streptococcus*

mutans, where deletion of *lytR* resulted in longer chain length despite an increase in autolysis (24), two traits typically considered to be the product of opposing processes. A similar phenomenon has been observed with insertional inactivation of *cpsA*, the putative regulator of the capsule locus in *S. iniae*, resulting in much longer chains of cocci (79) and increased autolysis when treated with non-ionic detergents or when grown in culture (B.H. and M.N., unpublished data). These seemingly contradictory findings emphasize the complexity of the PG cell wall network and its regulation by a number of factors, and indicate that unidentified targets of regulation likely exist for LytR family members beyond the PG hydrolase system that has been described thus far. A better understanding of the structural form of PG under conditions in which regulatory elements such as LytR are disrupted may provide insight on mechanisms responsible for controlling cell wall integrity.

In addition to regulation of synthesis and recycling of PG, a number of modifications can be made to PG, resulting in altered function. These include *O*-acetylation of NAM residues catalyzed by the protein OatA, and *N*-deacetylation of NAG through the functions of the PgdA protein. Both of these modifications confer resistance to lysozyme cleavage of the glycosidic bond between NAM and NAG residues (31). Regulation of OatA appears to be enacted through two-component systems that sense cell wall stress resulting in upregulation of *oatA* expression (66). Importantly, WTA is covalently attached to the same C-6 atom of NAM that is *O*-acetylated, suggesting there may be some cross-regulation of these processes (148). Expression of *pgdA* has been shown to be induced by oxidative stress in the Gram-negative pathogen *Helicobacter pylori* (158), and similar regulation may exist for PgdA homologues recently identified in Gram-positive pathogens (4). Taken together, a number of regulatory schemes

involved in PG synthesis, turnover through autolysin activity, and modification of PG residues converge to provide bacteria with a stable and functional cell wall.

Lipoteichoic acids and wall-teichoic acids

Teichoic acids of Gram-positive species represent an interesting subset of the cell surface. They exhibit a wide variety of functions including invasion of host tissue (36, 139), regulation of autolysis (3, 108, 115), and regulation of cell division (108, 128, 148). Cell wall-associated teichoic acid (WTA) and lipoteichoic acid (LTA) differ in overall structure, with WTA covalently attached to NAM (148) and LTA anchored to the membrane via a glycolipid (42). However, similar modifications are made to both WTA and LTA, such as D-alanylation (169), which has been shown to facilitate resistance to cationic antimicrobial peptides, glycopeptides, lytic enzymes produced by neutrophils (148) and reduced autolytic activity (115). The similar processing of WTA and LTA appears to provide some functional redundancy as disruption of both pathways simultaneously is lethal (108), but the phenotypes exhibited by individual disruption of LTA or WTA vary considerably. Disruption of LTA has been associated with a decrease in autolysis in *S. aureus*, and this seems to be associated with reduced levels of cell wall-associated hydrolases (108). This is consistent with the prediction that LTA actively recruits autolysins to septal regions during cell division to facilitate daughter cell separation (170). The relationship between LTA and autolysins is not currently known, but could include direct binding of autolysins to LTA, or enhanced substrate accessibility for autolysins in the presence of LTA.

The observations for LTA are in striking contrast to those found for WTA in which disruption results in increased autolysis (129) and a concomitant decrease in lysozyme resistance

(3). In *S. aureus*, WTA is hypothesized to indirectly mediate targeting of autolysins to newly synthesized PG by excluding its access to older PG where WTA is present, thus promoting its access to septal PG where WTA is absent and assisting with daughter cell separation (129). This hypothesis is supported by the observation that loss of WTA results in indiscriminate binding of autolysins to the cell surface instead of preferential localization to the septum (129). In addition to spatial regulation of autolysin activity, WTA has been shown to regulate peptidoglycan crosslinking in a spatial and temporal manner (3). Localized synthesis of intermediate forms of WTA at the septum appears to indicate the presence of a mature cell wall, and temporally triggers penicillin-binding proteins (PBP) to initiate crosslinking. Temporal regulation of this process may be important in permitting the introduction of proteins and glycopolymers that may not be able to penetrate a highly crosslinked cell wall (3).

Taken together, LTA and WTA appear to exert opposing and complementary functions during daughter cell separation with LTA promoting autolysis at the septum while WTA selectively blocks autolysis elsewhere on the cell. WTA intermediates subsequently accumulate at the septum, recruiting PBPs which crosslink the PG, forming a mature cell wall. These observations suggest a tight regulatory scheme over the localization of both elements during cell division, the mechanism of which is still not fully described. This principle may explain observed abnormalities in *Bacillus subtilis* morphology (128) and *S. aureus* cell division (108) when LTA is disrupted. Similarly, CPS and WTA have been demonstrated to have direct effects on each other. Phase variation in *S. pneumoniae* to a form that results in increased virulence relies on a switch from relative low levels of CPS and high levels of WTA to relative high levels of CPS and low levels of WTA (70). Whether this results from direct competition for covalent attachment to PG, or if it is due to a regulatory pathway that co-regulates levels of both CPS and

WTA is not understood. Clearly, WTA and LTA exert a fine tuned control over a number of important processes, including cell division and resistance to cell wall reactive antimicrobial agents. The effect that differing levels of CPS has on these traits has not been explored in depth, and it may be that the absence or presence of CPS contributes to the dynamic equilibrium experienced by components of the cell surface. Evidence for this is presented in both Chapters 1 and 2.

Capsule

The CPS of Gram-positive organisms can be covalently linked to a variety of surface structures, with attachment to the peptide moiety of PG for *Bacillus anthracis* (21), attachment to *N*-acetylglucosamine of PG for *Streptococcus agalactiae* (33), and covalent attachment to the PG or membrane for *S. pneumonia* (173). The enzyme that catalyzes the covalent addition of CPS to these locations has not been determined for many Gram-positive species, including *S. agalactiae* and *S. pneumoniae* (173). The location of CPS linkage is important to consider in the context of the cell surface as WTA may compete for these ligation sites, or experience steric hindrance in the presence of CPS (173). CPS appears to be generally linked to NAG instead of NAM in *S. agalactiae* and *S. pneumoniae* (173), therefore steric limitation may explain the relative balance between WTA and CPS described above for *S. pneumoniae*. An understanding of how CPS ligation is controlled may shed light on regulation of the other modifications that occur at or near this location.

Recent reports indicate that the presence or absence of CPS has a significant effect on minimum bactericidal activity of a number of cell wall reactive agents for *S. suis* (149) as well as vancomycin resistance in *S. pneumoniae* (101). Insertional inactivation of *cpsA,* which encodes

the putative regulator of capsule synthesis in *S. iniae,* results in various changes to antimicrobial sensitivity from cell wall-targeted compounds, with the *cpsA* mutant demonstrating decreased capsule levels in conjunction with increased resistance to lysozyme and bacitracin, and decreased resistance to ampicillin and methicillin (B.H. and M.N., unpublished data). These results indicate a clear association between expression of CPS and cell wall integrity, which may be mediated by the CpsA regulator protein. The exact mechanism mediating these events is unclear, though reasonable suggestions have been proposed based on simple occlusion of antibiotics via capsular stereochemistry or its contribution to structural stability (101, 149). These effects are most likely the result of more specific actions associated with perturbation of the cell surface, and these phenotypes may be explained by considering the relative changes in PG, WTA, and LTA and the pleiotropic effects that may occur with loss of CPS. Whether these regulatory events happen in response to the host environment is currently not known; however, evidence exists for the regulation of CPS expression *in vivo*, with the observation that levels of CPS are decreased when *S. pneumoniae* cocci come in contact with the surface of epithelial cells (51). This scenario suggests that bacteria may dynamically regulate levels or processing of CPS, WTA, and LTA in response to host signals, essentially altering the cell surface from prevalent CPS and immune evasion function to prevalent D-alanylated WTA and LTA with attachment and colonization function, as WTA and LTA have been shown to mediate adhesion to host cells (159). Supporting this model is the previously mentioned observation that CPS and WTA levels are coordinately altered during phase variation in *S. pneumoniae* (70).

CPS in *S. pneumoniae* also has a direct effect on the number of bacterial cells present in a single chain (8), with the presence of capsule generally leading to longer chains in *S. pneumoniae*. The observation that this trait varies when secondary mutations are made to genes

responsible for regulation of cell division further underscores the complexity of the cell surface and its regulation (8, 9). The disruption of *cpsA* in *S. agalactiae* and *S. iniae* results in decreased production of CPS (27, 79) which actually coincides with longer chains (79), (B.H. and M.N., unpublished). Whether this phenotype is a consequence of reduced capsule is unclear, or alternatively, CpsA may actively contribute to regulation of cell division. The discrepancy between these observations suggests that fundamental differences exist between *S. pneumoniae* and *S. agalactiae* concerning regulation of the cell surface, and may relate to the presence of multiple cell wall processing enzymes in *S. pneumoniae* that are absent in *S. agalactiae*, such as the PG hydrolases LytA and LytC. As with the other components of the cell surface, this indicates that the role of CPS and its effects on regulation of the cell surface in Gram-positive pathogens may indeed be species-specific.

The future of cell surface analysis

Clearly, analysis of the bacterial surfome should include the contributions made by PG, WTAs, LTAs, and CPS as each plays an individual role in pathogenicity and can have profound effects on the associations occurring in the overall architectural network. So called "fingerprinting" methods for characterizing the cell surface for individual species and strains under specific conditions will become increasingly important as the intricate associations between cell surface components and the corresponding implications for virulence and antimicrobial treatment are unraveled. Accomplishing this goal necessitates technological enhancements in the methods currently used to probe the bacterial cell surface. The determination that PG, LTA, WTA, and CPS have a shared pool of precursors and, with the exception of LTA, also share the undecaprenyl-phosphate acceptor (Und-P) for repeat unit

synthesis (169, 173) raises interesting questions about how precursor fate and prioritization of Und-P for different substrates is controlled. Ostensibly, this series of interconnected pathways has important points of regulation, and work describing regulation of branch points in this network or the point at which precursor fate is decided is currently incomplete. A better understanding of how each of these components relates functionally to one another in Gram-positive bacteria and the coordinated control of the enzymes that facilitate their construction and eventual fate remains an exceedingly important task in a future beset by the onset of antimicrobial resistance and vaccine escape through serotype diversity.

Work presented in the first chapter characterizes the contribution of the streptococcal CpsA protein to regulation of CPS in *S. iniae*, as well as pleiotropic effects associated with insertional inactivation of *cpsA*, which include altered antimicrobial sensitivity and autolysis activity. Work presented in the second chapter characterizes how the different domains of GBS CpsA contribute to regulation of CPS and chain length determination, and in turn how these observations can be used to impact GBS virulence in a detrimental fashion. The identification of streptococcal CpsA as an anti-virulence therapeutic target indicates that other regulators of the pleiotropic cell surface may also be amenable to similar strategies. The serotype independence of targets such as CpsA makes them especially attractive, and future vaccination or elimination strategies may hinge on the characterization of these pleiotropic cell surface regulators.

CHAPTER 1

MEMBRANE TOPOLOGY AND REGULATION OF CAPSULE AND CELL WALL BY THE CPSA PROTEIN OF *STREPTOCOCCUS INIAE*

ABSTRACT

Many streptococcal pathogens require a polysaccharide capsule for survival in the host during systemic infection. The highly conserved CpsA protein is proposed to be a transcriptional regulator of capsule production in streptococci, although the regulatory mechanism is unknown. Hydropathy plots of CpsA predict an integral membrane protein with 3 transmembrane domains and only 27 cytoplasmic residues, whereas other members of the LytR_cpsA_psr protein family are predicted to have a single transmembrane domain. This unique topology, with the short cytoplasmic domain, membrane localization and large extracellular domain, suggests a novel mechanism of transcriptional regulation. Therefore, to determine the actual membrane topology of CpsA, specific protein domains were fused to beta-galactosidase or alkaline phosphatase. Enzymatic assays confirmed that the predicted membrane topology for CpsA is correct. To investigate how this integral membrane protein may be functioning in regulation of capsule transcription, purified full length and truncated forms of CpsA were used in electrophoretic mobility shift assays to characterize the ability to bind the capsule operon promoter. The latter assays revealed that full length, purified CpsA protein binds specifically to DNA containing the capsule promoter region. Furthermore, the large extracellular domain was not required for DNA binding, but all cytoplasmic regions of CpsA are necessary and sufficient for specific binding to

the capsule operon promoter. This is the first demonstration of a member of this protein family interacting with its target DNA. The conserved protein domains of CpsA have no current designated function, so truncated forms of CpsA were used to assess the contribution of conserved domains to regulation of capsule. Ectopic expression of truncated forms of CpsA lacking the LytR domain in a WT *S. iniae* background resulted in differences in capsule level, chain length, and antimicrobial susceptibility, suggesting that conserved domains of CpsA contribute to regulation of capsule and the cell wall. Taken together, CpsA, as well as other members of the LytR_cpsA_psr protein family, may utilize a unique mechanism of transcriptional regulation to control the macromolecular structure of the cell surface.

INTRODUCTION

Systemic pathogens require a repertoire of specialized mechanisms to survive in the various tissue environments encountered during dissemination. A collection of highly efficient and precisely regulated virulence factors utilized by streptococcal systemic pathogens has allowed them to become remarkably successful at causing disease. For example, the systemic pathogens *Streptococcus agalactiae* and *Streptococcus pneumoniae* continue to be major causes of life-threatening infections (106, 117). One of the most important virulence mechanisms shared by systemic pathogens is the production of a polysaccharide capsule, which is essential for systemic dissemination during infection. In addition to providing resistance to phagocytosis (78) and complement deposition (88), the levels of polysaccharide capsule produced influence bacterial binding to host cells and tissue invasion during infection, with high levels of capsule inhibiting adherence (1, 51, 82), presumably by masking adhesive elements. For *S. pneumoniae,* capsule levels are reduced after adherence to epithelial cells, leading to increased exposure of adhesive molecules that facilitate a stronger interaction allowing for colonization (51). This suggests that streptococci most likely regulate synthesis of the polysaccharide capsule to balance systemic dissemination with colonization in response to cues from the host environment.

Although components of the polysaccharide capsule produced by streptococci can differ greatly not only between species, but also amongst different strains, certain genes of the capsule synthesis operon are highly conserved in most streptococcal pathogens. There is greater than 60% amino acid similarity between the first four genes of the capsule operon when comparing *S. agalactiae* to *S. pneumoniae*, all of which have been shown to play a role in regulation of capsule

production (27). This implies that regulatory strategies used by these pathogens to control capsule synthesis are likely conserved as well.

Analysis of enzymatic regulation of capsule production has provided a great deal of information on streptococcal pathogenesis (27, 79, 98, 153), although very little information has been provided on how regulation occurs at the transcriptional level. However, the apparent response of streptococcal pathogens to the surrounding environment and resulting modification of capsule production cannot be fully explained by regulation of enzymatic activity alone. The most likely scenario would involve a combination of transcriptional and post-translational enzymatic regulation of capsule synthesis allowing the bacteria to modulate capsule level in response to surrounding conditions. Transcriptional regulation of capsule production in streptococcal pathogens appears to be enacted through the highly conserved putative transcriptional activator CpsA (27, 79). The *cpsA* gene is the first gene of the capsule operon in streptococci. A non-polar deletion of CpsA results in streptococcal mutants with decreased levels of polysaccharide capsule and operon transcript (27). This is consistent with a transcriptional activating function for CpsA, whether it be a direct interaction or through another upstream event. To date, very little work has been described characterizing the role of CpsA in pathogenesis or its mechanism of transcriptional activation. *Streptococcus iniae*, an aquatic pathogen of great economic importance, is able to cause systemic disease in both marine animals and humans (160, 161). A comparison of the capsule operon between *S. iniae* and *S. agalactiae* shows greater than 70% amino acid similarity for the first four genes of the operon, implying homologous control mechanisms for virulence. We utilize *S. iniae* as a model pathogen due to the availability of a natural host, the zebrafish (*Danio rerio*), to understand host-pathogen interactions during streptococcal pathogenesis. A strain of *S. iniae* with a mutation in the *cpsA*

gene is significantly attenuated compared to the wild type strain in a systemic infection of zebrafish (79), demonstrating its importance in virulence. The high similarity between the capsule operons of *S. iniae*, *S. agalacticae*, and *S. pneumoniae*, suggest that observations made with *S. iniae* could be extended to these other pathogens as well. Therefore, in this study we characterize the highly conserved CpsA protein of *S. iniae* to determine its role in transcriptional regulation of capsule in streptococcal systemic pathogens.

The streptococcal CpsA protein is a member of the LytR_cpsA_psr family of "cell envelope-related transcriptional attenuator domain" (pfam PF03816) proteins, but has several intriguing differences from other members. One difference is in the predicted topology where CpsA has three transmembrane domains instead of the single transmembrane domain predicted for LytR and PSR proteins. Additionally, CpsA is the only family member that possesses a DNA polymerase processivity factor domain (DNA_PPF, pfam PF02916) adjacent to the LytR domain. Lastly, the CpsA proteins, of which over 180 members have been annotated, were shown to be involved in transcriptional activation (27, 79), as opposed to an attenuator function described for LytR and PSR (74, 75, 90) even though less than 30 of its more than 450 amino acids are predicted to reside in the cytoplasm (28, 157). The predicted topological model for CpsA includes a short N-terminal cytoplasmic tail, a small extracellular loop, a small cytoplasmic loop, and a large extracellular C-terminus. This suggests a novel mechanism for transcriptional activation and confirmation of this topological prediction is an important prerequisite to analyzing CpsA function.

In this chapter, employing a commonly used method for confirmation of protein membrane topology in which fusions are made to reporter enzymes that reflect specific subcellular localizations, we determined the membrane topology of the streptococcal CpsA

protein. Furthermore we demonstrate that there is a direct interaction between purified CpsA and capsule operon promoter DNA, and that specific domains of the CpsA protein are required for this interaction. We have also characterized the specificity of this interaction and speculate as to what it infers about the regulatory role of CpsA regarding control of polysaccharide capsule synthesis. Additionally, we propose that CpsA of *S. iniae* coordinately regulates multiple aspects of the cell surface, including the polysaccharide capsule and cell wall maintenance. The studies in this chapter support this hypothesis by showing that removal of the conserved LytR domain from CpsA results in a dominant negative loss of capsule when ectopically expressed in the WT *S. iniae* background, and also results in altered antimicrobial sensitivity.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Plasmids were maintained in *Escherichia coli* T7 Express lysY/Iq cells (NEB). Luria-Bertani (LB) medium was used to culture *E. coli* strains. Antibiotics were added as necessary to LB medium at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; erythromycin, 750 μg/ml; and chloarmphenicol, 20 μg/ml, for *E. coli* strains. *E. coli* cultures were grown at 37°C with shaking. Solid media was generated by supplementing liquid media with 1.4% agar (Acumedia). The streptococcal strain used was *S. iniae* 9117, a human clinical isolate from the blood of a patient with cellulitis (44). *S. iniae* was cultured in Todd-Hewitt medium (BD) supplemented with 0.2% yeast extract (BD) and 2% proteose peptone (BD) (TP) in airtight conical tubes without agitation at 37°C. Antibiotics were added as necessary to TP medium at the following concentrations: kanamycin, 500 μg/ml; erythromycin, 2 μg/ml; and chloramphenicol, 3 μg/ml, for *S. iniae* strains. *S. iniae* grown on solid media supplemented with 1.4% agar (BD) were incubated in GasPak jars (BBL) with GasPak anaerobic system envelopes (BD).

Cloning of CpsA membrane topology fusions: The previously described *rofA* promoter from *S. pyogenes* (48) was amplified by PCR from chromosomal DNA using primers 5'*rofA*-ApaI and 3'*rofA*-PstI (see Table 1) and the resulting fragment was inserted into the ApaI/PstI sites of the pLZ12-Km vector (53) creating pLZ12-Km-*rofA*-pro. The *lacZ* gene was amplified from plasmid pTL61T (76) using primers 5'*lacZ*-BamHI-SmaI and 3'*lacZ*-BglII. The *phoZ* gene was amplified from *Enterococcus faecalis* genomic DNA using primers 5'*phoZ*-BamHI-SmaI and 3'*phoZ*-BglII, removing the signal sequence from *phoZ*. Each of these fragments was then digested with

BamHI and BglII and inserted into the corresponding BamHI and BglII sites of pLZ12-Km-*rofA*-pro. This led to the generation of the fusion vectors p*lacZ* and p*phoZ*. Construction of several CpsA-LacZ fusion vectors were generated by amplification of fragments of the *cpsA* gene using genomic DNA of *S. iniae* strain 9117 with the universal 5' primer 5'*cpsA*-PstI in conjunction with the following primers: 3'*cpsA*-cyto1-SmaI, 3'*cpsA*-ext1-SmaI, 3'*cpsA*-cyto2-SmaI, 3'*cpsA*-ext2-SmaI, and 3'*cpsA*-dna_ppf. Each of these *cpsA* gene fragments were then digested with PstI and SmaI and inserted into the corresponding PstI and SmaI sites of p*lacZ* in frame and upstream of the lacZ gene (Fig. 4A). This created the plasmids p*lacZ*-*cpsA*-cyto1, p*lacZ*-*cpsA*-ext1, p*lacZ*-*cpsA*-cyto2, p*lacZ*-*cpsA*-ext2, and p*lacZ*-*cpsA*-dna_ppf. Construction of the CpsA-phoZ fusion vectors were created in a similar manner by amplification of fragments of the *cpsA* gene using the universal 5' primer 5'*cpsA*-EcoRI in conjunction with the following primers: 3'*cpsA*-cyto1-SmaI, 3'*cpsA*-ext1-SmaI, 3'*cpsA*-cyto2-SmaI, 3'*cpsA*-ext2-SmaI, and 3'*cpsA*-dna_ppf. These fragments were then digested with EcoRI and SmaI and inserted into the corresponding EcoRI and SmaI sites of p*phoZ* in frame and upstream of the phoZ gene (Fig. 4B). This generated the plasmids p*phoZ*-*cpsA*-cyto1, p*phoZ*-*cpsA*-ext1, p*phoZ*-*cpsA*-cyto2, p*phoZ*-*cpsA*-ext2, and p*phoZ*-*cpsA*-dna_ppf. The vector strains contained the parent plasmid with no *cpsA* fragment fused to the reporter enzyme and thus no ribosome binding site or start codon. These plasmids were then transformed into *S. iniae* as previously described (94).

To create and purify the intein-tagged CpsA protein, we used the IMPACT[TM] Kit (NEB). Amplification of *cpsA* gene fragments for fusion to the intein chitin binding protein (CBP) epitope tag was accomplished using the primer 5'*cpsA*-short-NdeI in conjunction with either 3'*cpsA*-short-SapI or 3'*cpsA*-short-TM. The PCR products were digested with NdeI and SapI and inserted into the pTXB1 plasmid (NEB) creating the plasmids pTXB1-*cpsA*-cyto1 and

pTXB1-*cpsA*-ext1 with 3' fusions to the intein tag. These constructs were transformed into T7 Express (Invitrogen) *E. coli* cells.

Measurement of β-galactosidase activity: The p*lacZ*-*cpsA* expressing *E. coli* cells were grown overnight as described above, then sub-cultured 1:40 the next morning into new medium and grown at 30°C with shaking to an OD_{600} of 0.3. Chloramphenicol was added at a concentration of 200 ug/ml to 1 ml of culture and incubated on ice for 20 minutes. Each sample was prepared by adding 0.1 ml of chloramphenicol-treated cell suspension to 0.9 ml of Z buffer (95), then adding 20 μl of 0.1% (wt/vol) SDS and 40 μl of chloroform, and vortexing the mixture vigorously for 10 seconds. The samples were then incubated at 30°C for 15 minutes before 200 μl of the colorimetric substrate *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) (NPI) was added. Reactions were developed at room temperature for various times prior to terminating the reaction with the addition of 500 μl of 1 M Na_2CO_3. Samples were centrifuged briefly to pellet cellular debris and the OD_{420} and OD_{550} of each supernatant was measured. Miller units were calculated as: units = $1,000 \times [OD_{420}-(1.75 \times OD_{550})]/(time \times volume \times OD_{600})$ (95). Each assay was repeated a minimum of three times.

Determination of alkaline phosphatase activity: The p*phoZ*-*cpsA* expressing *E. coli* cells were grown overnight, then sub-cultured 1:40 the next morning into new medium and grown at 37°C with shaking to an OD_{600} of 0.3. Culture concentrations were then normalized to an OD_{600} of 0.75 in 1 ml of LB, and 50 μl of each normalized sample was placed into wells of a flat bottom 96 well plate in triplicate, followed by 200 μl of *p*-nitrophenyl phosphate (Sigma) suspended in 1 M Tris (pH 8). Plates were incubated in the dark for one hour prior to reading the

OD$_{405}$, OD$_{550}$, and OD$_{600}$. The p*phoZ-cpsA* expressing *S. iniae* strains were grown overnight, sub-cultured 1:40 the next morning into new medium and grown statically at 37°C to an OD$_{600}$ of 0.3. Cultures were then normalized to an OD$_{600}$ of 0.4 in 1 mL of 1M Tris (pH 8) containing *p*-nitrophenyl phosphate. These were incubated in the dark for 3 hours, and then briefly centrifuged to pellet cells prior to reading the OD$_{405}$, and OD$_{550}$. Activity for *E. coli* was calculated as follows: $1,000 \times [OD_{420}-(1.75 \times OD_{550})]/(time \times volume \times OD_{600})$. Activity for *S. iniae* was calculated as done for *E. coli*, multiplied by an additional factor of 10 and normalized to WT activity. Each assay was repeated a minimum of three times.

Whole cell ELISA: Analysis of the *cpsA*-intein fusion expressing strains (described above) was accomplished by growing the cells overnight as previously described (see above) and subculturing 1:40 the next morning into new medium with shaking at 37°C for 2 hours. Expression was induced by addition of 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (MP Biomedicals) for three hours. Cultures were then concentrated to an OD$_{600}$ of 0.75 and resuspended in 1 mL LB media containing a 1:10,000 dilution of anti-CBD rabbit serum (NEB) and incubated at 4°C on a rotating platform for 1 hour. After incubation, cells were pelleted by centrifugation at $8,000 \times g$ for 5 minutes and washed with 1 mL of LB three times. Cells were then pelleted again and resuspended in 1 mL of LB media containing a 1:10,000 dilution of goat-anti-rabbit antibody conjugated to alkaline phosphatase and incubated at 4°C on a rotating platform for 1 hour. Cells were subsequently pelleted and washed another three times, and finally resuspended in 1 ml of LB for detection of alkaline phosphatase activity as described above.

Cloning of Maltose Binding Protein-CpsA fusions: The full-length *cpsA* gene was amplified from *S. iniae* 9117 genomic DNA using the primers 5'*cpsA*-SmaI and 3'*cpsA*-PstI. Various 3' truncations of the *cpsA* gene were amplified from *S. iniae* 9117 genomic DNA using the primer 5'*cpsA*-SmaI in conjunction with the following primers: 3'*cpsA*-del1-PstI-stop, 3'*cpsA*-del2-PstI-stop, and 3'*cpsA*-del3-PstI-stop. Each of these products was digested with SmaI and PstI and cloned into the corresponding SmaI and PstI sites of pMAL-c2x (NEB) leading to in-frame fusion of *cpsA* fragments downstream of the *malE* gene. This generated the following plasmids: pMAL-*cpsA*-full, pMAL-*cpsA*-116, pMAL-*cpsA*-78, and pMAL-*cpsA*-23. These constructs were transformed into T7 Express (Invitrogen) *E. coli* cells.

Protein purification: The pMAL-*cpsA* expressing *E. coli* strains were grown overnight as described above and the following morning sub-cultured 1:40 into 500 ml new medium and grown at 37°C shaking until reaching an OD_{600} of 0.5, at which point protein expression was induced by addition of 0.3 mM isopropyl-β-D-1-thiogalactopyranoside followed by incubation for 3 hours. Cells were then harvested by centrifugation at 4000 × g for 20 minutes, the supernatant discarded, and the cells resuspended in 20 ml of Column Buffer (20 mM Tris-HCl, 200 mM NaCl, and 1mM EDTA) and stored at -20°C. Frozen culture was thawed on ice and sonicated to lyse cells. The lysate was then diluted to a total volume of 100 ml using Column Buffer and loaded onto a column containing amylose beads (NEB) and eluted according to the manufacturer's specifications. Purified protein concentration was determined using the BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions. Protein purity was assessed with SDS-PAGE.

Though no advanced preparative techniques were required for purification of the MBP-CpsA fusions, those constructs containing transmembrane domains preferentially fractionated into the insoluble lysate fraction after sonication. The degree of insolubility of these proteins was also evidenced by minimal migration in non-denaturing polyacrylamide gels during EMSAs, a property that was somewhat mitigated by the addition of non-ionic detergents to binding reactions (see below).

Generating digoxigenin-labeled DNA probe: A 182 bp probe emcompassing the *cpsA* promoter was amplified from *S. iniae* 9117 genomic DNA using the primers 5'*cpsA*-pro and 3'*cpsA*-pro. This product was then labeled using the DIG Gel Shift Kit, 2nd Generation (Roche) and labeling efficiency determined according to manufacturer's instructions.

Electromobility Shift Assays (EMSA): The digoxigenin labeled *cpsA* promoter was used as the probe for all gel shift assays. To conduct the EMSA, varying amounts of the MBP–CpsA protein fusions were incubated with a constant amount of labeled probe (17 fmol) in a binding buffer containing 100 mM HEPES pH 7.2, 1 mM EDTA, 50 mM KCl, 50 mM $MgCl_2$, 1 mM DTT, 25% (v/v) glycerol, 1% (v/v) NP40, and 1% (v/v) CHAPS for 30 minutes at room temperature. For reactions requiring competitor DNA, a 20-fold excess of either unlabelled *cpsA* probe or sheared salmon sperm DNA was included in the binding reaction. The samples were loaded onto a 6% polyacrylamide native gel consisting of 6% (v/v) polyacrylamide, 44.5 mM Tris base, 44.5 mM boric acid, and 1mM EDTA. Electrophoresis was performed at 4°C. The gel was then transferred to a nylon membrane (Micron Separations Inc.) using a semi-dry transfer apparatus (Bio-Rad). Chemiluminescent detection of DIG-labeled DNA on membranes was

accomplished with a commercial reagent (DIG Gel Shift Kit, 2nd Generation, Roche) according to manufacturer instructions, followed by exposure to X-ray film.

Cloning truncated forms of *S. iniae* CpsA: A gene encoding kanamycin resistance was amplified from the plasmid pABG5 (48) using the primers 5'*kan*-BamHI and 3'*kan*-EcoRI. This product was digested with BamHI and EcoRI and cloned into the corresponding BamHI and EcoRI sites of the plasmid pOri23 (122), creating the plasmid pOri23-kan. The *cpsA* promoter and a truncated form of *cpsA* lacking the LytR domain were amplified together from 9117 genomic DNA using the primers 5'*cpsA*-pro-BamHI and 3'*cpsA*-ΔLytR-PstI-stop. This product was digested with BamHI and PstI and cloned into the corresponding BamHI and PstI sites of the plasmid pOri23-kan. This generated the plasmid pCpsA-ΔLytR. A truncated form of *cpsA* entailing only the DNA_PPF domain was amplified from 9117 genomic DNA using the primers 5'*cpsA*-DNA_PPF-BamHI and 3'*cpsA*-DNA_PPF-FLAG-PstI-stop. This product was digested with BamHI and PstI and cloned into the corresponding BamHI and PstI sites of the plasmid pLZ12-*rofA*-pro (48, 103). This generated the plasmid pCpsA-DNA_PPF.

Measurement of *S. iniae* capsule levels with Percoll buoyant density assays: Buoyant density centrifugation was performed similarly to previous work (32), (79), but with modification. Linear density gradients of Percoll (GE Healthcare) were generated by diluting Percoll to a high density limit (1.120 g/cm^3) and low density limit (1.0 g/cm^3) with a final concentration of 0.15 M NaCl according to the manufacturer's instructions, and carefully layering 5 mL of the low density solution on top of 5 mL of the high density solution in a 10 mL conical tube (BD). These tubes were then set horizontally at a 15° angle to the benchtop, and left

overnight. The next morning tubes were set upright and allowed to settle for 30 minutes prior to use. Bacterial cultures were grown overnight as described above, and cultures were normalized to an OD_{600} of 0.6 in 1 mL of TP medium, pelleted by centrifugation, resuspended in 50 μl of PBS, and added directly to the top of the Percoll gradients. Tubes were then centrifuged for 40 minutes at 5000 rpm in a swinging bucket Eppendorf Centrifuge 5403 with Rotor 16A4-44 at room temperature. Measurements were then taken from the meniscus to the bottom of the cell band in each tube, and compared to a set of colored beads of known density (GE Healthcare) to determine bacterial cell density. These experiments were performed at least three times, with results reported using a representative experiment. Measurement of capsule for time course experiments was identical to that given above, except that WT 9117 bacteria were taken at different time points from separate cultures after inoculating with a 1:10 dilution from an overnight culture.

Measurement of antimicrobial resistance: *S. iniae* strains were grown overnight, subcultured into fresh medium with a 1:25 dilution, and grown to an OD_{600} of 0.3. Cultures were normalized and serially diluted to 1 x 10^4 CFU/mL and 10 μl added to individual wells of a 96 well plate, with a final volume of 200 μl including antimicrobial agents. Antimicrobial concentration ranges were as follows: lysozyme, 0 mg/mL to 52 mg/mL; bacitracin, 0 μg/ml to 6.6 μg/ml; and methicillin, 0 μg/ml to 1.65 μg/ml. Subsequent to addition of bacteria, plates were incubated at 37° C overnight and OD_{600} measured with a VERSAmax microplate reader (Molecular Devices) the following day.

Measurement of autolysis: *S. iniae* strains were grown overnight and normalized to an OD_{600} of 0.3. Bacteria were then pelleted and resuspended in either PBS, or 0.6% triton X-100 or 0.6% tween-20 (v/v) in PBS. Resuspended bacteria were placed into a 96 well plate, incubated at incubated at 37° C for 24 hours and OD_{600} measured with a VERSAmax microplate reader (Molecular Devices) the following day. Lysis was calculated as ((OD_{600}final/OD_{600}initial) x 100%).

Table 1: Primers used in this chapter.

Primer name	Sequence 5' – 3'
5'*rofA*-pro-ApaI	TCC GGG CCC ATT TTC TCT CCT CTC AAA AAC ATA TAT GAG C
3'*rofA*-pro-PstI	AAA ACT GCA TCA CAA TAA TGG TTT AGT TGT AAA AAG GTT AAG CC
5'*lacZ*-BamHI-SmaI	CGC GGA TCC GCG TCC CCC GGG GTC GTT TTA CAA CGT CGT GAC
3'*lacZ*-BglII	GGA AGA TCT CTG CCC GGT TAT TAT TAT TTT TGA C
5'*phoZ*-BamHI-SmaI	CGC GGA TCC GCG TCC CCC GGG CAA AAA AGC GGC GAA AAA C
3'*phoZ*-BglII	GGA AGA TCT CGT TCT GCT TTT TCT TCA TTT TG
5'*cpsA*-PstI	AAA ACT GCA GGT AAA CTA GAT GAT TTG GAG
3'*cpsA*-cyto1-SmaI	TCC CCC GGG GCT TAG ATT ATG TTT CTC TTT CG
3'*cpsA*-ext1-SmaI	TCC CCC GGG CTG CCT AAA AGC AAG GAA G
3'*cpsA*-cyto2-SmaI	TCC CCC GGG CTT TTT CTT GAT AAC GAG
3'*cpsA*-ext2-SmaI	TCC CCC GGG AGC TGT AAA GTC AAT TGT TG
3'*cpsA*-dna_ppf-SmaI	TCC CCC GGG CAT AAC CAT TGC TTG AGA CTG
5'*cpsA*-EcoRI	CCG GAA TTC GAT AGC TAT AAG TCT AAT AGA TGA AG
5'*cpsA*-short-NdeI	GGA ATT CCA TAT GGC ACA TTC CAG AAG TAA ACG
3'*cpsA*-short-SapI	GAG GGC TCT CCG CAC GTA TTT ATT AAG CTT AG
3'*cpsA*-TM-SapI	GAG GGC TCTTCC GCA ATA GAG GTA CAT CCA AAA GC
5'*cpsA*-SmaI	TCC CCC GGG ATG GCA CAT TCC AGA AGT AAA CG
3'*cpsA*-PstI	AAA ACT GCA GTC ACC TGT TGA ACC TGT CC
3'*cpsA*-del1-PstI-stop	AAA ACT GCA GCT ATC ACA ATC TCT GAG AAA GAG GCT G
3'*cpsA*-del2-PstI-stop	AAA ACT GCA GCT ATC ACA ATC CCT TAA GCT TTT TCT TG
3'*cpsA*-del3-PstI-stop	AAA ACT GCA GCT ATC AGA TAT TTA TTA AGC TTA GAT TAT G
5'*cpsA*-pro	CTC ATA ATG ACA GTC TAT C
3'*cpsA*-pro	CCA TCA ATA TCA TTT AAG TC
5'*kan*-BamHI	CGC GGA TCC CAG GAC AAT AAC CTT ATA GC
3'*kan*-EcoRI	CCG GAA TTC GAC ATC TAA ATC TAG GTA C
5'*cpsA*-pro-BamHI	CGC GGA TCC GTT GTA TTC TCA TAA TGA CAG TC
3'*cpsA*-ΔLytR-PstI-stop	AAA ACT GCA GCT ATC AGA AAT CGG ACC ATA AGT ATC
5'*cpsA*-DNA_PPF-BamHI	GCG GGA TCC GAG GAG TAG TAA AGG GAT TGA CCA GTC
3'*cpsA*-DNA_PPF-FLAG-PstI-stop	AAA ACT GCA GTC ACT TGT CGT CAT CGT CCT TGT AGT CCA TAT AAG CAC TGT TCA TAA CC

RESULTS

CpsA membrane topology verification. Gene fusions made to either *lacZ* (encoding β-galactosidase) or *phoZ* (encoding alkaline phosphatase) provide a powerful framework for characterizing membrane protein topology. Fusion proteins containing β-galactosidase display high enzymatic activity only when β-galactosidase is attached to cytoplasmic domains (83, 85). In contrast, fusion proteins containing alkaline phosphatase exhibit high enzymatic activity only when alkaline phosphatase is fused to extracellular or periplasmic protein domains (84). If alkaline phosphatase is fused to a cytoplasmic protein domain then it remains enzymatically inactive (85). Therefore, high enzymatic activities for β-galactosidase fusions indicate a cytoplasmic location while high enzymatic activities for alkalkine phosphatase fusions indicate an extracellular orientation. Fusions of various truncated forms of CpsA were made to either β-galactosidase or alkaline phosphatase for confirmation of membrane topology, generating the plasmids p*cpsA-lacZ* (Fig. 4A) and p*cpsA-phoZ* (Fig. 4B). These fusions were made in-frame at five different locations of the *cpsA* gene resulting in a truncated protein containing the first 19, 50, 74, 105, or 188 amino acids. The location of each of these fusions relative to the three transmembrane domains is illustrated in Fig. 5. Colorimetric assays were performed with strains harboring each of the CpsA fusion plasmids as described in Materials and Methods.

Fusions to the predicted cytoplasmic regions gave the highest β-galactosidase activity in *E. coli* at 3440 Miller units (MU) for CpsA-cyto1-βgal and 5127 MU for CpsA-cyto2-βgal, while fusions to putative extracellular regions gave much lower activity at 86 MU for CpsA-ext2-βgal and 101 MU for CpsA-dna_ppf-βgal (Fig. 6A). Although less than half of that observed for the two predicted cytoplasmic regions, there was a higher than expected activity observed for CpsA-

ext1-βgal (1687 MU), as it was predicted to be in an extracellular loop. We attempted to perform the same β-galactosidase assays in *S. iniae*, but found that all constructs gave less than 1 MU of activity, a problem that persisted even with a positive control consisting of the native *lacZ* gene positioned behind the same promoter, which demonstrated only 8 MU of activity (data not shown). We speculate that this may be due to a problem with permeabilization of the cells, as in our experience it is especially difficult to achieve cell permeabilization with *S. iniae* compared to other Gram positive organisms. We adapted a variety of permeabilization methods corresponding to successful protocols in a number of other Gram positive species, including *S. pyogenes* (43), *S. agalactiae* (121), and *Enterococcus faecalis* (52), without any success.

Colorimetric assays were also performed with *E. coli* strains harboring each of the CpsA alkaline phosphatase fusion plasmids (Fig. 6B). Fusions to predicted extracellular domains gave the highest alkaline phosphatase activity with 807 MU for CpsA-ext2-Phos and 109 MU for CpsA-dna_ppf-Phos, while fusions to predicted cytoplasmic regions gave lower activity at 10 MU for CpsA-cyto1-Phos and 5 MU for CpsA-cyto2-Phos. Once again the activity of CpsA-ext1-Phos did not match its putative extracellular location with only 10 MU of alkaline phosphatase activity. The lower than expected activity for CpsA-dna_ppf-Phos was shown to be due to protein degradation (Fig. 6C), a phenomenon also observed with another member of the LytR_CpsA_psR family when fusions were made closer to the C-terminus (58). The stability of all CpsA-PhoZ fusions was confirmed by Western blot (Fig. 6C) with antibodies to the PhoZ alkaline phosphatase. Western blots also confirmed the stability of all CpsA-β-galactosidase fusions (data not shown). Alkaline phosphatase assays were also performed on *S. iniae* harboring each of the CpsA alkaline phosphatase plasmids. Results from these assays corresponded exactly to those observed in *E. coli* with predicted extracellular regions giving the

highest activity with 490 units of activity for CpsA-ext2-Phos and 210 units of activity for CpsA-dna_ppf-Phos, while fusions to putative intracellular regions gave lower activity at 40 units for both CpsA-cyto1-Phos and CpsA-cyto2-Phos (Fig. 6D). As seen in *E. coli*, activity for the CpsA-ext1-Phos fusion did not correlate with its predicted extracellular position at only 90 units of activity, indicating that this discrepancy was species independent (Fig. 6D).

We hypothesized that the unexpected results observed with CpsA-ext1 may have been due to masking of the hydrophobic region of the first transmembrane domain and prevention of membrane association by the large reporter enzymes fused to the C-terminus. Therefore, an alternative method was to use a construct with a smaller chitin binding domain (CBD) tag fused in-frame at amino acids 22 and 43 (Fig. 7A). If the CBD is indeed located extracellularly for CpsA-ext1-CBD, as predicted, then it will be accessible for antibody binding during whole cell ELISA and exhibit higher activity than vector alone or the cytoplasmic CpsA-cyto1-CBD fusion. Phosphatase activity, using a colorimetric assay from the whole cell ELISA, was measured for each strain (Fig. 7B). As predicted, CpsA-ext1-CBD demonstrated the highest enzymatic activity at 1543 MU, indicating an extracellular CBD location. The CpsA-cyto1-CBD fusion expressed significantly lower activity at 168 MU (similar to vector alone), consistent with the cytoplasmic location of the CBD. The stability of each of these constructs was confirmed by Western blot, using antibodies generated against the CBD (data not shown).
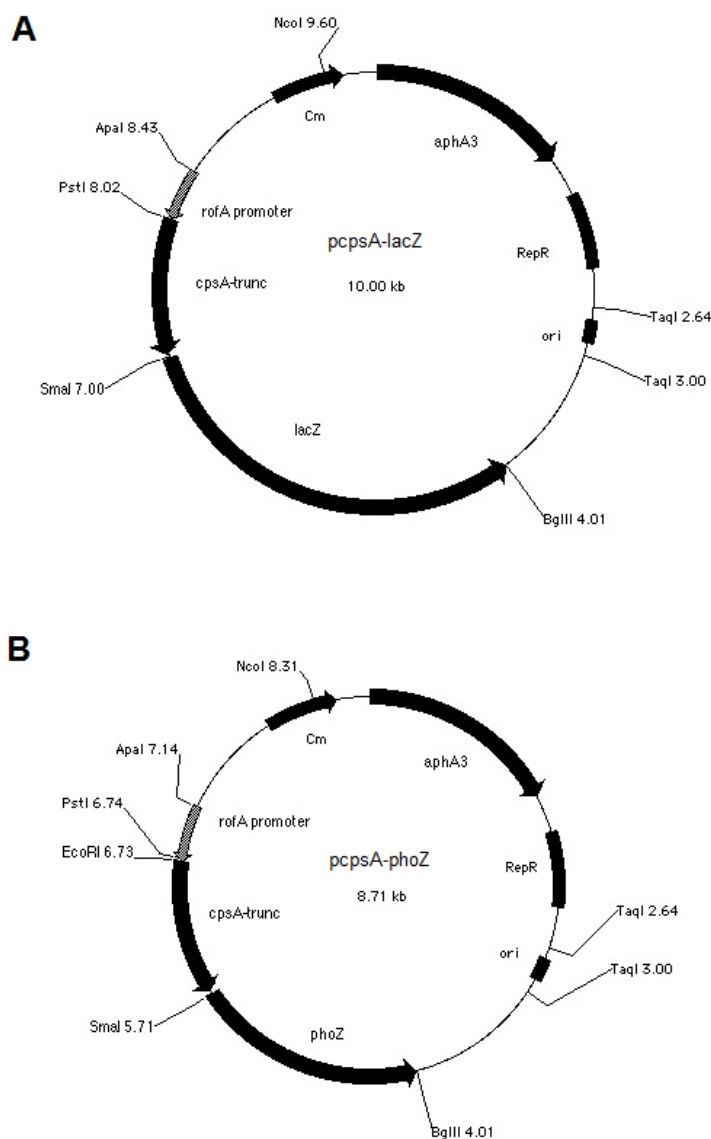
Figure 4: Plasmids constructed for CpsA membrane topology verification. (A) Fusion of *cpsA* to *lacZ* and (B) *cpsA* to *phoZ*. Plasmid pLZ12-*rofA*-pro was used to construct in-frame translational fusions of *cpsA* to the reporter enzymes. The chimeric gene products are under control of the mid-level constitutive *rofA* promoter. The size of the *cpsA*-trunc fragment varies in relation to the truncations used. The locations of genes encoding resistance to kanamycin (aphA3), chloramphenicol (Cm), origins of replication and restriction site locations are shown.
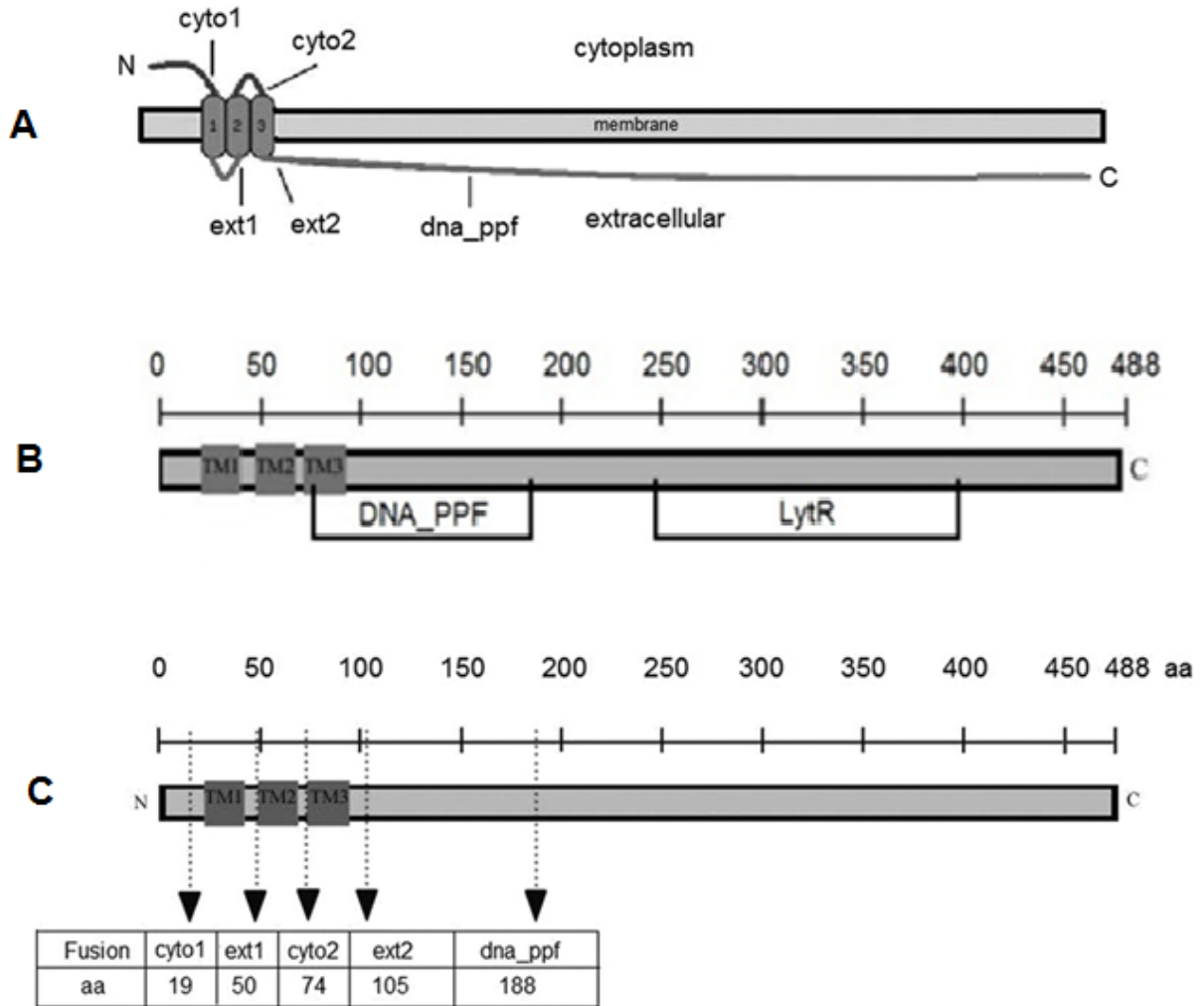
Figure 5: (A) CpsA membrane topology. (B) Conserved protein domains of CpsA. (C) Location of enzymatic fusions to CpsA to determine membrane topology. Hydropathy plots were used to predict the specific membrane topology for each fusion: TM (transmembrane), cyto1 or cyto2 (cytoplasm), ext1 or ext2 (extracellular), and dna_ppf (extracellular). aa, number of amino acids.

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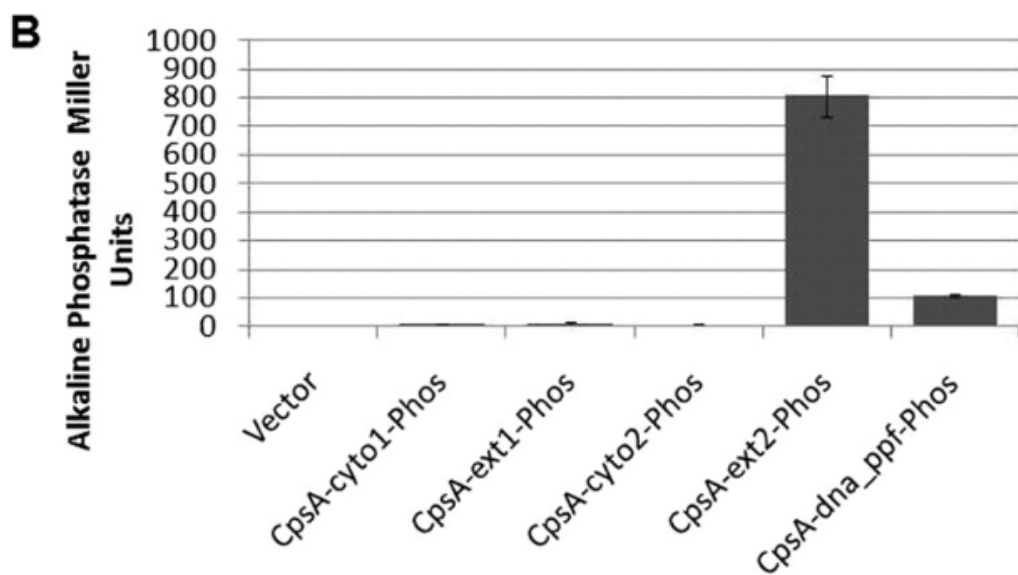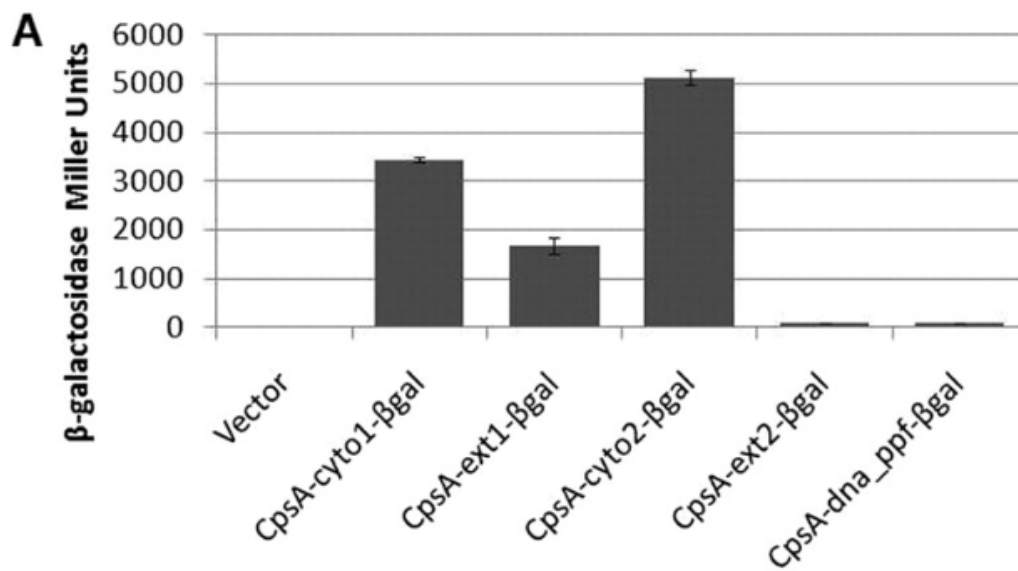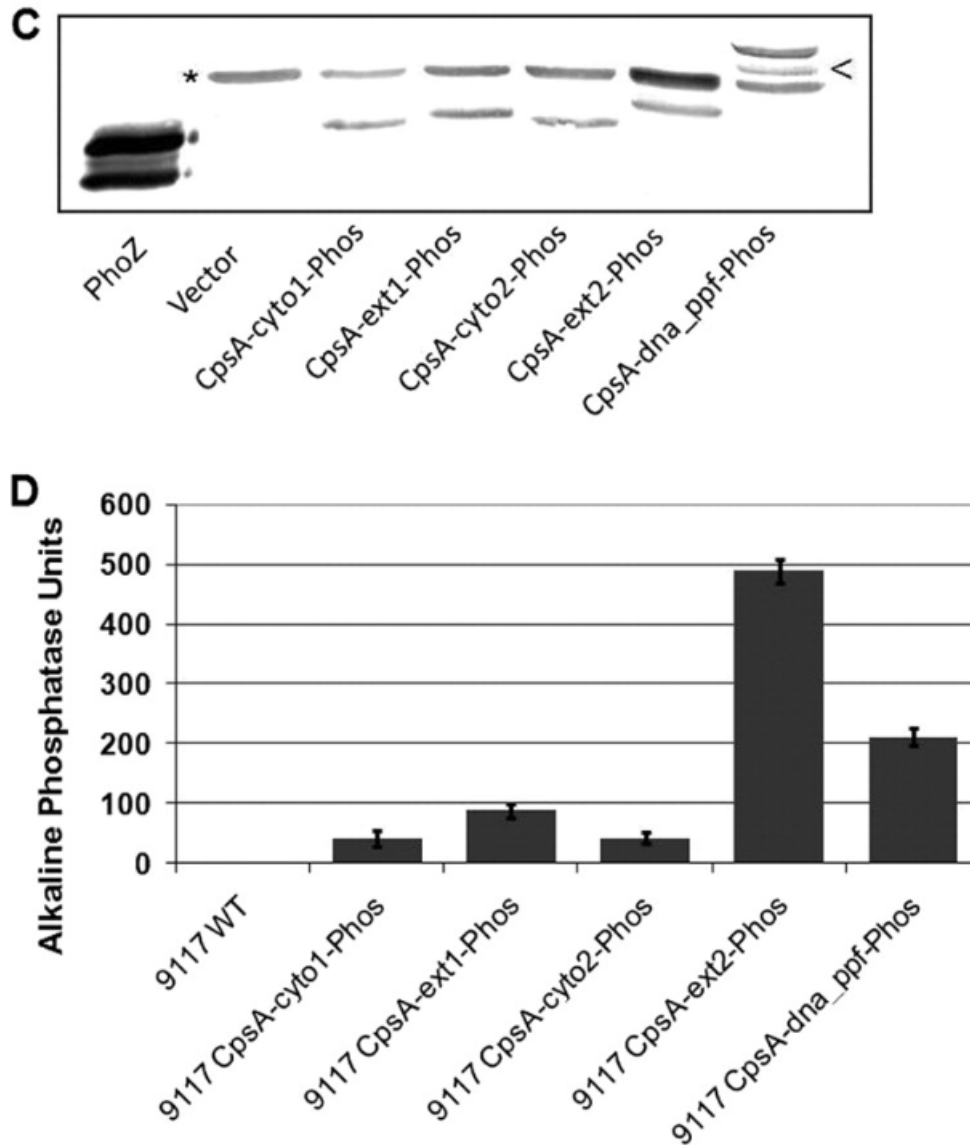
Figure 6: (A) Activity of CpsA-β-galactosidase *E. coli* fusion strains in colorimetric assays, expressed in Miller units. (B) Activity of CpsA-alkaline phosphatase *E. coli* fusion strains in colorimetric assays, expressed in Miller units. (C) Western blot for confirmation of stability of CpsA-alkaline phosphatase fusions. The asterisk indicates a nonspecific band. The arrowhead points to a degradation product. (D) Activity of CpsA-alkaline phosphatase *S. iniae* fusion strains in colorimetric assays. Error bars reflect the standard deviations.
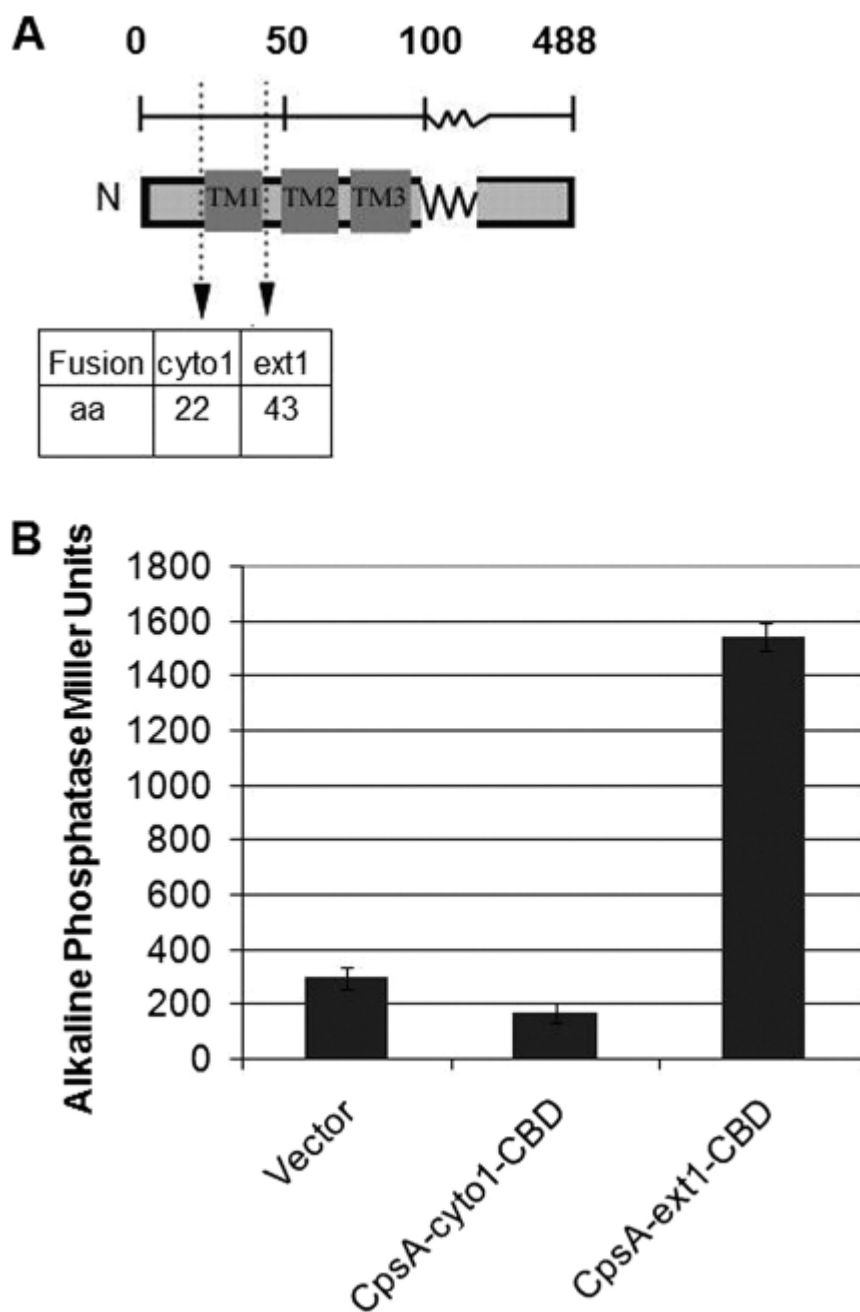
Figure 7: CpsA membrane topology verification using the chitin-binding domain (CBD) tag. (A) Location of CBD fusions to CpsA. (B) Alkaline phosphatase activity of each strain used in the whole-cell ELISA. Activity is expressed as Miller units. Error bars reflect standard deviations.

Analysis of CpsA promoter binding. All members of the LytR_cpsA_psr protein family are implicated in transcriptional regulation, but to our knowledge no direct DNA binding function for these proteins has been demonstrated. The CpsA protein has been shown to regulate capsule expression; *cpsA* is the first gene in the capsule operon and is adjacent to the putative capsule promoter (27, 79, 171). To determine if CpsA binds directly to the capsule operon promoter to influence transcription we performed electrophoretic mobility shift assays (EMSA) with purified CpsA and digoxigenin (DIG) labeled promoter DNA as a probe. The use of EMSA provides the ability to discern direct interactions between proteins and specific DNA sequences, as well as the ability to assess the specificity of any observed interaction. Sequence analysis of the cytoplasmic regions of CpsA revealed a high density of positively charged amino acids, with 8/22 on the cytoplasmic N-terminus and 4/5 on the cytoplasmic loop (Fig. 8). This led us to hypothesize that CpsA may be able to bind directly to the negatively charged phosphate backbone of DNA. To this end, we generated maltose-binding-protein (MBP) tagged versions of CpsA, with full length CpsA as well as various C-terminal truncations (Fig. 9). Following purification on an amylose column, EMSAs were performed with each of the MBP-CpsA fusion constructs. An 182 bp DNA fragment containing the *cpsA* promoter was labeled with DIG and used as a probe.

EMSA results demonstrated that full length MBP-CpsA was capable of binding specifically to the *cpsA* promoter probe (Fig. 10A). The smallest concentration of protein (25 pmol) was able to shift the probe completely (Fig. 10A, lanes 2-4). Addition of 20-fold excess unlabeled *cpsA* promoter DNA to the binding reaction led to competition for CpsA protein binding with the DIG-labeled probe, resulting in an increase in unbound labeled probe (Fig. 10A,

lane 5). Addition of excess non-specific competitor DNA led to minimal competition with the *cpsA* promoter DNA (Fig. 10A, lane 6).

To confirm that the large extracellular C-terminus of CpsA was not playing a role in the observed DNA binding, MBP-CpsA-116, which removes greater than 90% of the extracellular domain (Fig. 9), was constructed and analyzed by EMSA. Results with MBP-CpsA-116 revealed similar binding kinetics to those seen for the full length MBP-CpsA (Fig. 10B). Increasing the concentration of protein in the binding reactions led to an increased shift of free probe to the bound state (Fig. 10B, lanes 2-4). As seen with the full length form, excess unlabeled *cpsA* promoter led to a large shift of probe from the bound state to the unbound state (Fig. 10B, lane 5) while excess non-specific competitor led to only moderate competition with labeled probe (Fig. 10B, lane 6).

The contribution of the cytoplasmic loop between transmembrane domains 2 and 3 to DNA binding was analyzed by testing the MBP-CpsA-78 and MBP-CpsA-23 constructs with EMSA. The MBP-CpsA-78 truncation left the cytoplasmic loop intact, but removed the transmembrane domain that anchored it to the membrane, likely disrupting its native structure. The MBP-CpsA-23 truncation completely removed the cytoplasmic loop, as well as the first transmembrane domain, leaving just the cytoplasmic N-terminus intact. Results of the EMSA with MBP-CpsA-116 indicated efficient binding to the labeled probe as illustrated in Fig. 10B (Fig. 11, lane 1), as did MBP-CpsA-78 (Fig. 11, lane 2), demonstrating that the cytoplasmic loop transmembrane anchor was not necessary for CpsA to bind to the DNA probe. Excess unlabeled *cpsA* promoter DNA effectively competed with labeled probe for protein binding for both MBP-CpsA-116 (lane 4) and MBP-CpsA-78 (lane 5). The major difference between MBP-CpsA-116 and MBP-CpsA78 was observed when non-specific competitor DNA was used. Only moderate

competition was observed with MBP-CpsA-116 when excess non-specific DNA was added (lane 7) while significantly more competition was observed when excess non-specific DNA was incubated with MBP-CpsA-78 (lane 8), indicating loss of the native structure of the cytoplasmic loop through removal of the third transmembrane domain may result in a loss of specificity. Lastly, the full removal of the cytoplasmic loop in MBP-CpsA-23 completely abrogated the ability of CpsA to bind to DNA (Fig. 11, lanes 3, 6 and 9). This construct also served to show that the MBP tag alone was not responsible for the observed DNA binding in the other fusion constructs.

Since we could not rule out that the N-terminally-located MBP tag may be responsible for blocking DNA binding of MBP-CpsA-23 through steric hindrance, we used constructs in which a small chitin-binding domain (CBD) tag was attached to the C-terminus of the protein (CpsA-cyto1-CBD and CpsA-ext1-CBD) in EMSA analysis, leaving the N-terminus of CpsA untagged. Neither of these purified constructs was able to bind to the *cpsA* promoter probe (data not shown), indicating that the cytoplasmic N-terminus of CpsA, in the absence of the cytoplasmic loop, is not sufficient for DNA binding at this promoter.
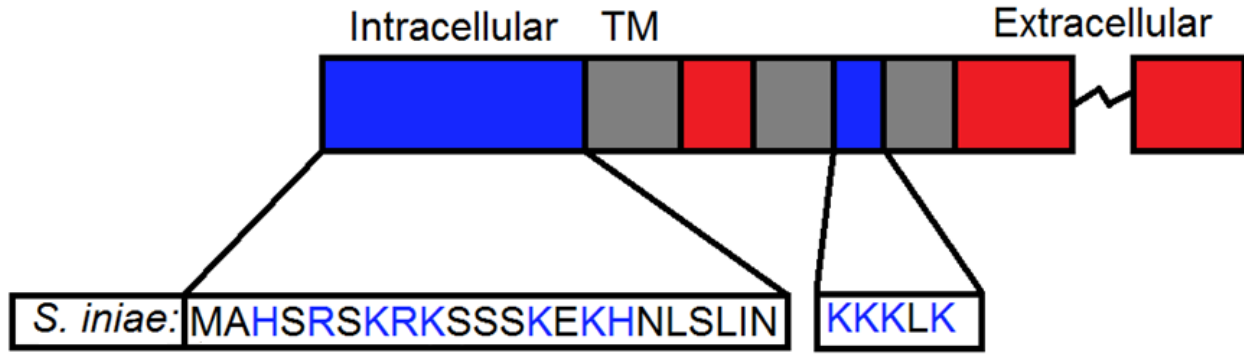
Figure 8: Cytoplasmic protein sequence of CpsA from *Streptococcus iniae* with positively charged amino acids in blue text.
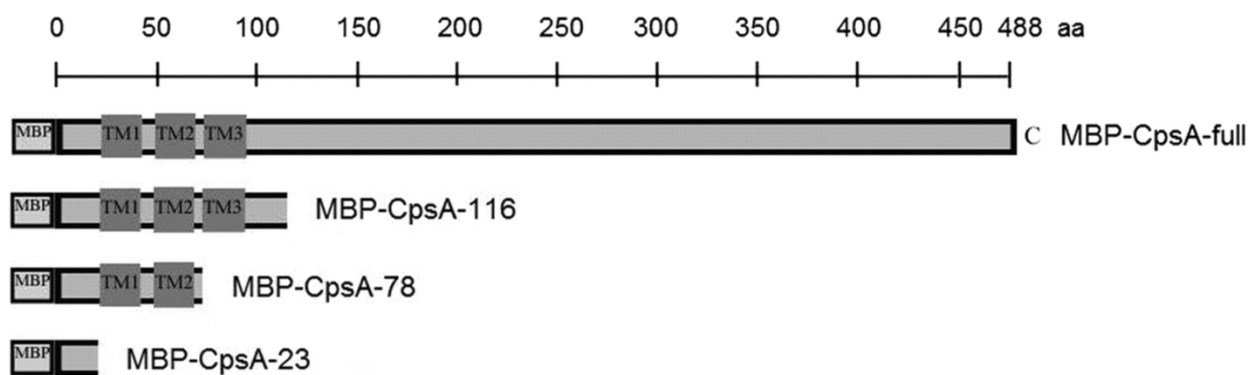
Figure 9: MBP-CpsA constructs used for DNA-binding assays. Full-length and sequential C-terminal truncations of CpsA were generated for analysis of DNA-binding properties. Truncated forms of CpsA contained the first 116, 78, or 23 amino acids of the protein, removing the large extracellular C terminus, the third transmembrane domain, and the cytoplasmic loop and the first transmembrane domain, respectively.
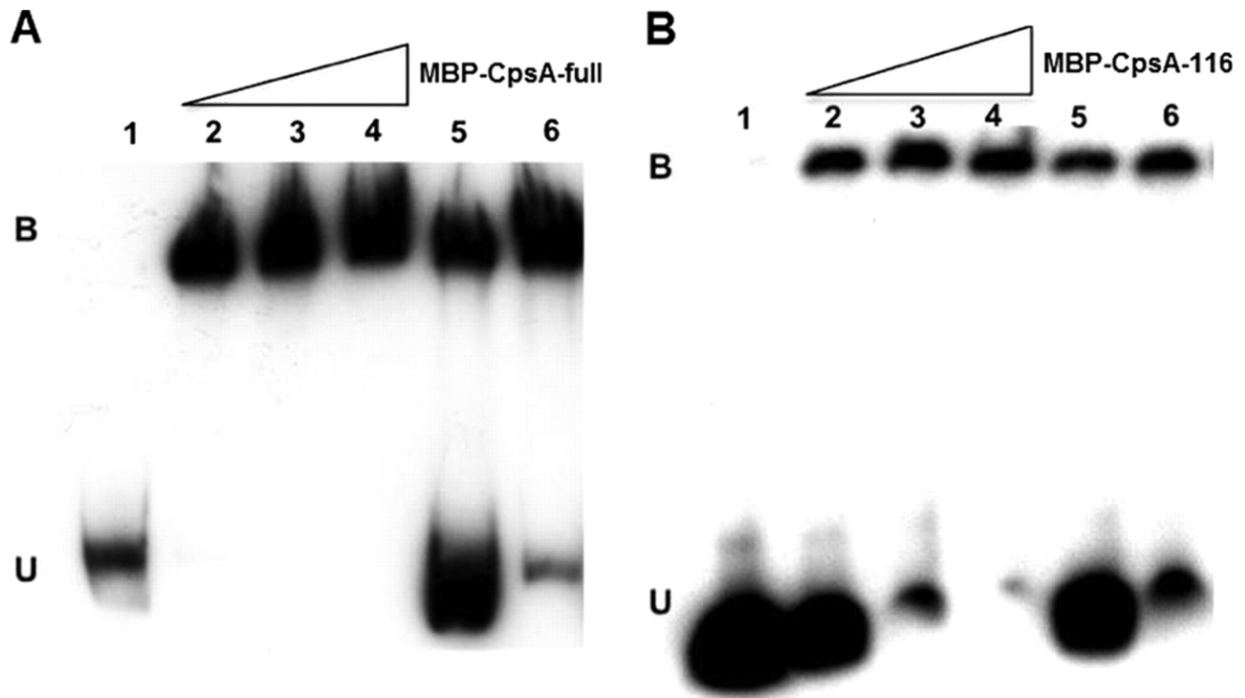
Figure 10: EMSA using digoxigenin-labeled *cpsA* promoter DNA as a probe. (A) EMSA with full-length MBP-CpsA. Lanes: 1, free probe; 2, 25 pmol MBP-CpsA; 3, 50 pmol MBP-CpsA; 4, 75 pmol MBP-CpsA; 5, 75 pmol MBP-CpsA and 20x unlabeled *cpsA* promoter DNA; 6, 75 pmol MBP-CpsA and 20x nonspecific DNA. (B) EMSA with MBP-CpsA-116. Lanes: 1, free probe; 2, 25 pmol MBP-CpsA-116; 3, 50 pmol MBP-CpsA-116; 4, 75 pmol MBP-CpsA-116; 5, 75 pmol MBP-CpsA-116 and 20x unlabeled *cpsA* promoter DNA; 6, 75 pmol MBP-CpsA-116 and 20x nonspecific DNA. B, bound probe; U, unbound probe.
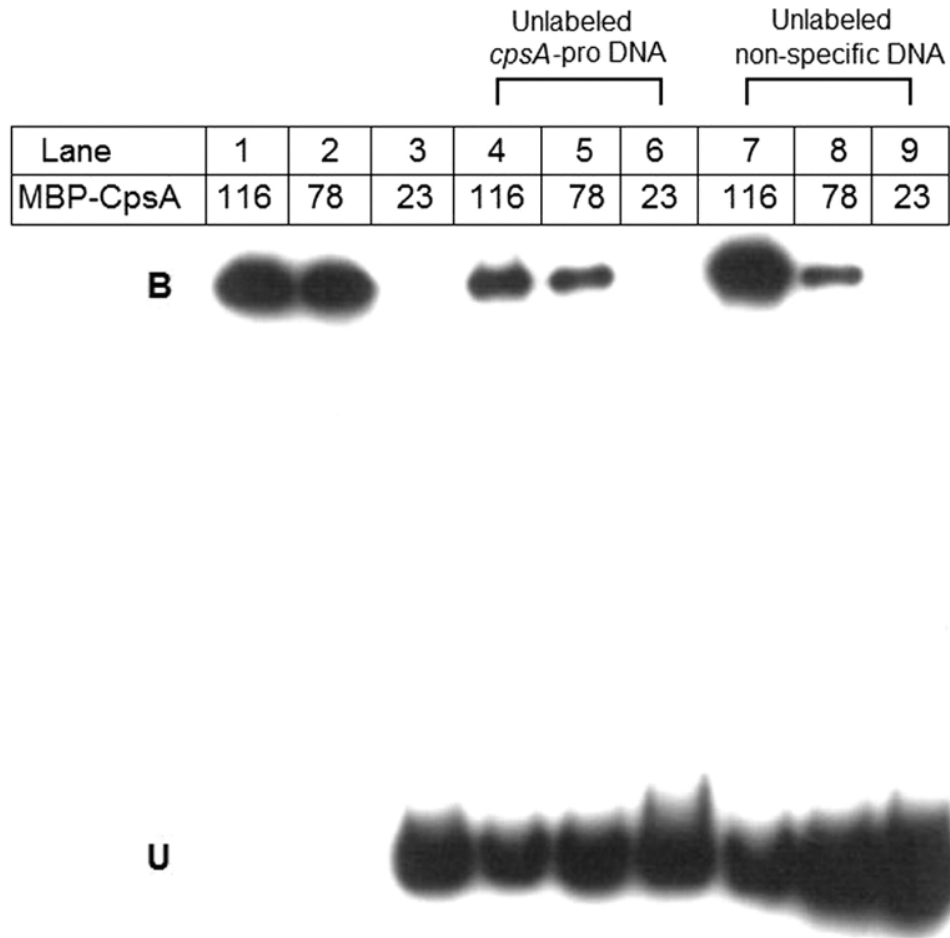
Figure 11: EMSA with MBP-CpsA truncations. All lanes contain 75 pmol of protein and DIG-labeled probe. Lanes: 1, MBP-CpsA-116; 2, MBP-CpsA-78; 3, MBP-CpsA-23; 4, MBP-CpsA-116 and 20x unlabeled *cpsA* promoter DNA; 5, MBP-CpsA-78 and 20x unlabeled *cpsA* promoter DNA; 6, MBP-CpsA-23 and 20x unlabeled *cpsA* promoter DNA; 7, MBP-CpsA-116 and 20x nonspecific DNA; 8, MBP-CpsA-78 and 20x nonspecific DNA; 9, MBP-CpsA-23 and 20x nonspecific DNA. B, bound probe; U, unbound probe.

The conserved domains of *S. iniae* CpsA are involved in regulation of capsule. Previous observations in our lab documented that a *cpsA* insertion mutant of *S. iniae* produced less capsule and was attenuated for virulence (79). The *S. iniae cpsA*-ins mutant effectively transcribes and translates a truncated form of the protein lacking the LytR domain that was initially thought to be inactive; however, the *cpsA*-ins mutant is unable to be complemented with ectopic expression of the full length native *cpsA* (data not shown). To assess the nature of these phenotypes and the role of the DNA_PPF and LytR domains of CpsA in capsule regulation, truncated forms of CpsA lacking the LytR domain, or containing just the DNA_PPF domain, were generated and ectopically expressed in the WT *S. iniae* background (Fig. 12).

Capsule production in the WT strain demonstrated growth phase dependency, with highest levels of capsule produced during exponential growth (3-4 hours) (Fig. 13). To assess capsule levels without variability due to growth phase, overnight cultures were used. Production of a form of CpsA lacking the LytR domain (CpsA-ΔLytR) in the WT *S. iniae* background resulted in a drastic reduction in capsule levels as measured by buoyant density centrfiguation, to levels comparable to those observed for the *S. iniae cpsA*-ins mutant (Fig. 14). The loss of capsule experienced with removal of the LytR domain could be due to either an induced dominant negative function or the adoption of a repressive function for CpsA in the absence of the LytR domain. Production of a truncated form of CpsA entailing just the DNA_PPF domain with a transmembrane anchor was also sufficient to produce the same phenotypic loss of capsule (Fig. 14), indicating that this phenotype was facilitated by the DNA_PPF domain and does not appear to require DNA-binding by the truncated form of the protein, as the DNA-binding domain of CpsA was absent for this construct (Fig. 12). Additionally these observations explain our inability to complement capsule production in the *cpsA*-ins mutant of *S. iniae* with native CpsA,

as the *cpsA*-ins mutant is producing a dominant negative or repressing form of the protein that somehow prevents the native CpsA from activating capsule synthesis.
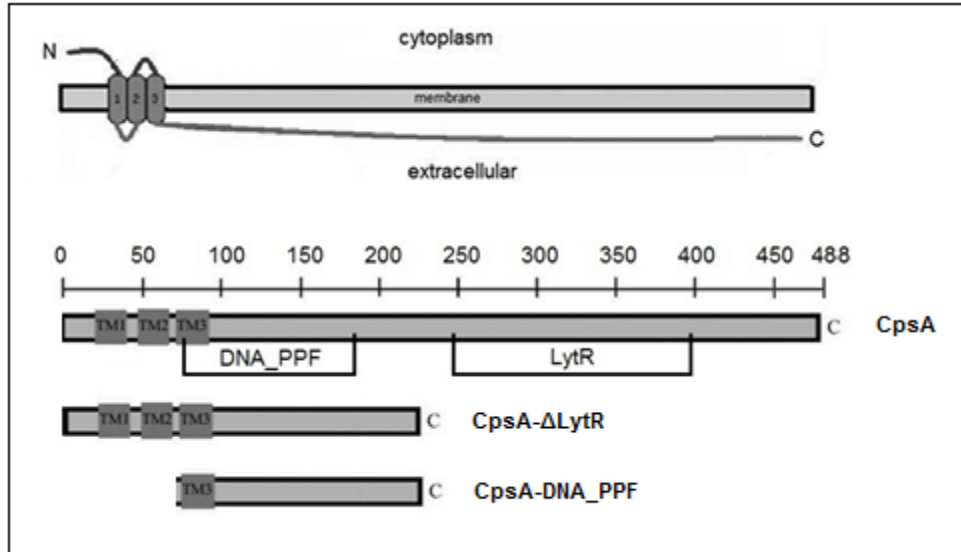
Figure 12: Membrane topology and conserved domains of *S. iniae* CpsA with truncated forms of CpsA lacking the LytR domain, and containing just the DNA_PPF domain.
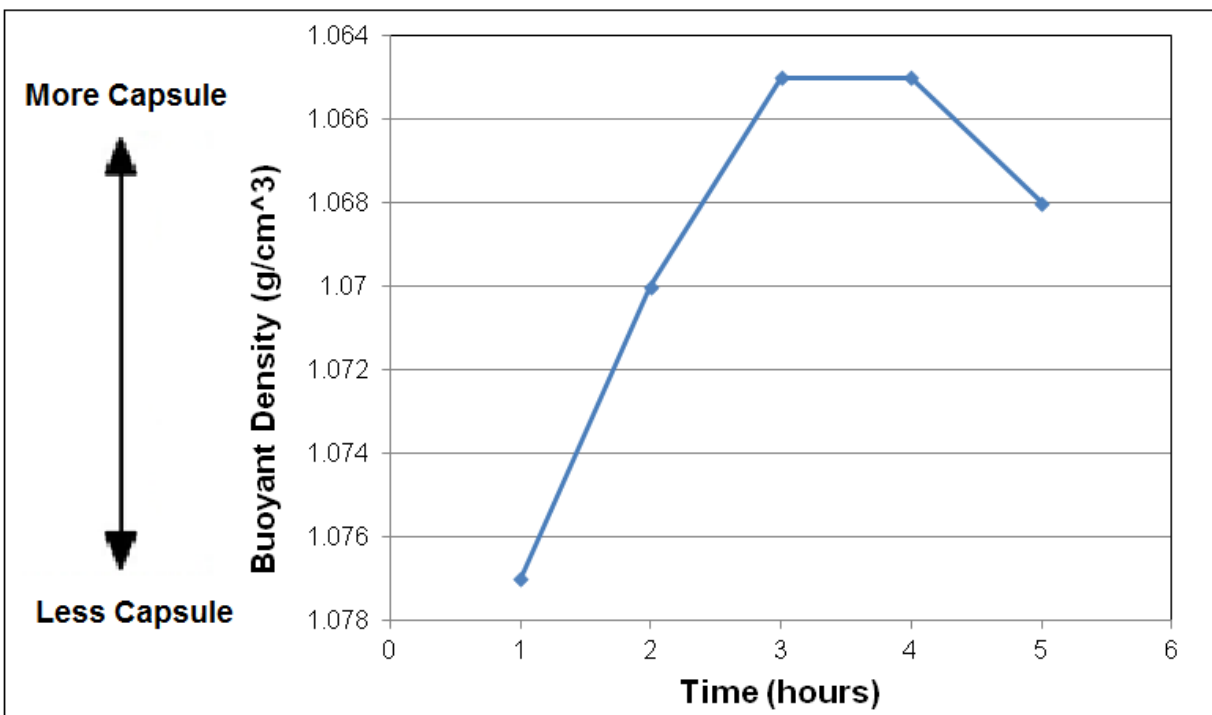
Figure 13: Production of capsule over time by WT 9117 *S. iniae* when growing in fresh medium.
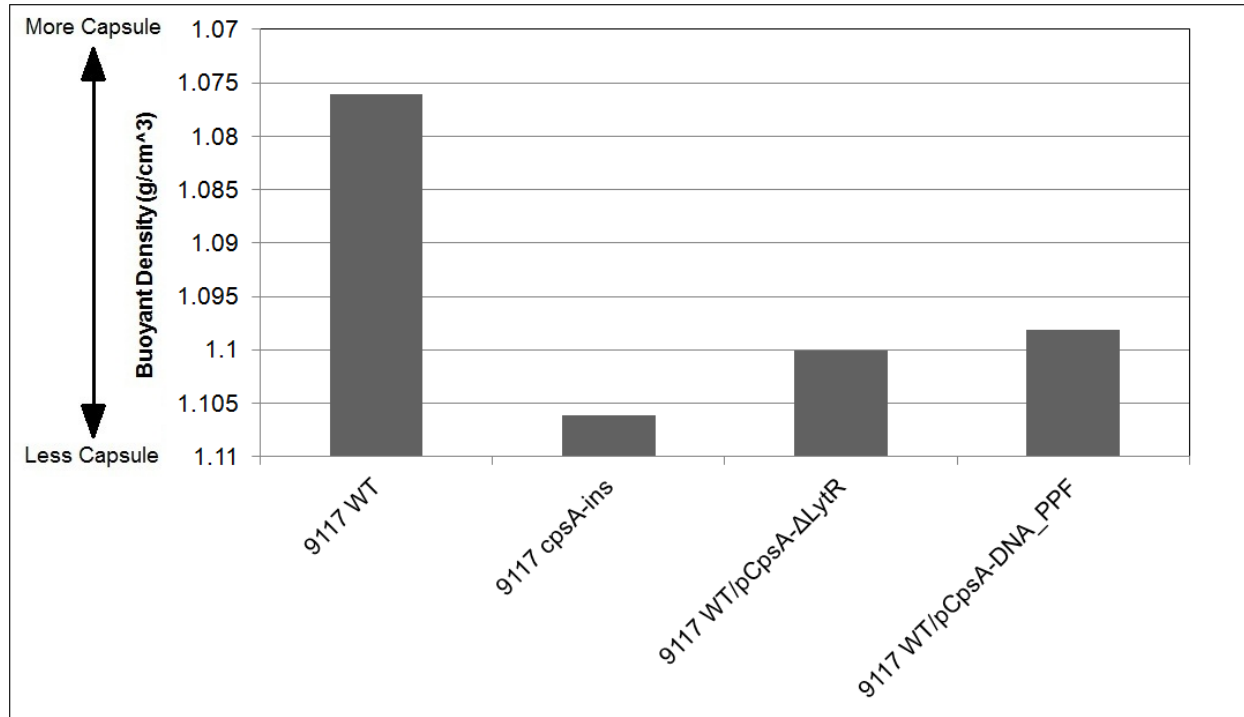
Figure 14: Capsule levels of *S. iniae* WT, *cpsA*-ins, and WT strain with either pCpsA-ΔLytR or pCpsA-DNA_PPF plasmids.

The conserved domains of *S. iniae* CpsA are involved in regulation of cell wall stability.
Other proteins containing the LytR domain have been shown to be associated with regulation of
cell wall maintenance, including autolysis and cell division (24, 58, 62, 74, 110). In addition to a
decrease in capsule, previous observations in our lab documented that the *cpsA*-ins mutant of *S.
iniae* formed significantly longer chains of cocci (79), a phenotype associated with altered cell
wall maintenance (8, 9, 24). To determine the role of CpsA and the LytR domain in regulation
of cell wall stability, a variety of cell wall active antimicrobial agents that target specific steps in
cell wall biosynthesis and turnover were used in conjunction with the *S. iniae* WT, *cpsA*-ins, and
WT/pCpsA-ΔLytR strains.

Lysozyme specifically cleaves the glycosidic bond between NAM and NAG of
the cell wall PG, and is used by host organisms to enhance clearance of pathogenic bacteria. In
response to this selective pressure, bacterial systems have arisen that modify the PG backbone
through *O*-acetylation of NAM and *N*-deacetylation of NAG to provide resistance to lysozyme
cleavage of PG (31). While growth of the WT *S. iniae* strain was ablated even at low levels of
lysozyme, both the *cpsA*-ins and WT/pCpsA-ΔLytR strains grew to similar levels across a broad
range of lysozyme concentrations (Fig. 15), possibly indicating that PG modifications are altered
in the absence of the CpsA LytR domain.

Bacitracin inhibits recycling of Und-P, which is the lipid acceptor required for PG and
CPS synthesis and transport to the cell exterior. The inability to form a cell wall is bactericidal
as bacteria easily lyse from osmotic pressure in the absence of the cell wall. Interestingly, both
the *cpsA*-ins and WT/pCpsA-ΔLytR strains were about two to three-fold more resistant to growth
in bacitracin than the WT strain of *S. iniae* (Fig. 16), suggesting differential usage of the Und-P
acceptor.

B-lactam antibiotics like methicillin inhibit crosslinking of the peptide side chains of PG, causing a bacteriostatic effect, but this indirectly results in an accumulation of PG precursors within the bacterial cell that positively activates the production of autolysins which ultimately cause bactericidal cell lysis. In contrast to what was observed for lysozyme and bacitracin, the *cpsA*-ins and pCpsA-ΔLytR strains of *S. iniae* demonstrated an approximately two-fold decrease in resistance to β-lactams, including methicillin (Fig. 17) and ampicillin (data not shown), when compared to the WT strain. Taken together, these results suggest that the cell wall architecture of *S. iniae* is altered when the LytR domain of CpsA is removed. This observation is consistent with a regulatory function for CpsA that includes not only capsule, but the cell wall as well.
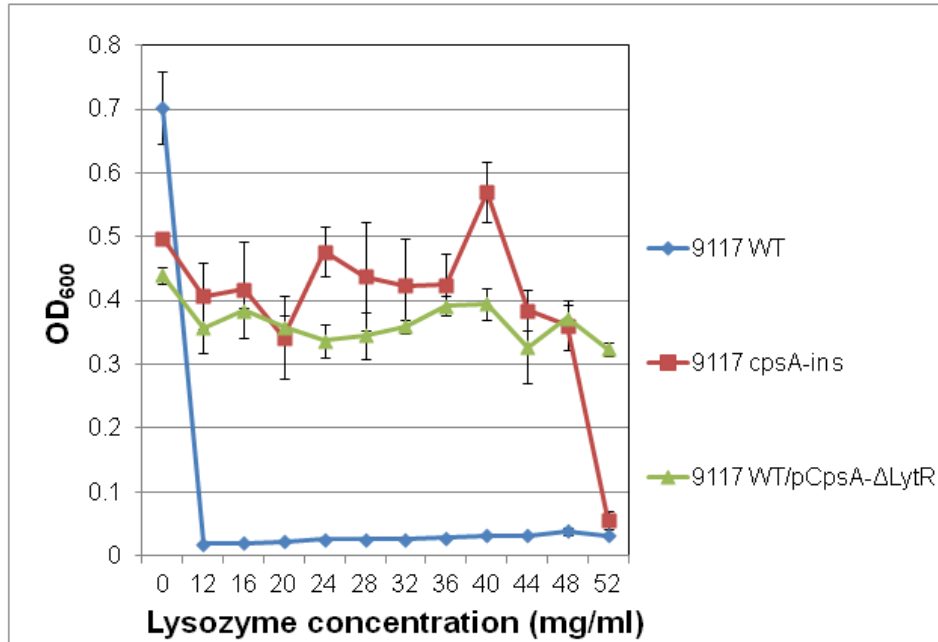
Figure 15: Growth profiles for strains of *S. iniae* grown in the presence of various lysozyme concentrations.
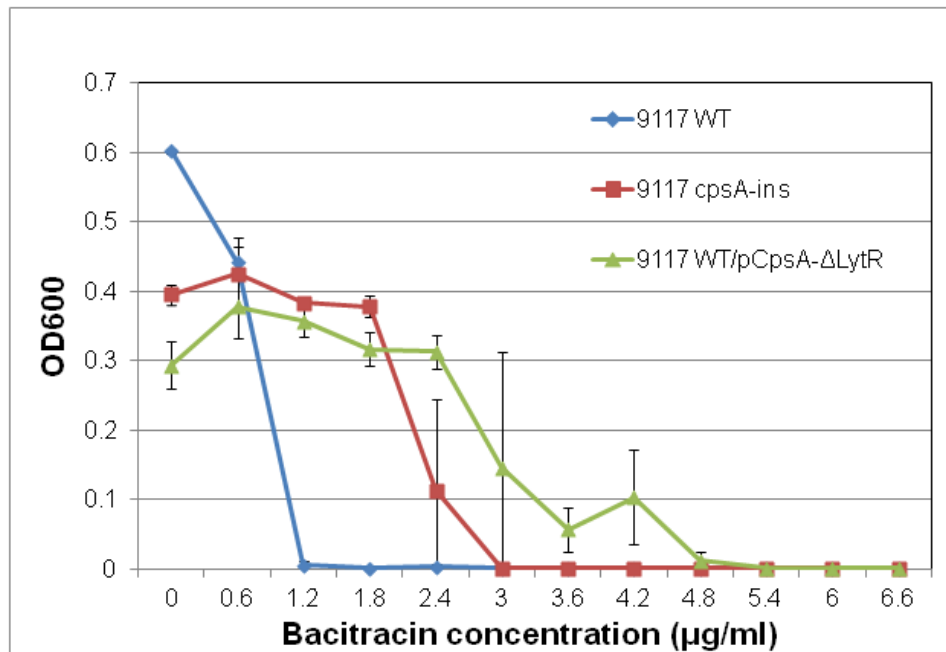
Figure 16: Growth profiles for strains of *S. iniae* grown in the presence of various bacitracin concentrations.
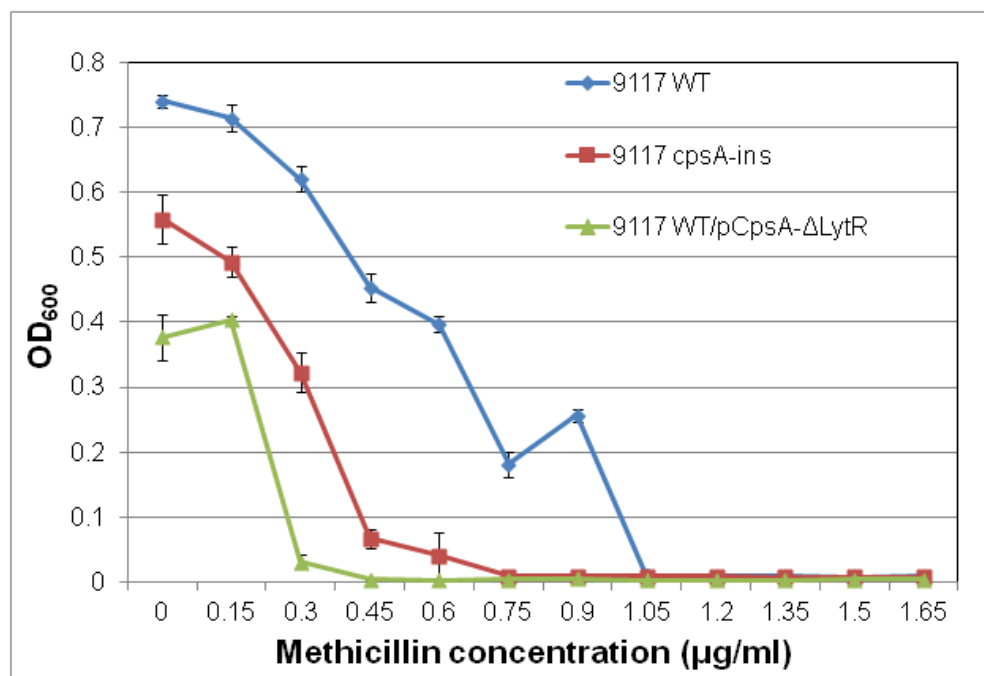
Figure 17: Growth profiles of *S. iniae* strains grown in the presence of various concentrations of methicillin.

The LytR domain of CpsA contributes to differences in autolysis in *S. iniae*. In addition to the differences in antimicrobial resistance observed for the different strains of *S. iniae*, we also observed that in the absence of antibiotics both the *cpsA*-ins and WT/pCpsA-ΔLytR strains exhibited a lower OD_{600} value after overnight growth (Figs. 15-17). We determined that this was not due to a growth defect by performing time-dependent growth curves, and observed that the OD_{600} value decreased during late stationary phase to a level much lower than that observed for the WT strain of *S. iniae* (data not shown). A decrease in OD_{600} values is generally representative of cell lysis, leading to decreased absorption at the 600 nm wavelength.

To measure the increased autolysis associated with the *cpsA*-ins and pCpsA-ΔLytR strains, bacteria were grown overnight and resuspended in PBS or the nonionic detergents triton X-100 and tween-20, which have been shown to induce autolysis (29). The percent lysis was determined by comparing the final OD_{600} value to the initial OD_{600} value. When strains were incubated in PBS alone, the *cpsA*-ins strain exhibited an approximately two-fold increase in autolysis compared to the WT strain (Fig. 18). The WT/pCpsA-ΔLytR strain demonstrated a more subtle increase in autolysis with an approximately 1.5 fold increase over that observed for the WT strain (Fig. 18). Surprisingly, none of the strains showed a significant increase in autolysis when incubated with the nonionic detergents triton X-100 (Fig. 19) or tween-20 (Fig. 20), but the same relative increase in autolysis was apparent for both the *cpsA*-ins and pCpsA-ΔLytR strains when compared to the WT strain. Taken together, the data suggests that the LytR domain of CpsA contributes either directly or indirectly to regulation of autolysis, which is consistent with the observation that CpsA contributes to regulation of cell wall maintenance or stability as assessed by antimicrobial sensitivity above.
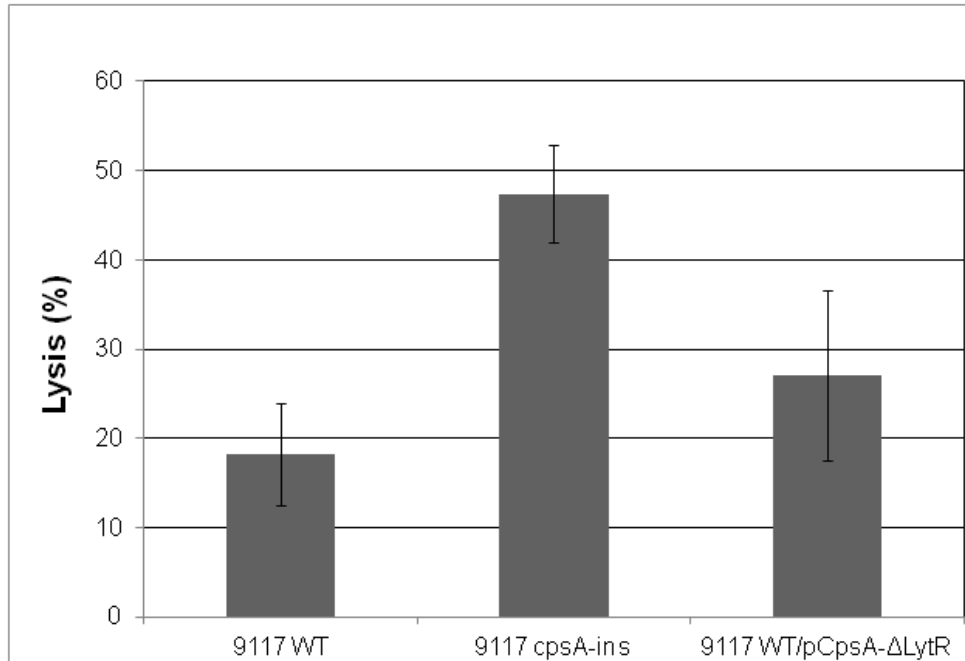
Figure 18: The amount of lysis observed for different strains of *S. iniae* incubated in PBS.
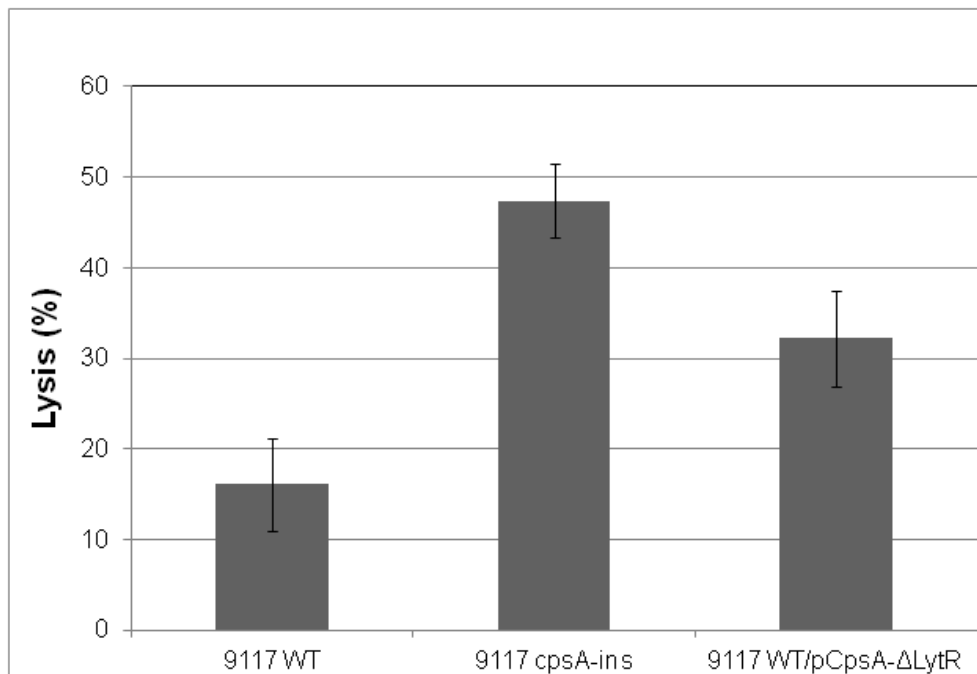
Figure 19: The amount of lysis observed for different strains of *S. iniae* incubated in 0.6% triton X-100.
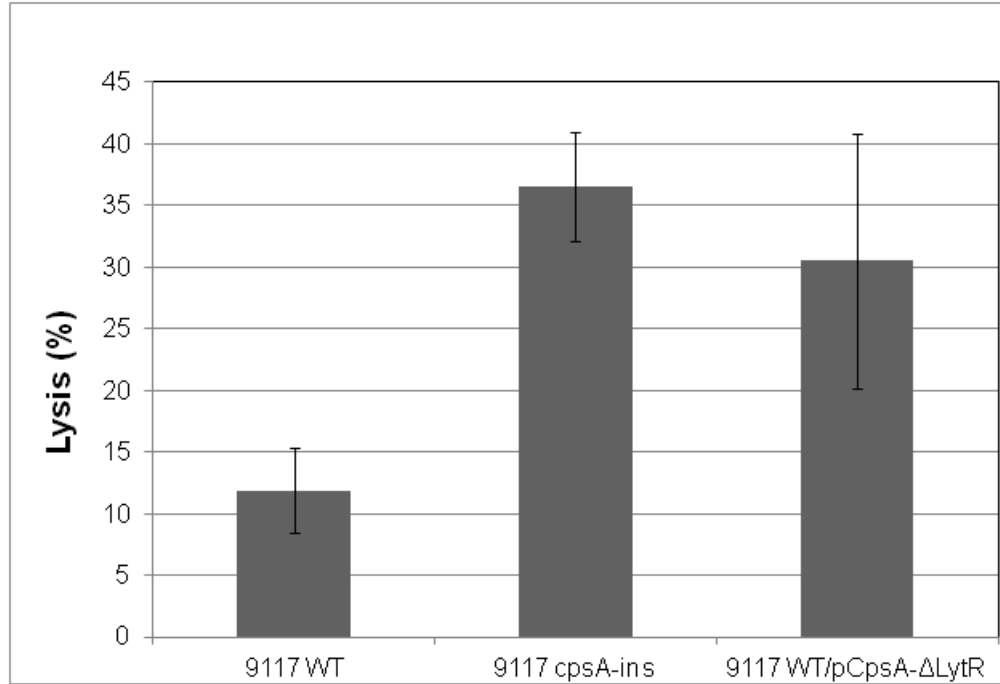
Figure 20: The amount of lysis observed for different strains of *S. iniae* incubated in 0.6% tween-20.

DISCUSSION

Many pathogens utilize a polysaccharide capsule to evade immune clearance, allowing for dissemination and development of systemic disease. Among these are a number of streptococcal pathogens that appear to use the CpsA protein to exert transcriptional control over capsular synthesis. Cieslewicz et al. demonstrated that a deletion of the *cpsA* gene resulted in a loss of capsule transcription in *S. agalactiae*, as well as decreased capsule production (27). Furthermore, mutation of *cpsA* in *S. iniae* resulted in an unencapsulated phenotype and led to a reduction in virulence and effective dissemination from the site of infection (79), underscoring the importance of capsule regulation in pathogenesis.

Though several members of the LytR_cpsA_psr protein family have been implicated in transcriptional regulation, a mechanism for how these proteins modulate transcription has not been determined. However, there appear to be several common themes of functionality within this protein family. The LytR protein of *Bacillus subtilis* demonstrates transcriptional attenuator activity of its own promoter and the promoter of the divergent operon of *lytABC*, which encodes proteins involved in autolysin production (74). The PSR protein, originally analyzed in *Enterococcus hirae*, was found to be a repressor for penicillin-binding protein 5 (PBP5) as well as a regulator of other cell surface-related processes (75, 90). However, a subsequent report on PSR in *E. faecium* demonstrated that neither repressor or activator activities were associated with control of PBP5, but suggested that PSR may activate its own transcription during growth in the presence of ampicillin (124). Expression analysis of the LytR_cpsA_psr family member *msrR* in *S. aureus* revealed transcriptional activation in the presence of multiple cell wall-targeted antimicrobials including vancomycin and lysostaphin (125). An *msrR* mutant strain had a 4-fold

decrease in minimal inhibitory concentration (MIC) to oxacillin compared to the wild type strain, suggesting that this protein may be involved in sensing and responding to antimicrobials that target the cell wall. Furthermore, this report demonstrated that MsrR had attenuator activity against the *sarA* gene, which encodes a global regulator of virulence genes in *S. aureus* (125). However, a recent microarray analysis with a deletion mutant of *msrR* demonstrated only minor changes in the transcriptome, suggesting that the regulatory effects of MsrR may be indirect (59). Conversely, when the gene encoding the *S. mutans* LytR (BrpA) protein, which is involved in biofilm formation, autolysis, cell division and systemic virulence (24, 102, 164, 172) was deleted and subjected to a transcriptome analysis, changes in expression of a number of genes was observed (163). Taken together, members of this family of proteins appear to be involved with cell wall biogenesis, polysaccharide production, stress tolerance and transcriptional modulation of proteins involved in these processes.

Although the predicted membrane topology for the CpsA protein suggests a unique transcriptional regulatory mechanism, it was first necessary to confirm the topological prediction provided by hydropathy plots. Recently the topology of another member of the LytR_cpsA_psr family, MsrR, was reported, although this protein has only a single transmembrane domain, with a small cytoplasmic N-terminus and a large extracellular C-terminus (58). Translational fusions of various regions of the CpsA protein to either β-galactosidase or alkaline phosphatase enabled us to verify which regions of the protein are cytoplasmic or extracellular. We found that the predicted cytoplasmic regions (cyto1 and cyto2) gave the highest β-galactosidase activity of all fusions tested, consistent with a cytoplasmic location. Enzymatic fusions to predicted extracellular locations (ext2 and dna_ppf) gave the highest alkaline phosphatase activity, confirming an extracellular location. The decreased alkaline phosphatase activity given by

CpsA-dna_ppf-Phos was shown to be due to increased protein degradation. Moreover, the extracellular location of this domain is consistent with the high alkaline phosphatase activity demonstrated by the adjacent CpsA-ext2 domain and the absence of a predicted transmembrane domain between the two locations. Though we were unable to confirm the CpsA β-galactosidase fusions in *S. iniae*, our results with the CpsA alkaline phosphatase fusions in *S. iniae* demonstrated identical trends to those seen in *E. coli*. We are therefore confident that the results obtained in *E. coli* accurately represent the membrane topology that CpsA manifests in *S. iniae*. The only CpsA fusion location that did not correspond to the predicted membrane topology was at amino acid 50 (ext1), a predicted extracellular loop of only 7 amino acids. We speculate that these results may have been due to misfolding or interference with membrane association caused by the large reporter enzymes. Subsequent construction with the smaller chitin-binding domain (CBD) tag allowed us to circumvent this problem, validating its extracellular location. Therefore, our results confirm the predicted membrane topology of the streptococcal CpsA protein with a short (~22 amino acids) cytoplasmic N-terminus, 3 transmembrane domains and a large extracellular domain (>400 amino acids).

As mentioned above, CpsA plays a role in transcriptional regulation of the capsule operon. To identify a regulatory mechanism, we utilized purified proteins with targeted truncations to determine whether direct binding to the promoter occurs, the protein domains responsible for binding to DNA and the specificity of the interaction between CpsA and the capsule operon promoter. Analysis by EMSA with purified MBP-CpsA-full showed a direct interaction between the CpsA protein and the *cpsA* promoter DNA, indicating that CpsA may regulate transcription through direct DNA binding. Furthermore, our analyses demonstrate that the interactions are specific for the capsule promoter DNA. To our knowledge this is the first

report demonstrating binding of any LytR_cpsA_psr family member to its target DNA. EMSA analysis with a CpsA protein that has the entire C-terminal extracellular domain removed (MBP-CpsA-116) confirmed that this domain does not contribute to the observed DNA binding, also consistent with the finding that this region is extracellular. However, these results do not rule out a possible contribution of this domain in transcriptional activation through possible conformational changes. Therefore, although the CpsA protein has less than 30 amino acids in the cytoplasm, its cytoplasmic regions are sufficient for binding to DNA. The ability of the membrane-bound CpsA protein to bind to a specific DNA target is not without precedent. The well-studied virulence protein ToxR of *Vibrio cholerae* is a transmembrane protein proven to bind to specific promoters to regulate transcription (55, 96). Therefore, the highly conserved CpsA protein present in the majority of streptococcal capsule operons appears to be modulating transcription of polysaccharide capsule through direct binding to the capsule promoter. Further experiments are necessary to determine if this interaction is sufficient for transcriptional activation and what other factors may contribute to DNA target specificity.

Notably, although the cytoplasmic regions of the CpsA protein bind to the *cpsA* promoter in a specific manner, there is no evidence of a typical DNA binding domain. A possible leucine zipper domain which may facilitate protein dimerization exists in the N-terminal portion of the protein, spanning the cytoplasmic N-terminus and first transmembrane domain, however, a single leucine in the heptad repeat of this motif is replaced with a tryptophan. It is possible that this still represents a functional leucine zipper domain as it has been shown that substitution of a single leucine in the repeat with a tryptophan does not necessarily abrogate the function of the motif (57), however, verification is required. Results of the topological analysis combined with the protein sequence suggest that the DNA binding may be influenced by an interaction with the

positively charged residues present in the small cytoplasmic N-terminus (8 of 22 residues) and the cytoplasmic loop between transmembrane domains 2 and 3 (4 of 5 residues), although this would not necessarily provide specificity. Therefore some additional component may be required to enhance CpsA binding specificity and transcriptional activation in vivo. Many transcriptional activators require a co-inducer for enhanced binding to their target DNAs such as what is observed for the LysR family of transcriptional regulators (81). The co-inducer causes a conformational change in the tertiary structure of the protein that allows specific binding to its DNA target. Alternatively, binding may require interactions with another protein to induce a conformational change that will enhance binding to the DNA target. The ToxR transmembrane transcriptional regulator of *V. cholerae* mentioned above interacts with another membrane protein, ToxS, through specific residues in the periplasm (35). Although ToxR can bind to its target DNA in the absence of ToxS, the specific interactions between the two proteins in their periplasmic domains enable transcriptional activation (116). As described above, several members of the LytR_cpsA_psr family can modulate transcription of multiple genes, however, the short cytoplasmic domains of many of these proteins suggest that the mechanism of transcriptional activation may also require interaction with other proteins to alter their transcriptional profile. In the case of the CpsA proteins, such an interaction may occur through the extensive extracellular domain and may also influence DNA binding specificity. Absence of both the DNA_PPF domain and the LytR domain was shown to not significantly affect the ability of CpsA to bind DNA, indicating that these domains are likely not involved in facilitating the DNA binding event, but serve some other function that may be linked to transcriptional activation. One possibility could be through protein-protein interactions with other membrane-bound proteins encoded by the capsule operon.

The presence of the DNA polymerase processivity factor domain (DNA_PPF, pfam PF02916) in the CpsA protein alone out of all members of the LytR_cpsA_psr family is particularly intriguing since we have determined that it is located extracellularly. DNA_PPF proteins function to bind to and tether their cognate polymerases to the duplex DNA template so that the polymerase stays firmly in contact with the DNA during high-speed replication (73). Clearly, this domain in CpsA is not binding to a polymerase, but instead may have been co-opted for another specific protein-protein interaction. Interestingly, although absence of the DNA_PPF and LytR domains (MBP-CpsA-116) did not significantly alter binding to the *cpsA* promoter *in vitro*, ectopic expression of a construct lacking the LytR domain in the wild type background results in a dominant negative (non-encapsulated) phenotype, suggesting that the DNA_PPF domain, expressed in the absence of the LytR domain, can interfere with regulation by the wild type CpsA protein. This assertion is further supported by the observation that expression of just the DNA_PPF domain of CpsA in the wild type background is sufficient to incur the dominant negative phenotype. Although the LytR domain is not required for DNA binding, it may be involved in facilitating protein-protein interactions through the DNA_PPF domain, or it may act to modulate DNA binding ability through a ligand or environmental sensing function as demonstrated for the extracellular portion of *V. cholerae's* ToxR protein (142).

In addition to alteration of capsule levels, insertional inactivation of *cpsA* and ectopic expression of *cpsA* lacking the LytR domain in a WT background result in changes to antimicrobial sensitivity for lysozyme, bacitracin, and methicillin. The dominant negative phenotype induced in the *cpsA*-ins and WT/pCpsA-ΔLytR strains results in an increase in resistance to lysozyme. The degree to which resistance is increased, approximately 10 fold, is highly suggestive of modifications to the PG backbone that confer resistance to lysozyme,

including *O*-acetylation of NAM and *N*-deacetylation of NAG (31). Additionally, both strains are also about 2 fold more resistant to bacitracin when compared to the WT strain. Both PG and CPS require the Und-P lipid acceptor that bacitracin inhibits (173), and it may be that the reduction in capsule experienced by these strains results in an increase in free Und-P that allows for viability at higher concentrations of bacitracin. Both the *cpsA*-ins and WT/pCpsA-ΔLytR strains also exhibited a 2 fold reduction in resistance to the β-lactam methicillin. The cause for this decrease in resistance is unclear, but the increase in autolytic activity observed for these strains suggests that they would be more sensitive to the autolytic bactericidal activity of β-lactams. LytR proteins are generally associated with transcriptional attenuation of autolysin genes (24, 58, 62, 74, 110), and have also shown functional redundancy in *Staphylococcus aureus* (110), therefore it could be that CpsA also contributes in some way to regulation of autolysins in manner similar to that observed for other LytR proteins. A decrease in capsule alone is unlikely to be responsible for the increase in autolysis, as it has been shown for *S. pneumoniae* that less capsule is associated with an increase in wall teichoic acids (70), which act to inhibit autolysis (129). However, an isogenic acapsular mutant of *S. iniae* is required as a control to determine the extent to which a decrease in capsule alone causes these phenotypes.

There is currently little information on the environmental signals that are important for influencing capsule levels. Exposure to aerobic conditions has been shown to cause a reduction in capsule levels in *S. pneumoniae*, which was initially attributed to increased phosphorylation of the tyrosine kinase CpsD, a predicted negative regulator of capsule production (162). However, it has since been shown that phosphorylation of CpsD instead correlates with increased capsule levels (11), leaving the mechanism of response to oxygen levels unexplained. In *S. agalactiae*, growth in acidic media leads to an increase in buoyant density, indicative of reduced capsule

levels (136). However, the authors point out that the terminal sialic acids of the polysaccharide capsule are acid labile, and go on to show that sialic acid levels are indeed decreased in acidic media (136), indicating that this observed phenotype is likely a more general molecular response than an active regulatory phenomenon. Additionally, no change in *S. agalactiae* capsule levels in response to differing levels of oxygen tension or temperature (136) was observed in contrast to the oxygen responsiveness of *S. pneumoniae*. Other factors that have been shown to modulate polysaccharide capsule expression in *S. agalactiae* type III strains are growth rate (112) and growth in human serum (120). In the context of commensal colonization, it is likely that each streptococcal species downregulates capsule in response to different signals, representative of the different colonization niches they inhabit, such as that seen for oxygen levels (nasopharynx) for *S. pneumoniae* and pH levels (vaginal canal) for *S. agalactiae*. Most likely, similarities in virulence related transcriptional control of capsule would become more apparent in the context of systemic infection, a commonality amongst the different streptococcal pathogens possessing the conserved capsule operon. One could speculate that the large extracellular domains of CpsA are responsible for environmental signal sensing, which is then conveyed through conformational changes or differential protein associations into a direct transcriptional response and experiments addressing those possibilities are currently in progress.

In this chapter, we have confirmed the predicted membrane topology of the streptococcal CpsA protein and demonstrated its DNA binding ability; providing a glimpse into the mechanism by which CpsA modulates capsule gene transcription in streptococci. Additionally, we have identified that the DNA_PPF domain of *S. iniae* CpsA induces a dominant negative loss of capsule in the absence of the LytR domain, suggesting the possible presence of a repressing domain and a control domain for CpsA. The phenotypes that result from expressing a dominant

negative form of CpsA include altered resistance profiles to antimicrobials and increased autolysis, suggesting that CpsA directly or indirectly contributes to regulation of cell wall turnover or stability. The CpsA proteins of *S. iniae* and *S. agalactiae* are highly homologous with 55% identity and 74% similarity at the amino acid level, so the observations made with *S. iniae* CpsA are likely applicable to GBS CpsA as well. Additionally, observations made for the CpsA protein also shed light on other members of the LytR_cpsA_psr family, present in a number of medically and economically important bacteria. The importance of capsule in virulence makes CpsA a promising therapeutic target, but further characterization of its two extracellular protein domains to determine functional properties in capsule regulation is required.

CHAPTER 2

REGULATION OF CAPSULE AND CELL WALL BY THE CPSA PROTEIN OF *STREPTOCOCCUS AGALACTIAE*

ABSTRACT

 Pathogenic streptococci such as *Streptococcus agalactiae* (GBS) are an important cause of systemic disease, which is facilitated in part by the presence of a polysaccharide capsule. The protein CpsA is a putative transcriptional regulator of the capsule locus in these pathogens, but its exact contribution to regulation is unknown. To address the role of CpsA in regulation, full-length GBS CpsA and two truncated forms of the protein were purified and analyzed for DNA binding ability.   These studies demonstrated that CpsA is able to bind specifically to two putative promoters within the capsule operon with similar affinity, and that the full length protein is required for specificity. Functional characterization of CpsA confirmed that the ∆*cpsA* strain produced less capsule than WT, and demonstrated that production of full length CpsA or the DNA-binding region of CpsA resulted in increased capsule levels. In contrast, production of a truncated form of CpsA lacking the extracellular LytR domain (CpsA-245) in the wild type background resulted in a dominant negative decrease in capsule level. GBS with CpsA-245, but not the ∆*cpsA* strain, were attenuated in human whole blood. However, the ∆*cpsA* strain showed significant attenuation in a zebrafish infection model. Furthermore, chain length was observed to be variable in a CpsA-dependent manner, but could be restored to WT levels with growth in the presence of lysozyme. Despite obvious differences in cell wall related phenotypes, GBS ∆*cpsA*

and GBS with CpsA-245 did not exhibit differences in antimicrobial sensitivity for lysozyme, bacitracin, or ampicillin compared to the WT GBS strain. Taken together, these results suggest that CpsA is a modular protein with different levels of regulatory capacity, and that this regulation may include not only capsule synthesis, but also cell wall associated factors.

INTRODUCTION

Streptococcal pathogens capable of causing systemic disease utilize a number of strategies for survival in the host. The most important of these is the polysaccharide capsule that is produced to shield the pathogens from clearance by components of the immune system, including complement deposition (88) and phagocytosis (67). The production of a polysaccharide capsule has proven to be a successful strategy for a number of human-specific pathogens, including the Group B Streptococcus (GBS) *Streptococcus agalactiae*, as well as *Streptococcus pneumoniae*. GBS has long been a significant cause of neonatal mortality (34), and long term sequelae (37), with intrapartum antibiotic prophylaxis still the recommended measure taken to combat incidence of infection (154). GBS remains a pathogen of significant import in developing countries, and the preemptive use of antibiotics to combat this disease is not ideal, as the development of drug resistance is a major concern (154). Though rare in adults, recent work has revealed an alarming trend of increased incidence of GBS infection in the United States in non-pregnant adults (117) (141), particularly in elderly patients with at least one underlying health issue, illustrating that GBS remains an important problem for adults as well. These observations demonstrate the need for further characterization of targets for antimicrobial therapy or vaccine generation, and the unequivocal importance of the polysaccharide capsule during infection makes it a prime candidate for disruption and subsequent alleviation or prevention of disease.

The production of a polysaccharide capsule by GBS and *S. pneumoniae* both rely on a number of shared components, with the first four genes of the capsule operon the most highly conserved between the two species with greater than 60% similarity (27). These genes are

annotated as *cpsA*, *cpsB*, *cpsC*, and *cpsD* for both species. The gene *cpsA* encodes a putative membrane-bound transcriptional regulator of the capsule operon (49), and contains a small intracellular domain and two conserved extracellular protein domains; the DNA Polymerase Processivity Factor (DNA_PPF; Pfam accession no. PF02916) domain and the LytR_cpsA_psr (LytR; Pfam accession no. PF03816) domain. The genes *cpsB*, *cpsC*, and *cpsD*, constitute a phospho-relay system that regulates polymerization and ligation of the capsular polysaccharide to the cell wall peptidoglycan (11, 20, 98, 99), with the encoded proteins CpsB as a phospho-tyrosine protein phosphatase, CpsD as a tyrosine kinase, and CpsC as a membrane tether and accessory protein for CpsD.

The presence of the DNA_PPF domain is curious for two reasons: first, although this region of the protein is categorized as belonging to a family of sliding clamp proteins that bind directly to DNA, the DNA_PPF domain of CpsA has been shown to reside extracellularly (54). Second, despite this region of the protein being classified with the DNA_PPF designation, the protein sequence of the DNA_PPF region of CpsA proteins diverges a great deal from traditional DNA_PPF sliding clamp proteins, with a BLAST alignment of the GBS CpsA DNA_PPF domain to the bacteriophage RB69 DNA_PPF domain giving no significant similarity. Therefore, we propose that the DNA_PPF designation of this portion of the protein does not correspond to a function consistent with sliding clamps, and that streptococcal species utilize the DNA_PPF domain of CpsA for some other as yet unknown function, which has been suggested previously (58).

In contrast to the DNA_PPF designation, the LytR designation of the CpsA protein is much more robust, with a sequence alignment of the GBS CpsA LytR domain to the *Bacillus subtilis* LytR protein's LytR domain giving 38% identity and 58% similarity (E-value = 1e-25),

indicating possible functional conservation. LytR proteins have been associated with transcriptional attenuation of the *lytRABC* divergon (74), which encodes cell-wall modifying enzymes. In contrast to the transcriptional attenuation observed for LytR, CpsA appears to have a transcriptional activation function for the capsule locus (27). The mechanism by which the LytR domain functions remains unclear. It may act as an environmental sensor that modulates the activity of the CpsA protein, thereby controlling the cytoplasmic domain, or in the case of CpsA altering the function of the DNA_PPF domain. In addition to regulation of capsule, CpsA may prove to exhibit roles associated with cell wall regulation as well, which would be congruent with the function assigned to LytR proteins. Although GBS lacks the *lytABC* operon of *Bacillus* and other species, CpsA may represent a regulatory module at the crossroads of capsule and the cell wall, two of the major surface components of streptococcal cells.

The presence of a small N- terminal cytoplasmic domain is common to both GBS CpsA and *B. subtilis* LytR proteins with 26 and 11 amino acids respectively. We have previously shown that this small cytoplasmic region in its entirety is sufficient for *S. iniae* CpsA to bind to the promoter upstream of *cpsA* (54), and it may be that LytR proteins function similarly. Both CpsA and LytR proteins contain a high density of positively charged amino acids in the intracellular domains with 11/26 amino acids for CpsA and 7/11 amino acids for LytR which may help facilitate interaction with specific DNA sequences. Additionally, the CpsA proteins of GBS and *S. pneumoniae* have possible leucine zipper domains extending from the cytoplasmic region into the first transmembrane region, which could also help facilitate DNA binding through dimerization. LytR proteins lack this property. Although the leucine repeat is present, there is no predicted coiled-coil sequence for CpsA, so the presence of a functional leucine zipper domain requires validation. In addition to the operon promoter upstream of the *cpsA* gene, a

second promoter element may also exist upstream of the *cpsE* gene in GBS, as a secondary transcription initiation site was identified in this region with upstream A-T rich repeats (171).

This study focuses on the function of each of the CpsA domains as they relate to the capsule locus promoters, actual capsule levels, and preliminarily, to cell wall stability. We demonstrate that the GBS CpsA protein is able to bind specifically to both the GBS *cpsA* and *cpsE* promoter elements, and define the regions of CpsA that facilitate binding to DNA and contribute to the specificity of the interaction. We have also shown that expression of these different domains in either the WT or a Δ*cpsA* strain of GBS alters capsule level and the capacity of the bacteria to survive in human whole blood. Additionally, we present data implicating CpsA in modulating the cell wall.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Plasmids were maintained in *Escherichia coli* electro-competent Top 10 cells (Invitrogen). Luria-Bertani (LB) medium (BD) was used to culture *E. coli* strains. Antibiotics were added as necessary to LB medium at the following concentrations: chloramphenicol, 20 μg/ml, and ampicillin, 100 μg/ml for *E. coli* strains. *E. coli* cultures were grown at 37°C with shaking. When growing *E. coli* cultures for protein purification, LB media was supplemented with 0.2% glucose (w/v). Solid media was generated by supplementing the liquid media with 1.4% agar (Acumedia). The streptococcal strain *Streptococcus agalactiae* Group B Strep (GBS) 515, a human clinical isolate from the blood of a patient with neonatal septicemia and GBS 515 ΔcpsA were generously provided by M. Wessels. GBS 515 was cultured in Todd-Hewitt medium (BD) supplemented with 0.2% yeast extract (THYB) (BD) in airtight conical tubes without agitation at 37°C. When transforming GBS 515 by electroporation, bacteria were grown on solid media supplemented with 1.4% agar (BD) and incubated in GasPak jars (BBL) with GasPak anaerobic system envelopes (BD).

Transformation of GBS 515. GBS 515 cultures were grown statically in THYB supplemented with 80 mM glycine overnight, diluted 1:20 in 25 mL of THYB supplemented with 80 mM glycine the following day and grown with shaking to an OD_{600} of 0.4. The cells were then harvested by centrifugation, washed 3 times with 10 mL of ice-cold 10% glycerol, and resuspended in 1 mL of ice-cold 10% glycerol. Plasmid DNA was mixed with 200 μl of cells, placed into an electroporation cuvette (DOT Scientific), and electroporated with a BIO-RAD Gene Pulser II at 25 μF, 2.0 kV, and 400 Ω. Cells were immediately transferred to 10 mL of

fresh THYB medium, and allowed to recover for 90 minutes at 37°C prior to plating on selective media.

Cloning of maltose-binding-protein (MBP)-CpsA fusions. The full length *cpsA* gene was amplified from GBS 515 genomic DNA using the primers 5' GBS-*cpsA*-SmaI and 3' GBS-*cpsA*-full-stop-PstI. Truncations of the 3' end of *cpsA* were amplified from GBS 515 genomic DNA using the primer 5'GBS-*cpsA*-SmaI in conjunction with the primers 3'GBS-*cpsA*-245-stop-PstI, 3'GBS-*cpsA*-117-stop-PstI or 3'GBS-*cpsA*-39-stop-PstI. These products were digested with SmaI and PstI and cloned into the corresponding SmaI and PstI sites of pMAL-c2x (NEB) leading to in-frame fusions of *cpsA* fragments downstream of the *malE* gene. This generated the following plasmids: pMAL-GBS-*cpsA*-full, pMAL-GBS-*cpsA*-245, pMAL-GBS-*cpsA*-117, and pMAL-GBS-*cpsA*-39. These constructs were transformed into *E. coli* Top10 cells (Invitrogen).

Protein purification: Overnight pMAL-*cpsA* expressing *E. coli* strains were sub-cultured 1:40 into 300 mL new LB medium supplemented with 0.2% glucose and grown at 37°C with shaking until reaching an OD_{600} of approximately 0.5, and protein expression was induced by addition of 0.3 mM isopropyl-β-D-1-thiogalactopyranoside, followed by incubation for 3 hours. Cells were then harvested by centrifugation at $6500 \times g$ for 10 minutes, the supernatant discarded, and the cells resuspended in 30 ml of Column Buffer (20 mM Tris-HCl, 200 mM NaCl, and 1mM EDTA) and stored at -20°C overnight. The frozen cultures were thawed on ice, and 10 mL of lysis buffer was added (50 mM Tris-HCl, 150 mM NaCl, 1% Sarkosyl (w/v), 1% Triton-X 100 (v/v), 10 mM CHAPS, pH 7.4) along with 40 μl of 100X ProteoBlock protease inhibitor cocktail (Fermentas). The mixture was then sonicated in 30 second bursts to lyse cells. The lysate was

centrifuged at 10,000 x g for 30 minutes and the clarified lysate diluted to a total volume of 100 ml using Column Buffer. This was run on a glass column containing amylose beads (NEB) and eluted according to the manufacturer's specifications. Purified protein concentration was determined using the BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions. Protein purity was assessed with SDS-PAGE.

Generating digoxigenin-labeled DNA probe and competitor DNA probes: Probes consisting of the GBS 515 *cpsA* promoter (217 bp) and GBS 515 *cpsE* promoter (221 bp) were amplified from GBS 515 genomic DNA using the primers 5' GBS-*cpsA*-pro with 3' GBS-*cpsA*-pro, and 5' GBS-*cpsE*-pro with 3' GBS-*cpsE*-pro, respectively. The *S. iniae cpsA* promoter (182 bp) was amplified from *S. iniae* 9117 genomic DNA using the primers 5'*iniae-cpsA*-pro and 3'*iniae-cpsA*-pro. The 515 GBS promoter products were then labeled with digoxigenin using the DIG Gel Shift Kit, 2nd Generation (Roche) according to manufacturer's instructions.

Electromobility Shift Assays: To conduct the EMSA, constant amounts of the MBP –CpsA protein fusions were incubated with a constant amount of labeled probe (12 fmol) in a binding buffer containing 100 mM HEPES pH 7.2, 1 mM EDTA, 50 mM KCl, 50 mM MgCl$_2$, 1 mM DTT, and 30% (v/v) glycerol for 30 minutes at room temperature. For reactions with competitor DNA, an excess of unlabeled GBS 515 probe DNA (either *cpsA*-pro or *cpsE*-pro) was used as a specific competitor, and unlabeled *S. iniae cpsA*-pro was used as a non-specific competitor. The samples were loaded onto a 6% polyacrylamide native gel consisting of 6% (v/v) polyacrylamide, 44.5 mM tris base, 44.5 mM boric acid, and 1mM EDTA. Electrophoresis was performed at 4˚C. The gel was then transferred to a nylon membrane (Santa Cruz) using a semi-

dry transfer apparatus (Hoefer). Chemiluminescent detection of DIG-labeled DNA on membranes was accomplished with CDP-Star (Roche) according to manufacturer instructions, followed by exposure to X-ray film. Each EMSA was repeated at least twice, and was also repeated by using sheared salmon sperm DNA as a non-specific competitor to confirm results with the *S. iniae cpsA*-pro non-specific competitor. Only EMSAs using the *S. iniae cpsA*-pro as a non-specific competitor are reported in the results.

Cloning of MBP-*cpsA* fusions for complementation. The MBP-*cpsA* fusions MBP-*cpsA*-full, MBP-*cpsA*-246, and MBP-*cpsA*-117 were amplified from the plasmids generated above using the primer 5' MBP-RBS-BamHI in conjunction with the primers 3' GBS-*cpsA*-full-stop-PstI, 3' GBS-*cpsA*-245-stop-PstI, and 3'GBS-*cpsA*-117-stop-PstI respectively. These fragments were then digested with BamHI and PstI and ligated into the corresponding BamHI and PstI sites on the plasmid pLZ12-*rofA*-pro (103) behind the *rofA* promoter (12) creating the following plasmids: pGBS-*cpsA*-full, pGBS-*cpsA*-245, and pGBS-*cpsA*-117. These constructs were transformed into *E. coli* Top10 cells, propagated, and then transformed into GBS 515 as described above.

Measurement of GBS 515 capsule levels using buoyant density centrifugation. Buoyant density centrifugation was performed similarly to previous work (32), (79), but with modification. Linear density gradients of Percoll (GE Healthcare) were generated by diluting Percoll to a high density limit (1.120 g/mL) and low density limit (1.085 g/mL) with a final concentration of 0.15 M NaCl according to the manufacturer's instructions, and carefully layering 2 mL of the low density solution on top of 2 mL of the high density solution in a 5 mL

Falcon tube (BD). These tubes were then set horizontally at a 15° angle to the benchtop, and left overnight. The next morning tubes were set upright and allowed to settle for 30 minutes prior to use. Bacterial cultures were grown overnight as described above, and cultures were normalized to an OD_{600} of 0.6 in 1 mL of THYB medium, pelleted by centrifugation, resuspended in 50 μl of PBS, and added directly to the top of the Percoll gradients. Tubes were then centrifuged for 30 minutes at 5000 rpm in a swinging bucket Eppendorf Centrifuge 5403 with Rotor 16A4-44 at room temperature. Measurements were then taken from the meniscus to the bottom of the cell band in each tube, and compared to a set of colored beads of known density (GE Healthcare) to determine bacterial cell density. These experiments were performed at least six times, with results reported using a representative experiment.

Time course measurement of GBS 515 capsule as a function of growth phase. Measurement of capsule for time course experiments was identical to that given above, except that GBS bacteria were taken at different time points from separate cultures after inoculating with a 1:10 dilution from overnight cultures. These experiments were performed at least two times, with results collated from separate experiments and normalized to WT buoyant density.

Measurement of capsule after growth of GBS 515 in media of differing pH. THYB medium was adjusted to a pH of 5, 6, 7, 8, or 9, using liquid HCl or NaOH when appropriate. Bacterial strains were grown as previously described and capsule measured as given above, with the exception that Percoll gradients were generated in a 10 mL conical tube with 5 mL each of the high density and low density solutions.

Measurement of capsule after incubation in fresh medium or 100% human serum.
Bacterial strains were grown as previously described and the following day normalized to an
OD_{600} of 0.6 in 1 mL of THYB medium, pelleted with centrifugation, and resuspended in 50 μl
of PBS. This concentrate was then resuspended in either 1 mL of fresh THYB medium or 1 mL
of 100% human serum donated from a healthy volunteer, and incubated with rotation at 37° C
for 1 hour. Subsequently, bacteria were pelleted with centrifugation, resuspended in 50 μl of
PBS, and capsule measured as given above.

Incubation of GBS 515 in human whole blood. Human blood was obtained from healthy
volunteers and collected in heparinized vacuum tubes. Bacteria were grown overnight and
normalized to an OD_{600} of 0.3 in PBS, serially diluted in PBS, and 2.5×10^5 CFU were added to
1 mL of blood and incubated with rotation at 37°C for 3 hours. After incubation, 100 μl of the
inoculated blood was plated onto selective media, and incubated overnight at 37°C in a CO_2
incubator to determine colony forming units. Whole blood assays were repeated a minimum of
three times with results reported using a representative experiment.

Zebrafish survival assays with GBS 515. Assays were performed in a similar manner as
described previously (79). Briefly, bacteria were grown overnight, diluted 1:50 the following
day in fresh medium, and grown to an OD_{600} of approximately 0.3. Cultures were then
normalized to 1×10^8 CFU/mL and 10 μl of culture or media alone was injected into zebrafish
intramuscularly, resulting in an infectious dose of 1×10^6 CFU. A total of 25 fish were infected
with each strain over three experiments, and zebrafish survival monitored over a 6-day period,
after which all surviving fish were euthanized.

Quantification of GBS 515 chain length. Cultures were grown overnight and 6 μl of culture was directly placed onto a glass slide with a coverslip (Fisher) and viewed at 1000X magnification on a Zeiss AxioSkop 40. Pictures were taken of visual fields selected as randomly as possible with an attached Zeiss AxioCam MRc. Pictures were taken on two separate occasions and at least 250 chains were counted from each set, for a total of 500 or more chains counted for each strain. Chain length values were distributed between arbitrarily set numerical categories and calculated as a percentage of all counted chains. For analysis of lysozyme treated strains, cultures were grown overnight in the presence of a sub-inhibitory concentration of lysozyme (200 μg/mL) and pictures taken as above.

Measurement of antimicrobial resistance. GBS strains were grown overnight, subcultured into fresh medium with a 1:10 dilution, and grown to an OD_{600} of 0.3. Cultures were normalized and serially diluted to 1×10^4 CFU/mL and 10 μl added to individual wells of a 96 well plate, with a final volume of 200 μl including antimicrobial agents. Antimicrobial concentration ranges were as follows: lysozyme, 0 mg/mL to 3.65 mg/mL; bacitracin, 0 μg/ml to 1.65 μg/ml; and ampicillin, 0 μg/ml to 0.275 μg/ml. Subsequent to addition of bacteria, plates were incubated at 37° C overnight and OD_{600} measured with a VERSAmax microplate reader (Molecular Devices) the following day.

Table 2: Primers used in this chapter.

Primer	Sequence (5' - 3')
5' GBS-*cpsA*-pro	CGC GGA TCC GTT GAA TTC TCA TAA CTC TAG
3' GBS-*cpsA*-pro	CCG GAA TTC GCG AAT GAT TAG ACA TTG
5' GBS-*cpsE*-pro	GAA AAA GGA AGT AAG GGG CTC TTG
3' GBS-*cpsE*-pro	GCC ACG ACT CCA AAA GTC TC
5' *iniae-cpsA*-pro	CTC ATA ATG ACA GTC TAT C
3' *iniae-cpsA*-pro	CCA TCA ATA TCA TTT AAG TC
5' GBS-*cpsA*-SmaI	TCC CCC GGG TCT AAT CAT TCG CGC CGT C
3' GBS-*cpsA*-full-stop-PstI	AAA ACT GCA GTT ATT CCT CCA TTG TGT TC
3' GBS-*cpsA*-117-stop-PstI	AAA ACT GCA GTT ACT CAA TTT CAG AGT ATG AAG C
3' GBS-*cpsA*-39-stop-PstI	AAA ACT GCA GTT ACA TAA GAA ATA ATG AGA CTA C
3' GBS-*cpsA*-245-stop-PstI	AAA ACT GCA GTT ATG TTG ATA TAG AGC CAA AAG
5' MBP-RBS-BamHI	CGC GGA TCC GCG GAT AAC AAT TTC ACA CAG G

RESULTS

GBS CpsA binds to two separate putative promoters located in the capsule operon. All members of the LytR_cpsA_psr protein family have been connected to transcriptional regulation, but the precise method by which these proteins contribute to transcriptional regulation has not yet been elucidated. As a member of this protein family CpsA has been identified as a potential transcriptional activator of the capsule operon (27). Previous work in our lab has demonstrated that purified CpsA protein from *S. iniae* was capable of binding with specificity to the promoter region of the *S. iniae* capsule operon upstream of the *cpsA* gene (54), suggesting that *S. iniae* CpsA may modulate transcription by directly binding to promoter sequences. An alignment of the *cpsA* promoter DNA sequences of *S. iniae* and GBS using BLAST reveals that these promoters share no significant similarity. Additionally, despite the CpsA proteins of *S. iniae* and GBS sharing 57 % amino acid identity and 76 % amino acid similarity, the intracellular regions of CpsA responsible for binding to DNA in *S. iniae* share no significant similarity with the same regions on the GBS form of the protein (Fig. 21). These differences in sequence at the DNA and protein level necessitate confirmation of DNA binding by the GBS form of CpsA. To this end we performed electromobility shift assays using labeled probes reflective of two putative promoter regions within the GBS capsule operon, upstream of the previously identified transcriptional start sites of the *cpsA* gene and *cpsE* gene (Fig. 22A) (171). To determine the importance of different regions of the GBS CpsA protein in binding to DNA, we constructed and purified multiple maltose binding protein fusions of the CpsA protein including the full-length protein (MBP-CpsA-full) as well as two truncated forms of the protein, MBP-CpsA-117 and MBP-CpsA-39 (Fig. 22C).

Purified MBP-CpsA-full was incubated with either the DIG labeled GBS *cpsA* promoter or the DIG labeled GBS *cpsE* promoter. MBP-CpsA-full demonstrated the ability to bind to the labeled *cpsA* promoter with specificity (Fig. 23A). Lane 1 demonstrates the migration of unbound labeled *cpsA*-pro probe, lanes 2 and 3 demonstrate labeled probe bound by the protein in the presence of 25 fold specific or non-specific unlabeled competitor DNA, lanes 4 and 5 demonstrate labeled probe bound by the protein in the presence of 50 fold specific or non-specific unlabeled competitor DNA, and lanes 6 and 7 demonstrate labeled probe bound by the protein in the presence of 75 fold specific or non-specific unlabeled competitor DNA. In the same manner, using the same concentration of protein and probe, the full length GBS CpsA protein demonstrated specific binding to the labeled *cpsE* promoter as well (Fig. 23B).

After observing specific binding of MBP-CpsA-full to both the labeled GBS *cpsA* and GBS *cpsE* promoters, we proceeded to determine if there was a preference for binding to one of these promoters over the other by cross-competing each labeled probe with unlabeled probe (Fig. 24A). As shown above, full-lengh CpsA protein binds to the *cspA*-pro and *cpsE* pro probes (Fig. 24A, lanes 2 and 7). Competition with an excess of unlabeled probe of either *cpsA*-pro (lanes 3 and 9) or *cpsE*-pro (lanes 4 and 8), revealed that the full length CpsA protein was able to bind both labeled promoters and did not demonstrate a clear preference for either *cpsA* or *cpsE* probe to the exclusion of the other. Again, these interactions were specific as a 50-fold excess of unlabeled *S. iniae cpsA*-pro (nonspecific competitor) showed no competition (Fig 24A, lanes 6 and 10).

To determine what regions of the protein were required for DNA-binding, EMSAs were performed using both truncated forms of the GBS CpsA protein, MBP-CpsA-117 and MBP-CpsA-39. MBP-CpsA-117 truncated the CpsA protein after the third transmembrane domain

(see Fig 22C), thereby removing the large extracellular region to assess its contribution to binding or specificity. When full length GBS CpsA was replaced with the truncation MBP-CpsA-117 (Fig. 24B), the protein was still able to bind both labeled probes, and no clear preference for either the labeled *cpsA*-pro or *cpsE*-pro probes was observed when cross-competed, as seen for the full length CpsA. However, a reduction in specificity was observed for both the *cpsA* and *cpsE* labeled probes when comparing competition with unlabeled specific and non-specific DNA, though some level of specificity was still present. This indicated that the large extracellular portion of CpsA is not required for binding to DNA, but does affect the specificity with which CpsA is able to interact with DNA.

MBP-CpsA-39 has a truncation of the protein at the end of the first transmembrane domain, leaving the putative leucine zipper domain intact, but removing the cyoplasmic loop between the second and third transmembrane domains (see Fig. 22C). Thus, an EMSA using this protein fusion assessed the contribution of the cytoplasmic loop to binding ability and specificity. When the MBP-CpsA-39 truncation was used with the same parameters (Fig. 24C), the protein retained the ability to bind to both labeled probes, but lost all semblance of specificity for either the *cpsA*-pro or *cpsE*-pro when comparing competition for unlabeled specific and non-specific DNA. This confirmed that the cytoplasmic loop contributes to specificity, but is not required for binding ability. Taken together, these results demonstrate that only the cytoplasmic N-terminus of GBS CpsA is required for binding to DNA, but that the cytoplasmic loop and extracellular region of the protein both contribute to specificity of the interaction.
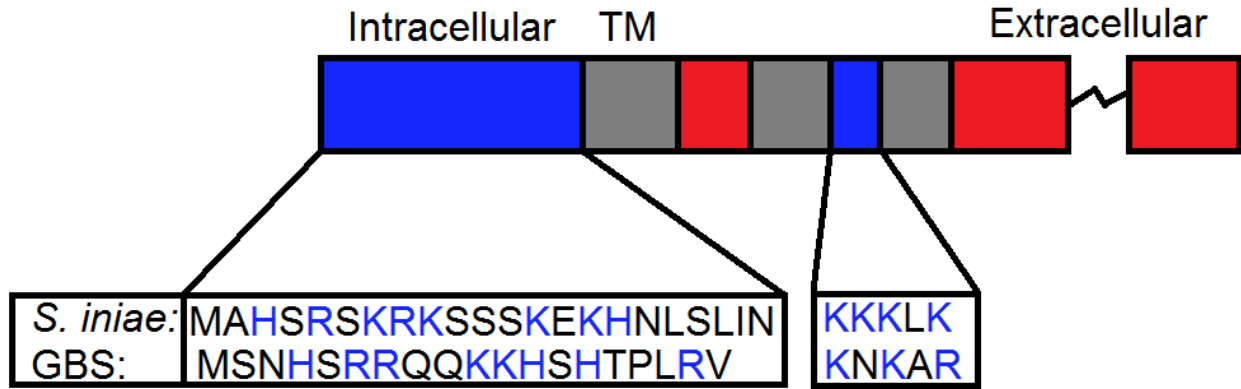
Figure 21: Comparison of the cytoplasmic amino acid sequence for CpsA from *S. iniae* and GBS with positively charged amino acids in blue text.
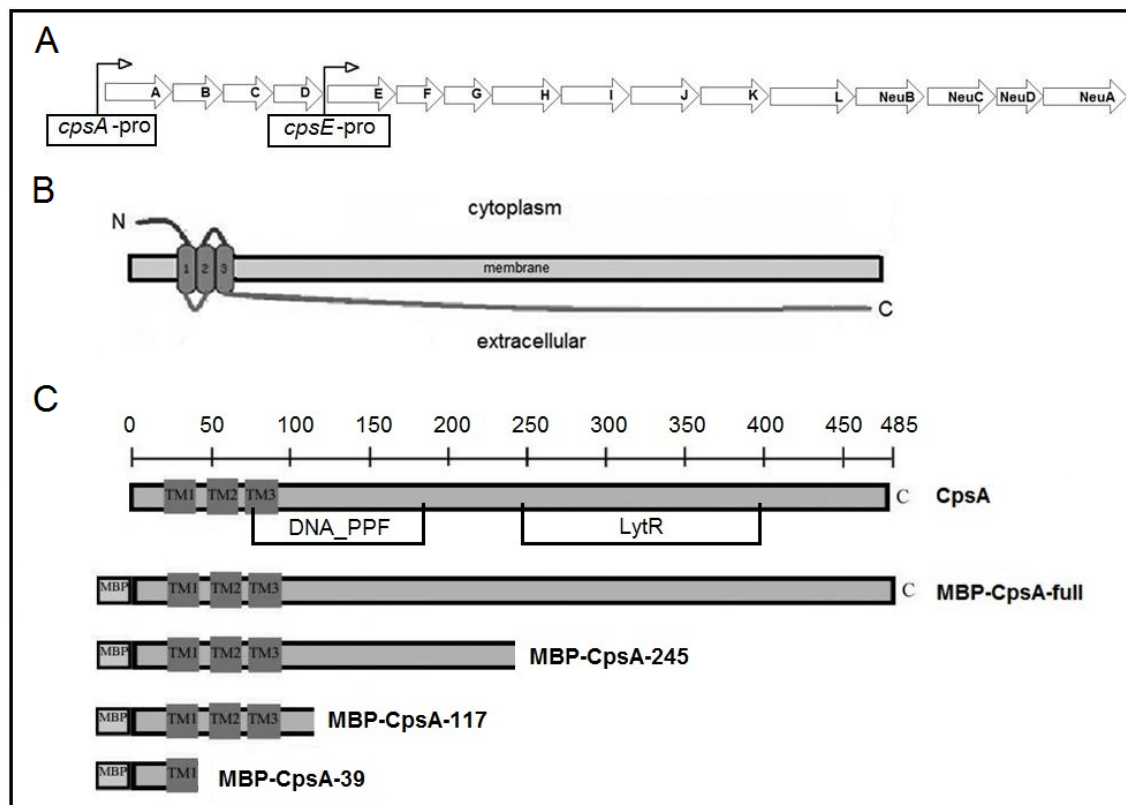
Figure 22: (A) Capsule operon of GBS. Putative promoter sequences within the capsule operon are indicated by directed arrows. (B) Membrane topology of GBS CpsA. (C) Arrangement of the GBS CpsA protein where domains are shown as TM (Transmembrane domains), DNA_PPF (DNA Polymerase Processivity Factor domain), and LytR (LytR_cpsA_psr family domain). Below are truncations made to MBP fusions of CpsA representing the full protein, a truncation at amino acid 245, a truncation at amino acid 117, and a truncation at amino acid 39.
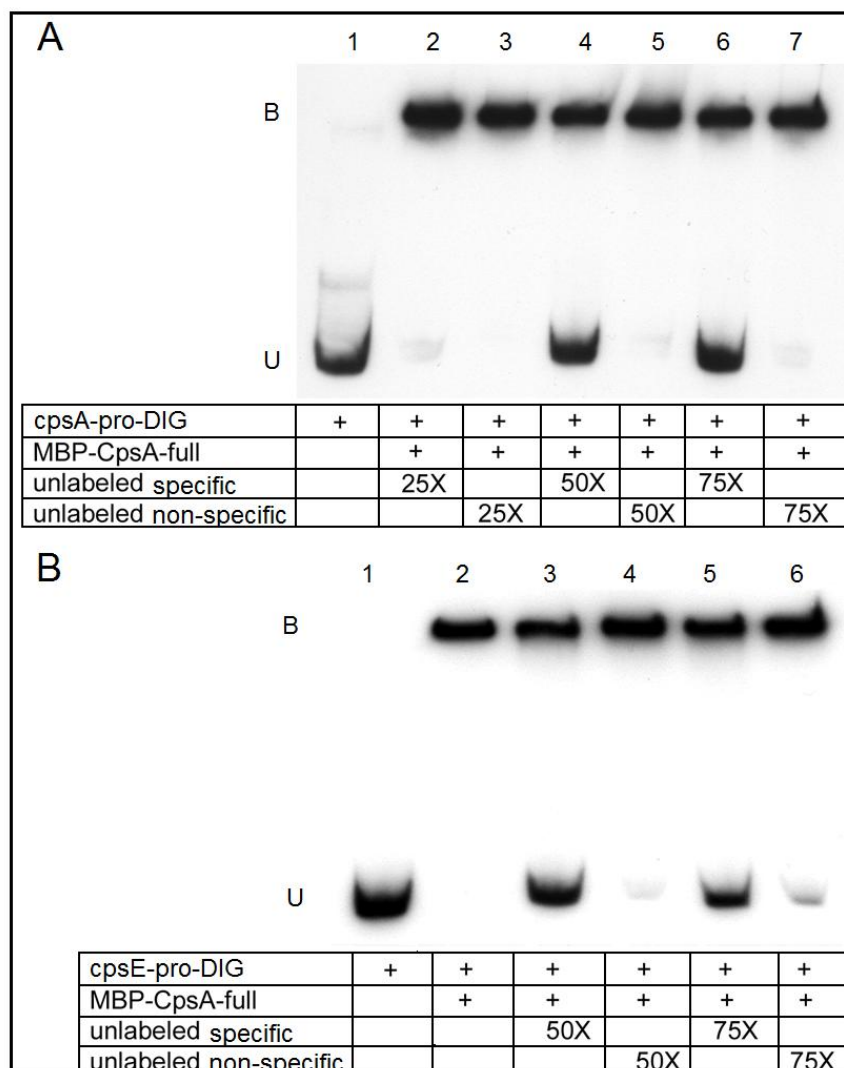
Figure 23: Electromobility shift assay demonstrating binding of GBS MBP-CpsA-full to either (A) the labeled GBS *cpsA*-pro probe or (B) the labeled GBS *cpsE*-pro probe. In both (A) and (B) 10 pmol of protein is used in lanes with protein added.
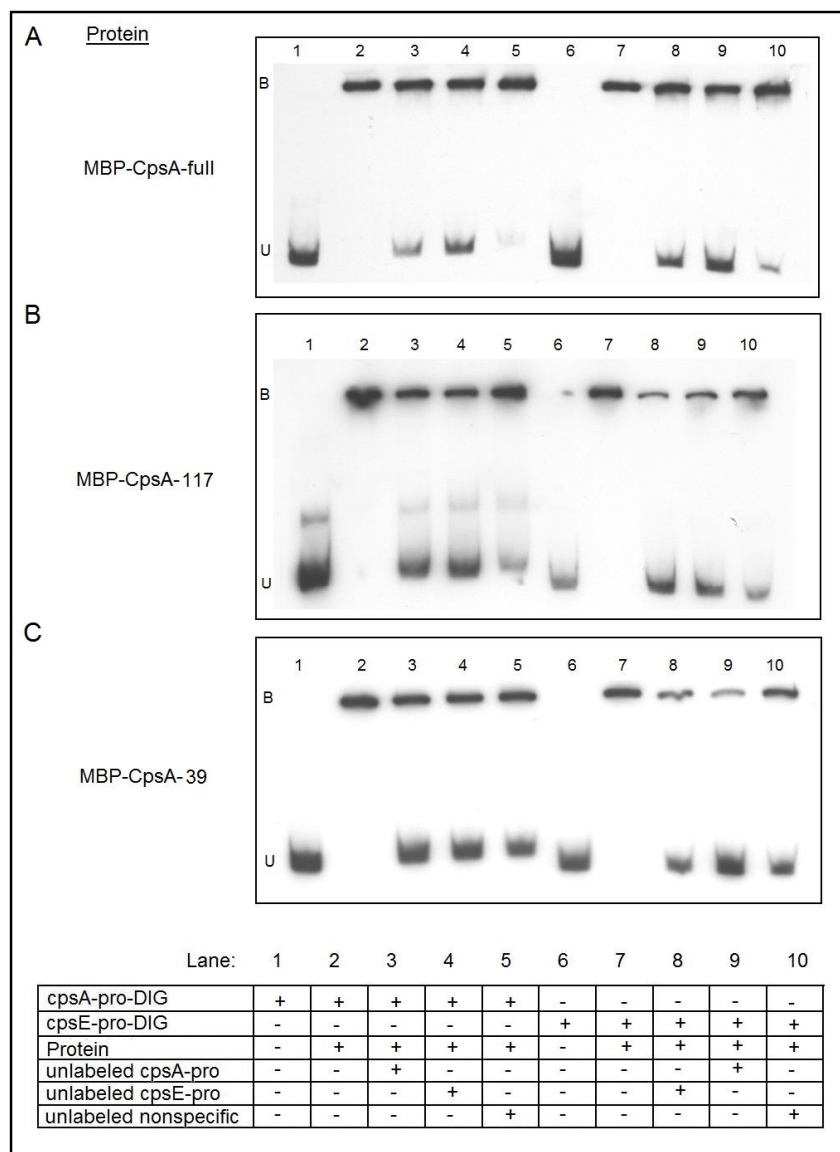
Figure 24: Electromobility shift assays showing binding of (A) MBP-CpsA-full (10 pmol), or (B) MBP-CpsA-117 (52 pmol), or (C) MBP-CpsA-39 (7 pmol), to either the labeled GBS *cpsA*-pro or labeled GBS *cpsE*-pro probes in the absence or presence of competitor DNA representing unlabeled GBS *cpsA*-pro, GBS *cpsE*-pro, or *S. iniae cpsA*-pro (unlabeled nonspecific). Unbound labeled probe is indicated by "U" and bound labeled probe is indicated by "B."

Ectopic expression of CpsA affects GBS capsule level. Deletion of the *cpsA* gene has previously been associated with decreased capsule production (27). To assess the contribution of each domain of the CpsA protein to capsule production in the bacterial cell, full length and truncated forms of CpsA (Fig. 22C) were constructed and placed on the plasmid pLZ12-*rofA*-pro (103), providing constitutive expression. These plasmids were then transformed into either the WT GBS 515 strain or a Δ*cpsA* GBS 515 strain. Buoyant density centrifugation was used to determine relative differences in the level of capsule produced by the strains created as described above. Measurement of capsule demonstrated that the Δ*cpsA* strain produced less capsule than the WT strain when both strains harbored the vector alone (Fig. 25). Expression of MBP-CpsA-full in both the WT and Δ*cpsA* background led to an increase in capsule over that produced normally by the WT strain, (Fig. 25), demonstrating that expression of the full length form of CpsA was able to complement the Δ*cpsA* strain, although the complemented strain still produced slightly less capsule compared to the WT strain with the same plasmid. When a truncated form of the protein lacking the LytR domain (MBP-CpsA-245) was expressed in either background a loss of capsule was experienced to levels below that of the Δ*cpsA* strain (Fig. 25), indicative of possible dominant negative or repression mechanism. The addition of just the N-terminal DNA-binding domain of CpsA (MBP-CpsA-117) resulted in an increase in capsule for the WT strain to the same level as expression of MBP-CpsA-full and an increase in capsule for the Δ*cpsA* strain to a slightly lesser degree (Fig. 25), showing complementation of the Δ*cpsA* mutant with a truncated form of CpsA missing the entire extracellular domain. Taken together, the data suggest that the various domains of CpsA contribute to regulation of capsule production in different ways.

To further investigate the role of CpsA in controlling capsule production, capsule levels were monitored over time as a function of growth phase in both the WT and ΔcpsA strains ectopically expressing different forms of CpsA. As observed for *S. iniae*, WT GBS demonstrated the highest levels of capsule during exponential growth, between hours 4 and 5, (Fig. 26). Expression of MBP-CpsA-full in the WT background did not significantly change the curve, but the decrease in capsule at early time points and the increase in capsule during later time points were both more pronounced (Fig. 26), suggesting that CpsA may contribute to both the initial decrease in capsule as well as the later increase during exponential growth. Expression of the dominant negative form MBP-CpsA-245 did not turn capsule fully off, and growth phase related changes were still observable (Fig. 26). However, capsule levels were decreased at almost all time points relative to the other strains (Fig. 26). Expression of the DNA-binding domain MBP-CpsA-117 resulted in a slight increase in capsule levels at all time points relative to the other strains (Fig. 26), consistent with its presumed activating function.

Investigation of the ΔcpsA strain revealed that capsule level is still modulated in response to growth phase, with a slight reduction in capsule compared to the WT strain at early time points (Fig. 27). Interestingly, at later time points the ΔcpsA strain induces capsule production to a higher level than that observed for the WT strain, but then promptly decreases to a level lower than the WT strain (Fig. 27), perhaps indicating dysregulation of other components involved in regulation of capsule synthesis. Expression of MBP-CpsA-full in the ΔcpsA background gave different results than those observed for the WT strain, with capsule levels generally higher at most time points relative to the other strains (Fig. 27). Production of MBP-CpsA-245 in the ΔcpsA background led to a decrease in capsule levels at almost all time points relative to the other strains (Fig. 27), consistent with a putative dominant negative function. The presence of

MBP-CpsA-117 did not exhibit the same activating function in the Δ*cpsA* background as that observed in the WT background, with lower levels of capsule at early time points relative to other strains, however, an increase in capsule levels relative to other strains was observed at later time points (Fig. 27). Taken together, the data suggest that in the presence of WT CpsA, expression of various forms of MBP-CpsA does not alter growth phase dependency for capsule production, but does modulate the level to which capsule is produced. However, when these same constructs are ectopically expressed in the Δ*cpsA* parent strain, growth phase dependency appears to be somewhat dysregulated.
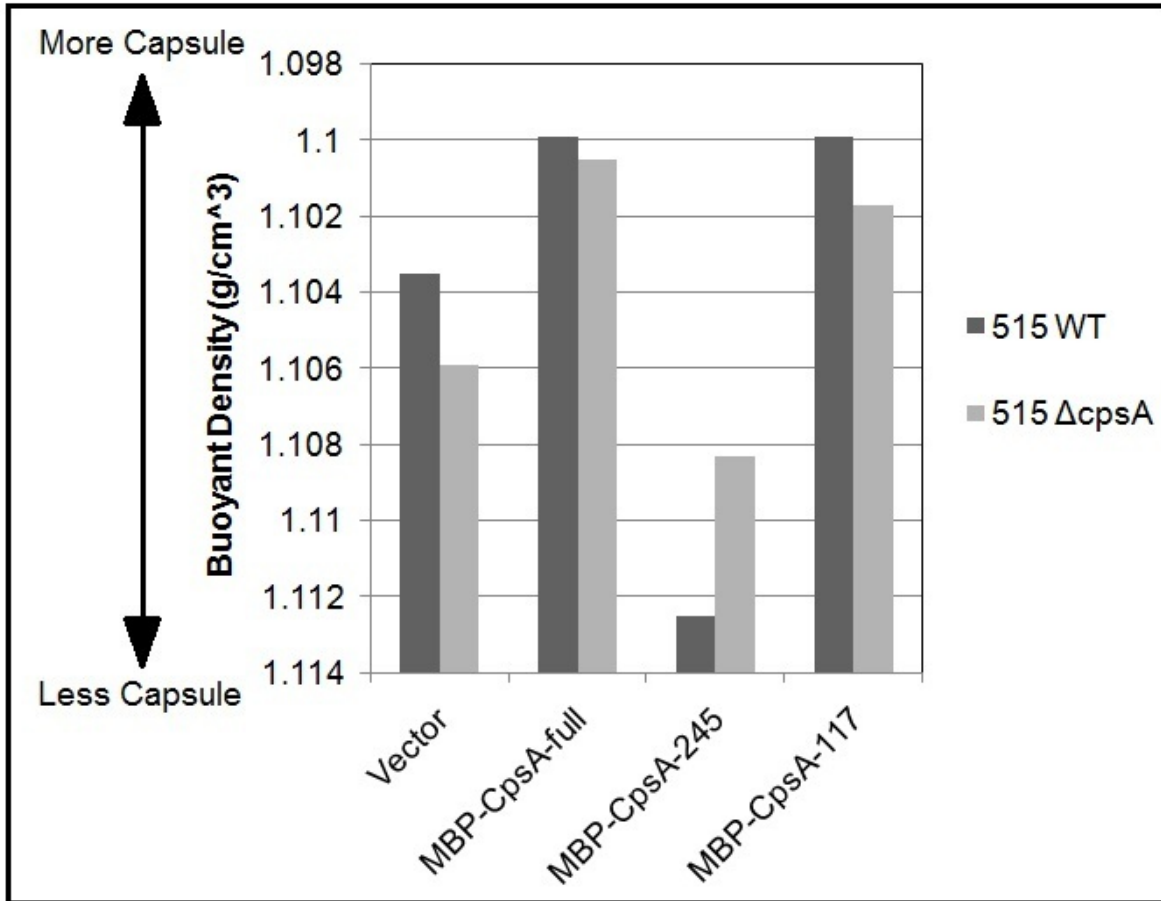
Figure 25: Percoll buoyant density assay reflecting capsule levels of the GBS WT or Δ*cpsA* strains harboring the vector plasmid, or a plasmid containing MBP-CpsA-full, MBP-CpsA-245, or MBP-CpsA-117.
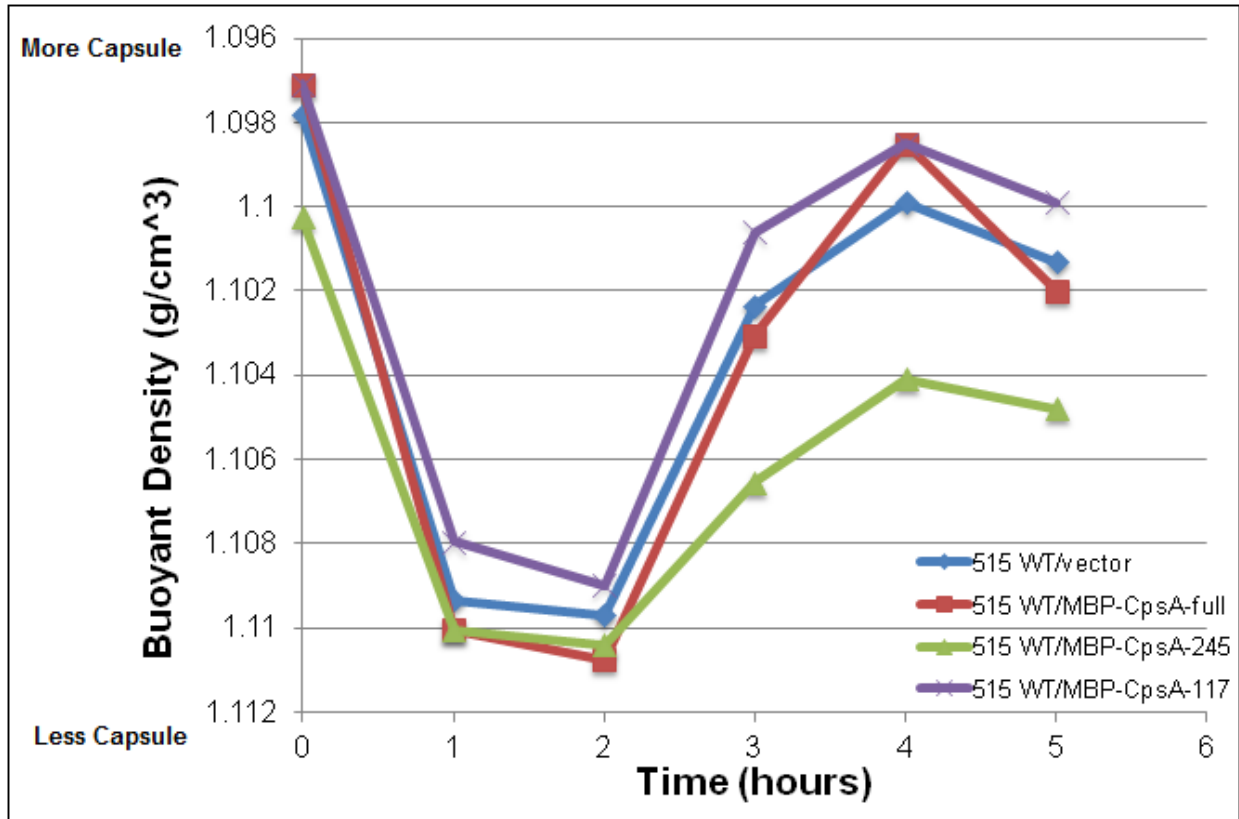
Figure 26: Changes in capsule as a function of growth phase for WT 515 strains carrying the vector plasmid, MBP-CpsA-full, MBP-CpsA-245, or MBP-CpsA-117.
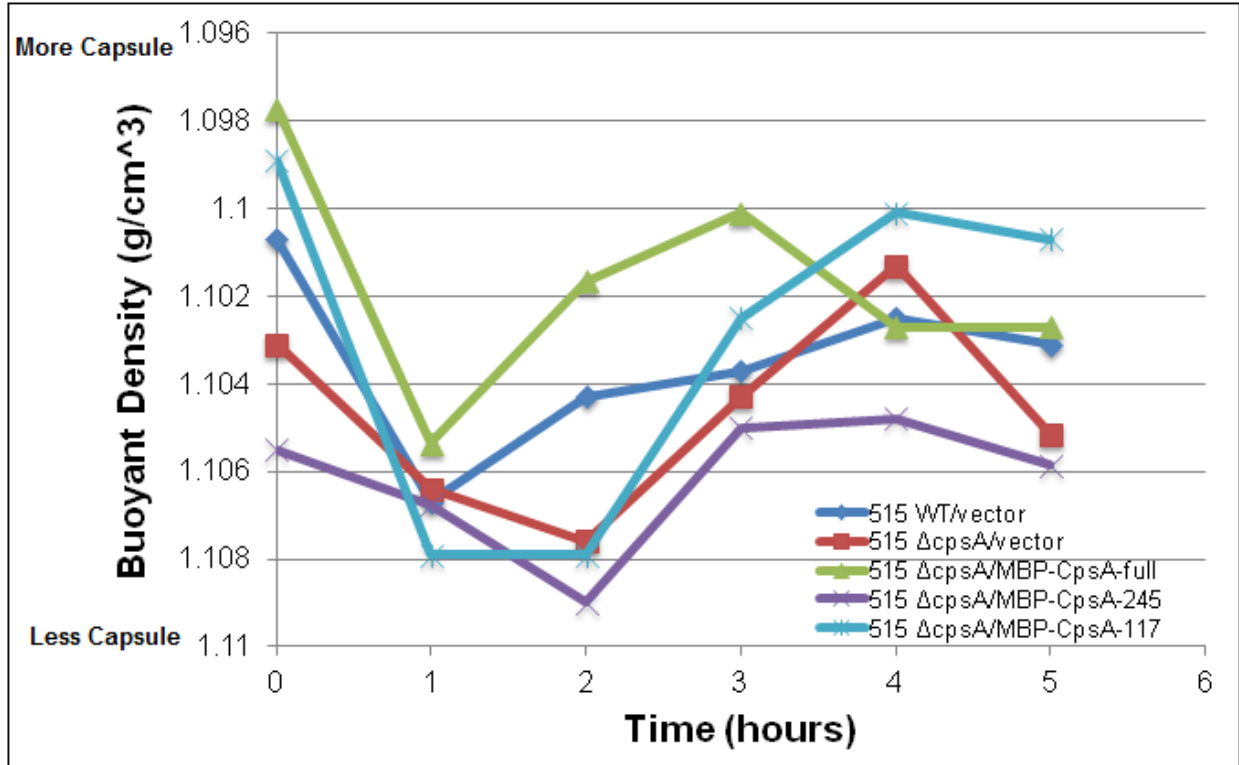
Figure 27: Changes in capsule as a function of growth phase for the 515 WT/vector strain and Δ*cpsA* 515 strains carrying the vector plasmid, MBP-CpsA-full, MBP-CpsA-245, or MBP-CpsA-117.

Capsule change associated with pH and human serum is not dependent on CpsA. Previous work in our lab showed that *S. iniae* capsule levels changed when bacteria were grown in media of differing pH, with an acidic pH leading to less capsule and a basic pH leading to more capsule (B. Lowe and M. Neely, unpublished data). Additionally, it has also been reported that GBS increases capsule levels in the presence of serum derived from human blood (119). One of our hypotheses is that CpsA integrates environmental signals within the host to regulate capsule levels, so we proceeded to determine the extent to which CpsA of GBS contributes to capsule regulation with regard to pH and human serum. Using GBS 515 WT and Δ*cpsA* strains we determined that GBS also demonstrates changes in capsule when grown in media of different initial pH levels, however, the observation that the Δ*cpsA* strain showed the same pH responsiveness ruled out a role for CpsA in facilitating changes in capsule level in response to pH (Fig. 28). Furthermore, we tested the same two strains by incubating in fresh media or in 100% human serum for one hour, and confirmed that GBS increased capsule levels in response to human serum, but again observed that this response was not dependent on the presence of CpsA (Fig. 29). While not ruling out an environmental sensing function for CpsA, these results do suggest alternate regulatory schemes employed by GBS to dynamically alter capsule levels in response to two physiologically relevant environmental signals encountered in the host, with changes in pH occurring during a switch from the genitourinary tract to the bloodstream, and components of serum encountered during systemic disease.
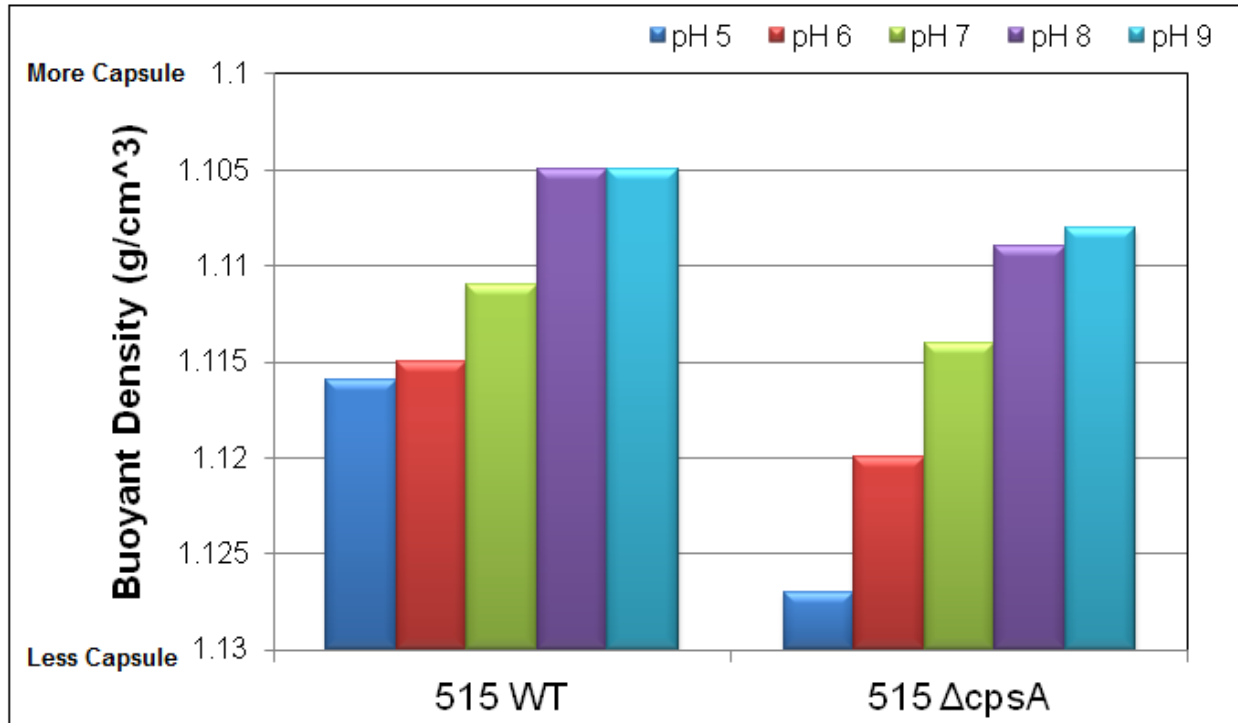
Figure 28: Capsule levels of GBS 515 strains grown in media of varying pH.
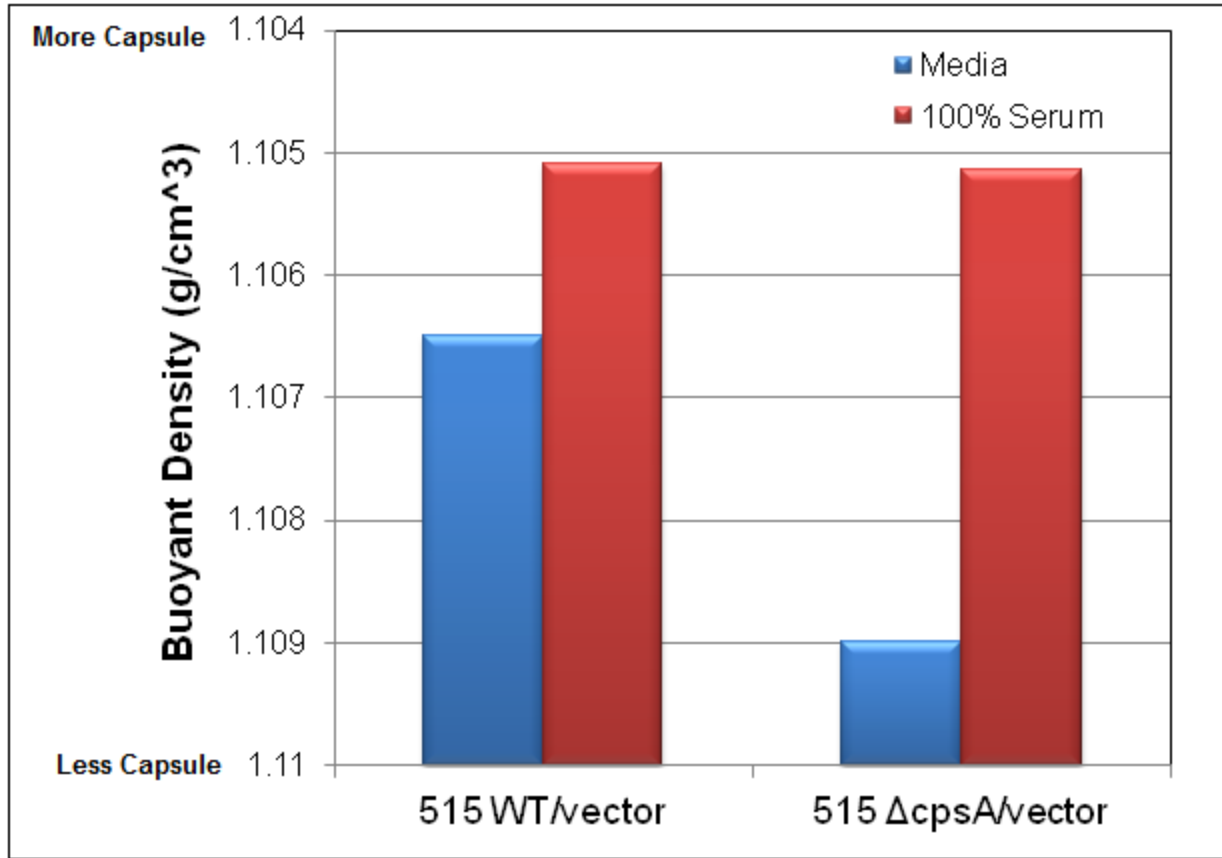
Figure 29: Capsule level of GBS 515 strains when incubated in THYB medium or 100% human serum for one hour at 37° C.

GBS survival in whole blood is altered by ectopic expression of CpsA. GBS virulence entails
dissemination through the bloodstream, an ability that relies on inhibiting phagocytic clearance,
which is primarily dependent on the presence of capsule (126). The variations in capsule
production observed when different domains of CpsA are ectopically expressed in GBS led to
the question of whether these variations corresponded to changes in survival in human blood.
Surprisingly, when incubated in human whole blood, the Δ*cpsA* strain of GBS shows no major
difference in the number of bacteria killed compared to the WT strain of GBS (Fig. 30), despite
the presence of less capsule as measured by buoyant density (Fig. 25). Expression of MBP-
CpsA-full or MBP-CpsA-117 in the WT background did not significantly alter the number of
bacteria killed (data not shown), despite the presence of more capsule than the WT strain with
vector alone as measured by buoyant density (Fig. 25). Alternatively, expression of MBP-CpsA-
full or MBP-CpsA-117 in the Δ*cpsA* background caused an approximately 0.4 log increase in
bacterial killing compared to the parent strain (Fig. 30), despite the production of more capsule
(Fig. 25). However, expression of MBP-CpsA-245 in both the WT and Δ*cpsA* background
resulted in an approximately 0.6 log increase in the number of bacteria killed compared to the
respective parent strain (Fig. 30), which may correspond to the loss of capsule these strains
exhibit as measured by buoyant density (Fig. 25).
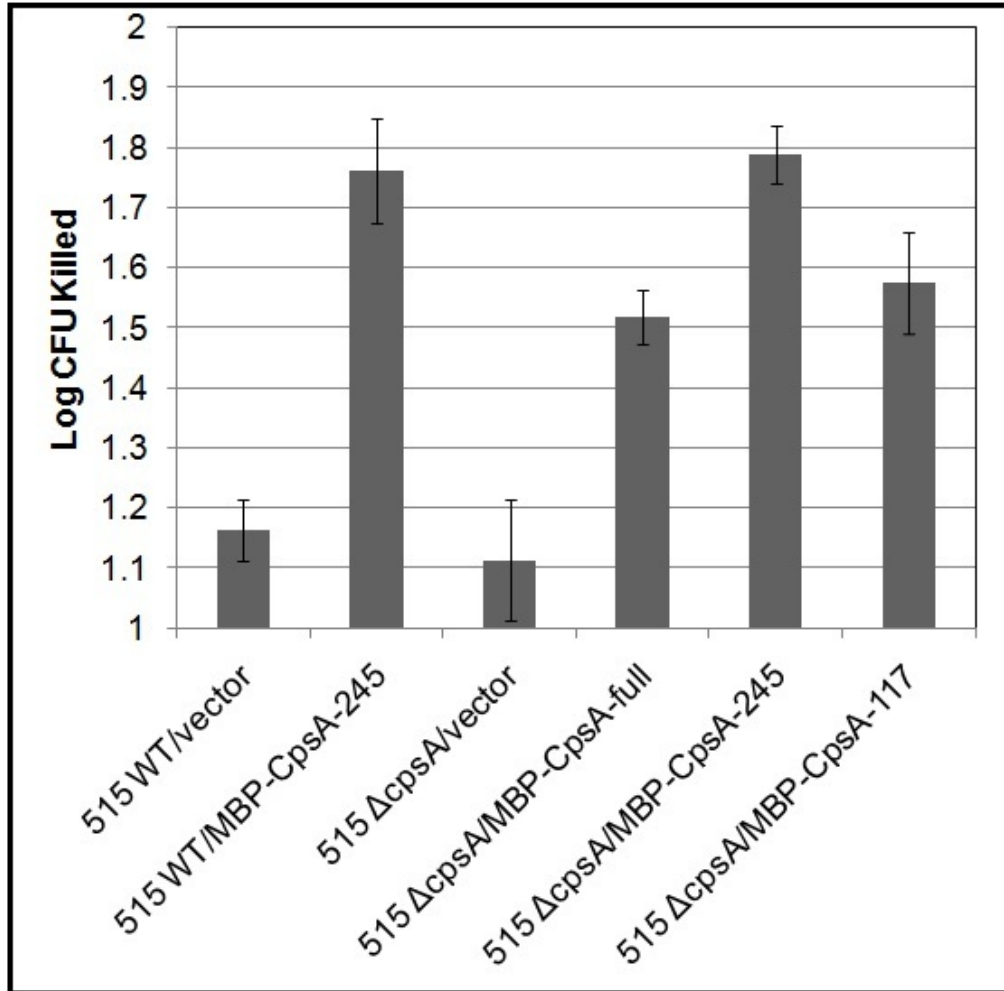
Figure 30: Whole blood assay measuring the Log_{10} level of CFU killed for bacterial strains incubated in human whole blood for 3 hours.

GBS virulence is attenuated in a zebrafish model of infectious disease in the absence of CpsA. The surprising result that the GBS Δ*cpsA* strain was not attenuated in human whole blood, despite the production of less capsule, led to an *in vivo* assessment of virulence for the GBS 515 strains using a zebrafish model of infectious disease. When zebrafish were inoculated with the WT strain of GBS, only 8% of zebrafish survived to day 6 (Fig. 31). In contrast, when zebrafish were inoculated with a Δ*cpsA* strain of GBS, 68% of fish were viable at day 6 (Fig. 31). The observed decrease in virulence of the Δ*cpsA* strain in an *in-vivo* model of pathogenesis, when compared to a lack of attenuation in human whole blood (Fig. 30), suggests that disruption of *cpsA* may lead to deficiencies that are only observable in the context of systemic disease. These deficiencies could be associated with synthesis of capsular polysaccharide, cell wall maintenance, or a combination of both. Alternatively, the amount of capsule production observed when grown in laboratory medium does not correlate to that produced in whole blood.
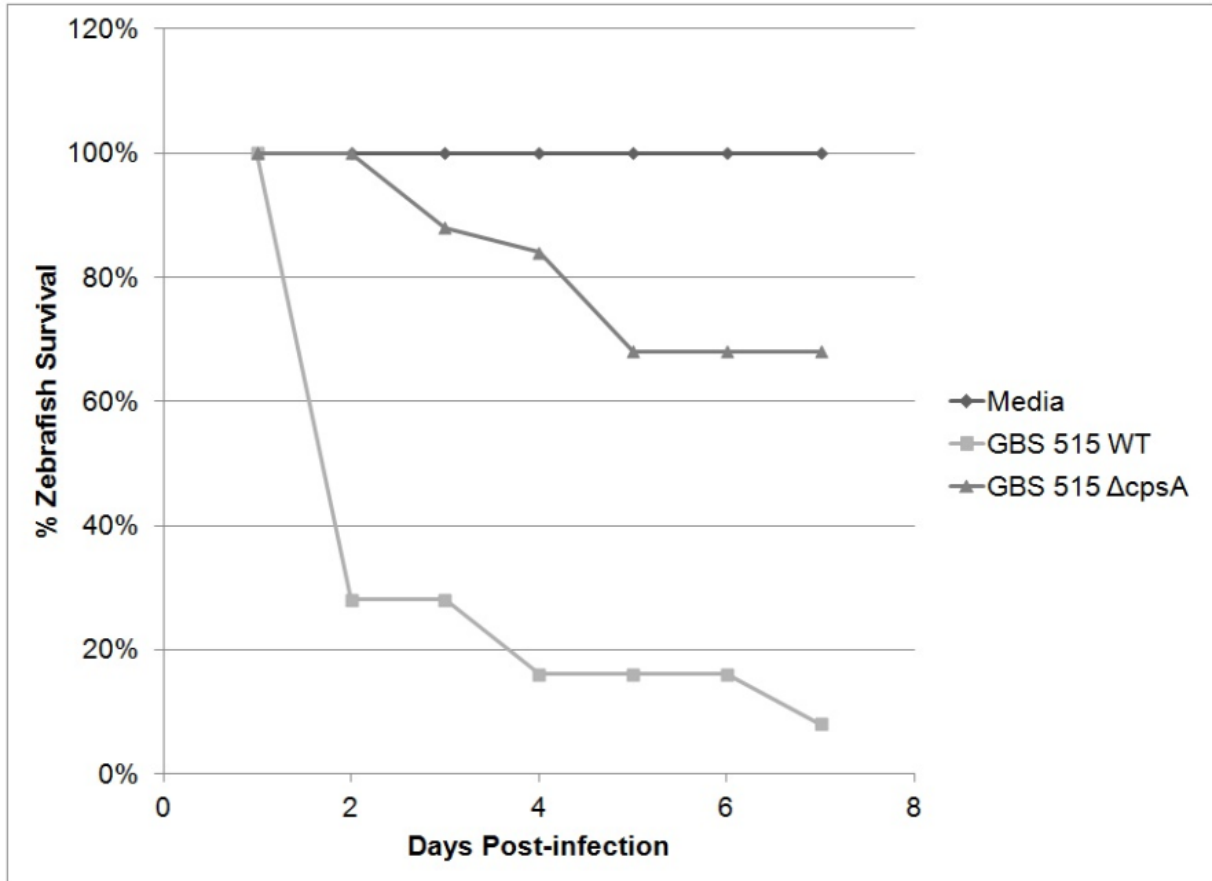
Figure 31: Zebrafish infection study tracking survival over time for zebrafish infected intramuscularly with either the GBS 515 WT or Δ*cpsA* strain and compared to media only mock infection.

CpsA affects chain length distribution. The chain length of cocci adopted by streptococcal species depends on a number of conditions, not all of which have been determined. Chain length is often controlled by production of cell wall amidases or other autolysins (24), but the presence of capsule has also been shown to affect chain length as well (8, 9), with the presence of capsule generally leading to longer chains. CpsA is a member of the same family of proteins as LytR, a group of proteins associated with attenuating expression of cell wall modifying enzymes (74), and various domains of CpsA also influence capsule levels (Fig. 25). Therefore, the effect of these associations on chain length in GBS was analyzed using microscopy. Despite a small relative difference in capsule level, the Δ*cpsA* mutant with vector alone produced considerably longer chains than the WT GBS strain with vector alone (Fig. 32). Complementation of the Δ*cpsA* strain with either MBP-CpsA-full or MBP-CpsA-117, both of which increased capsule, showed a shift to shorter chains, but did not fully restore the WT shorter chain phenotype (Fig. 32). Furthermore, the expression of the LytR deletion construct, MBP-CpsA-245, in the WT strain, which greatly decreased capsule, led to markedly longer chains than the WT strain (Fig. 32), while the Δ*cpsA* strain expressing MBP-CpsA-245 maintained the long chain phenotype (Fig. 32).

To confirm the microscopy observations, the number of cells per chain for each strain was calculated as described in the Materials and Methods, verifying the prevalence of short chains in the WT/vector strain with 1-2 cells per chain predominating at 70% of chains (Fig. 33A) as well as a preponderance of long chains for the Δ*cpsA*/vector strain with greater than 10 cells per chain the most numerous population at 45% of chains (Fig. 33B). Addition of MBP-CpsA-full to the WT background did not substantially alter chain length as short chains were still favored with 1-2 cells per chain at 66% (Fig. 33A). Addition of MBP-CpsA-full to the Δ*cpsA*

background did not fully alleviate the long chain phenotype, with greater than 10 cells and 3-4 cells per chain representing the largest populations at 27% and 26% respectively (Fig. 33B). When the LytR deletion strain, MBP-CpsA-245 was expressed in the WT background, the long chain phenotype predominated with the majority of chains showing greater than 10 cells per chain at 83% (Fig. 33A), while the presence of MBP-CpsA-245 in the Δ*cpsA* parent strain seemed to exacerbate the long chain phenotype with greater than 10 cells per chain predominating at 59% (Fig. 33B). Expression of MBP-CpsA-117 in the WT strain did not change the WT chain phenotype (Fig. 33A). Similar to MBP-CpsA-Full, expression of MBP-CpsA-117 did not fully alleviate the long chain phenotype of the Δ*cpsA* strain, with approximately equal chains at greater than 10 cells per chain (27%) and 3-4 cells per chain (28%) (Fig. 33B). These results suggest that CpsA either directly or indirectly influences chain length and that capsule level alone is not sufficient to explain chain length variance in these circumstances.

To determine if cell wall related factors were primarily responsible for the observed variances in chain lengths, each GBS strain above was cultured in sub-inhibitory concentrations of lysozyme. Lysozyme has muramidase activity and cleaves *N*-acetyl-D-glucosamine residues of the peptidoglycan cell wall. When grown in the presence of lysozyme, all strains existed almost exclusively as diplococci or single cells (Fig. 34), indicating that CpsA-dependent changes in the cell wall or associated enzymes may be responsible for the observed chain length variances.

In an attempt to determine the mechanism by which CpsA alters the cell wall architecture, a number of cell wall active antimicrobial agents were employed, including lysozyme, bacitracin, and ampicillin. In contrast to what was observed for *S. iniae* (Table 3),

parent strains of GBS, and GBS with MBP-CpsA constructs, did not demonstrate a difference in antimicrobial sensitivity for any of the agents tested, (Table 3), and data not shown respectively. This may be reflective of species-specific differences with regard to the macromolecular structure of the cell surface or the impact of relative capsule levels on the activity of these agents.
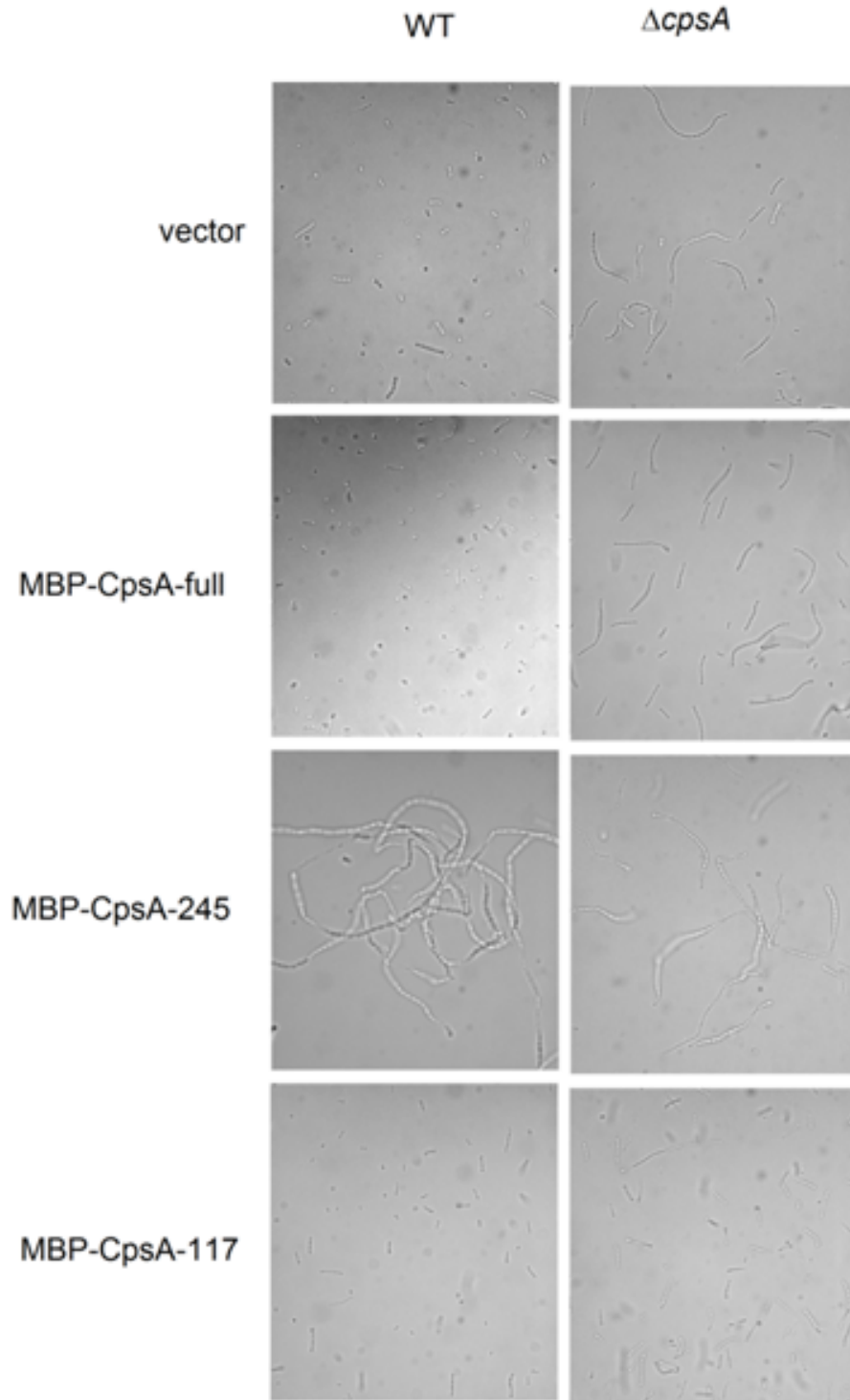
Figure 32: Visualization of chain length at 1000X magnification for GBS 515 WT and Δ*cpsA* strains, carrying the plasmid vector, MBP-CpsA-full, MBP-CpsA-245, or MBP-CpsA-117 as indicated on the top of the panel.
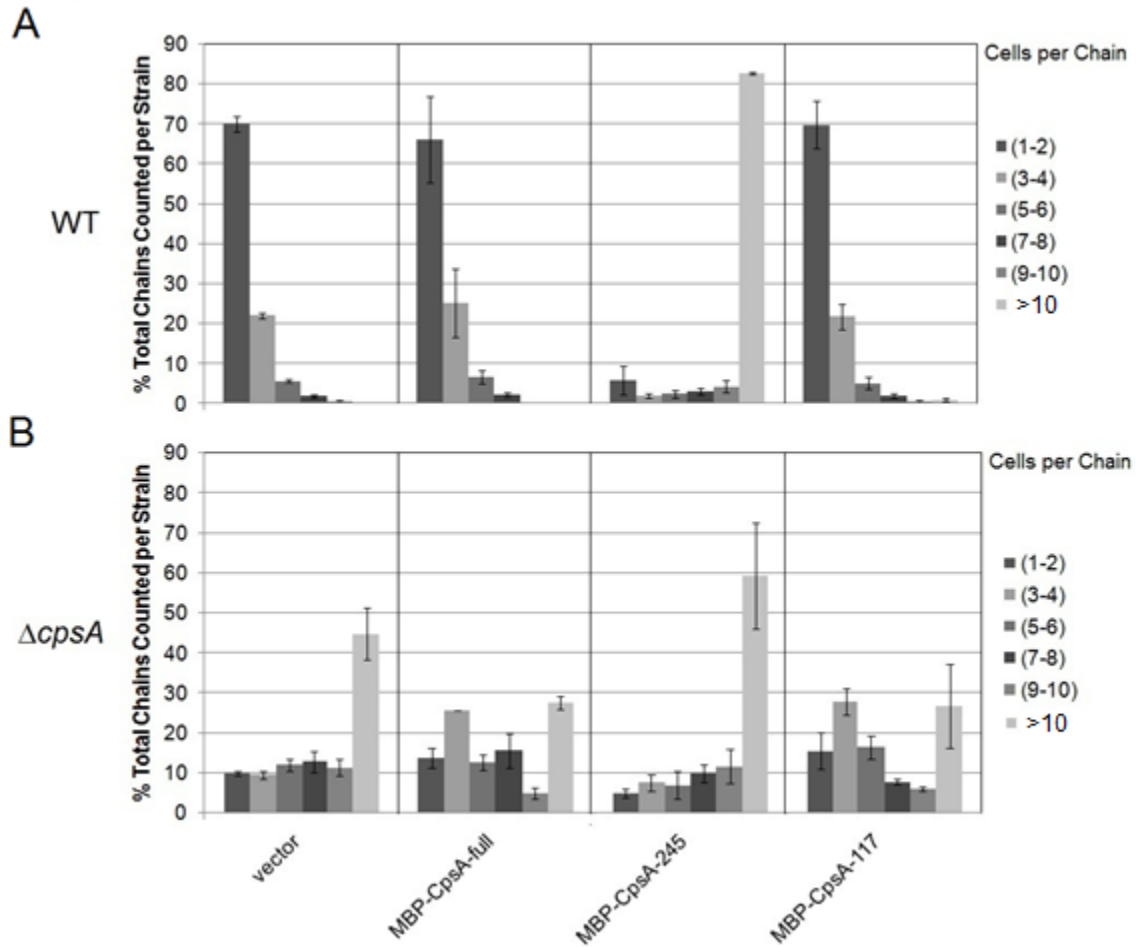
Figure 33: Quantification of chain length for parent strains of GBS 515, (A) WT and (B) Δ*cpsA*, carrying the plasmid vector, MBP-CpsA-full, MBP-CpsA-245, or MBP-CpsA-117 as indicated on the bottom of the panel.
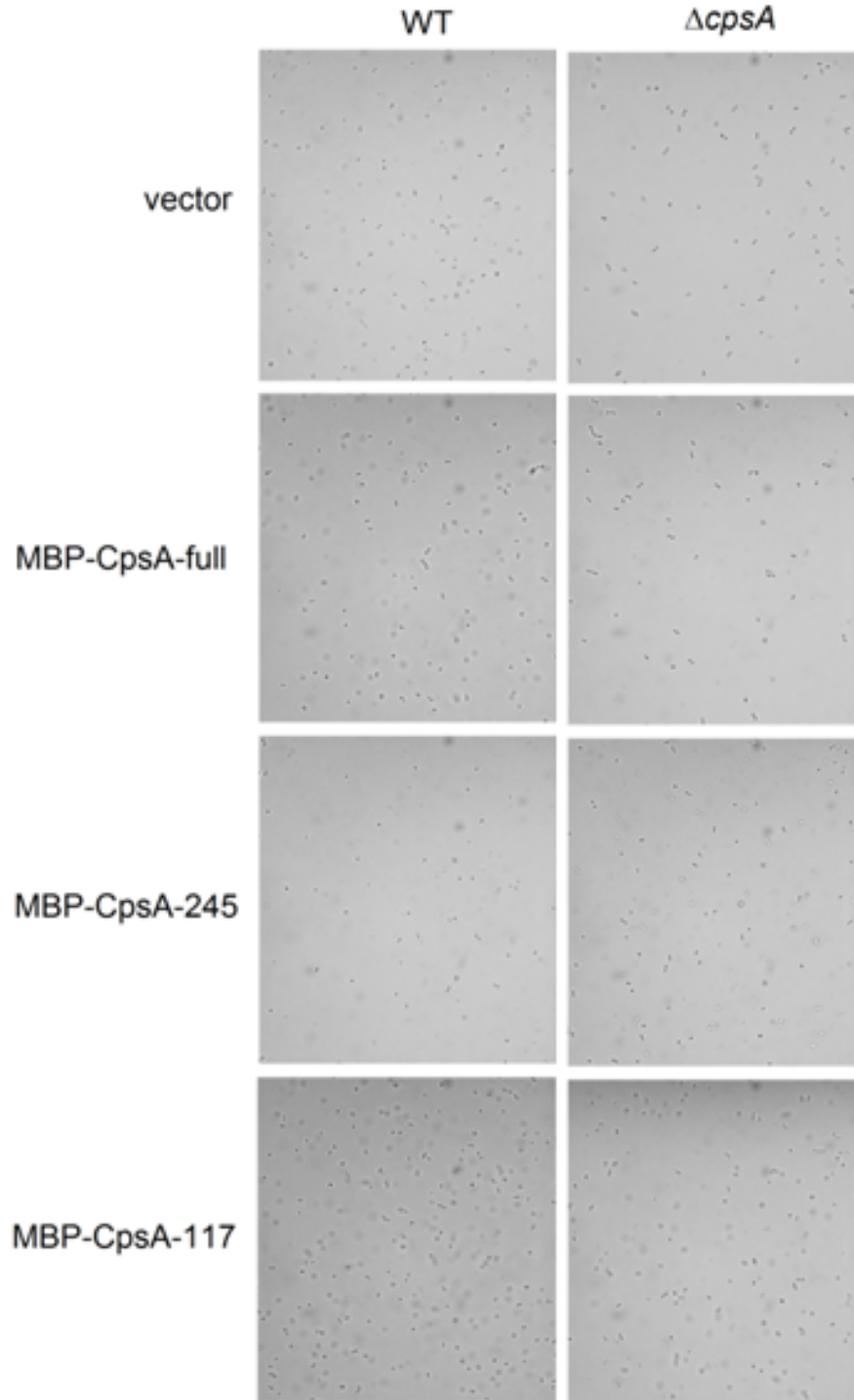
Figure 34: Visualization of chain length at 1000X magnification for GBS 515 WT and Δ*cpsA* strains, carrying the plasmid vector, MBP-CpsA-full, MBP-CpsA-245, or MBP-CpsA-117 as indicated on the top of the panel when grown in the presence of a sub-inhibitory concentration of lysozyme.

Table 3: Antimicrobial minimum inhibitory concentrations for *S. iniae* and GBS strains grown under conditions given in Materials and Methods.

	Lysozyme	Bacitracin	Ampicillin	Methicillin
S. iniae WT	<12 mg/mL	1.2 µg/mL	0.078 µg/mL	1.05 µg/mL
S. iniae cpsA-ins	>48 mg/mL	3.0 µg/mL	0.039 µg/mL	0.45 µ/mL
GBS WT	2.6 mg/mL	0.075 µg/mL	0.075 µg/mL	----
GBS Δ*cpsA*	2.6 mg/mL	0.075 µg/mL	0.075 µg/mL	----

DISCUSSION

Streptococcal pathogens capable of causing systemic disease remain a major health concern worldwide, and new strategies are currently being utilized to identify and exploit novel vaccine and antimicrobial targets (113, 150). The streptococcal CpsA protein is part of the LytR_cpsA_psr family of proteins associated with regulatory control over cell surface physiology, including polysaccharide synthesis (27), cell wall processing (24, 62, 74), and response to antimicrobial stress (152). The involvement of this protein family with these important virulence determinants highlights its potential as a possible target for virulence reduction, increased clearance by host immune function or antimicrobial therapy. Therefore, our aim was to characterize the functional properties of CpsA to better understand the role it may play during initiation and perpetuation of disease.

The streptococcal CpsA protein has been identified as a putative regulatory activator of capsule, with an in-frame deletion of *cpsA* resulting in reduced levels of transcript from the capsule operon as well as a concomitant loss in capsule level in GBS (27). Work on a strain of the aquatic pathogen *S. iniae* in which the *cpsA* gene was insertionally inactivated demonstrated that interruption of *cpsA* led to significant attenuation in a zebrafish model of infectious disease when compared to the WT *S. iniae* strain (79). This evidence suggests that CpsA is required for full virulence as a positive regulator of capsule, and that this may occur through direct interaction with DNA to facilitate transcriptional changes.

CpsA proteins effectively contain three discrete domains: a cytoplasmic N-terminal DNA-binding domain, an extracellular DNA_PPF domain, and an extracellular C-terminal LytR domain (54). The N-terminal DNA-binding domain is conserved in other streptococcal CpsA

proteins and contains a possible lecuine zipper motif, which may facilitate homo- or hetero-dimerization and DNA binding ability (18). The DNA_PPF domain function is canonically ascribed to sliding clamp structures that enhance the rate of DNA replication through association with DNA polymerases (97, 138). However, the protein sequence of the DNA_PPF domain of CpsA diverges considerably from that of traditional sliding clamp DNA_PPF domains, suggesting a different function for the DNA_PPF domain in the CpsA protein. This is also supported by the observation that the DNA_PPF domain of CpsA resides extracellularly where it would be unable to participate in DNA replication (6). Sliding clamps that contain the DNA_PPF domain typically participate in a number of protein-protein interactions that contribute to their function (138), and although it appears that the DNA_PPF domain of CpsA has been functionally redirected from traditional sliding clamps, it may be that the ability for facilitating protein-protein interactions has been retained.

The LytR domain of CpsA demonstrates a relatively high level of homology to traditional LytR proteins of Gram-positive species with a comparison of the GBS CpsA LytR domain and the *B. subtilis* LytR protein showing 38% identity and 58% similarity at the amino acid level. LytR proteins of Gram-positive species are generally associated with regulation of cell wall maintenance through transcriptional attenuation of autolysin genes, as well as their own sequence (74). Analysis of the LytR protein from *Streptococcus pneumoniae* demonstrated that LytR is required for normal septum formation during cell division (7). *lytR* null mutants divide non-symmetrically and have highly variable cell shape and size, with *lytR* mutants sometimes demonstrating much larger cell size (62). Similar results were reported for the LytR protein of *Streptococcus mutans*, with a *lytR* null mutant exhibiting cell division defects, including the production of significantly longer chains of bacteria (24). These reports are consistent with our

results with the CpsA truncation in which the LytR domain has been removed (MBP-CpsA-245). When this construct is expressed alone (in the ΔcpsA strain) or along with the WT CpsA strain, consistently longer chain lengths are observed. In addition, this construct produces cocci that are noticeably larger in size than the WT strain expressing full length CpsA. Increased autolysin production was also observed for the S. mutans *lytR* mutant, suggestive of a transcriptional attenuator role for LytR over autolysin genes (24). The homology between CpsA and LytR proteins indicates that some functional overlap may be present, and that in addition to regulation of capsule, CpsA may also contribute to regulation of cell wall maintenance. Evidence to support this hypothesis is found in *Staphylococcus aureus* where LytR_cpsA_psr family members have been shown to exhibit functional redundancy (110).

Members of the LytR_cpsA_psr family of proteins have generally been associated with a regulatory role at the transcriptional level, and it may be that these proteins are capable of binding directly to DNA to modulate transcription. Because CpsA has been associated with transcriptional activation of the capsule operon, our aim was to assess the ability of GBS CpsA to bind to two putative promoter sequences within the GBS capsule operon. A series of truncated CpsA proteins were constructed to analyze the contribution of different domains of the protein to DNA binding ability and specificity. Using EMSA, we found that the full length GBS CpsA protein was capable of binding to both the *cpsA* and *cpsE* promoters with specificity. Furthermore, we determined that the CpsA protein had the same apparent affinity for both the *cpsA* and *cpsE* promoters. Removal of the large extracellular portion of the CpsA protein immediately after the third transmembrane domain did not preclude CpsA from binding to the *cpsA* and *cpsE* promoters, but a decrease in both the specificity and affinity of binding to both promoters was observed. When the CpsA protein was truncated to a region encompassing the

cytoplasmic N-terminus and first transmembrane domain, binding to both capsule operon promoters was still observed, however all apparent specificity was abolished.

These results indicate that GBS CpsA is able to bind directly to both the *cpsA* and *cpsE* promoters of the capsule operon, perhaps providing a mechanism for the transcriptional changes associated with deletion or interruption of the *cpsA* gene. The observation that specificity of DNA binding decreases with sequential truncation from the C-terminus of the protein suggests that both the large extracellular region of the protein and the cytoplasmic loop between transmembrane domains 2 and 3 likely contribute to specificity either structurally or through direct interaction, much like what has been demonstrated with *S. iniae* CpsA (54). The observed DNA binding ability for GBS MBP-CpsA-39 is in contrast to what was found with *S. iniae* CpsA, in which truncation to the cytoplasmic N-terminus abolished DNA binding ability (54). This discrepancy could be explained by the inclusion of the first transmembrane domain for GBS MBP-CpsA-39 (including the putative leucine zipper), which was removed from the comparable *S. iniae* form of the protein. Another possibility is that differences in sequence between the GBS and the *S. iniae* proteins and promoter DNA may result in different functional interactions between CpsA and capsule promoter DNA in each species. At present, it is unclear what the relative contributions of the *cpsA* and *cpsE* promoters are to eventual capsule levels, or how they may be differentially regulated. Also unclear is whether the internal *cpsE* promoter exists in the capsule operon of other streptococcal systemic pathogens.

After determining that GBS CpsA is able to bind directly to the capsule operon promoters with specificity *in vitro*, we proceeded to analyze the function of different domains of CpsA when ectopically expressed in either a WT or Δ*cpsA* GBS background. To do this, we truncated CpsA to remove the LytR domain (MBP-CpsA-245) and both the LytR and DNA_PPF domain

(MBP-CpsA-117) and expressed these forms of CpsA, as well as the full length CpsA (MBP-CpsA-full) and the vector plasmid in both parent GBS backgrounds. When each of these strains was assayed for capsule level with percoll buoyant density gradients, we found that the ΔcpsA/vector strain produced less capsule than WT/vector, and that both parent strains could be induced to produce higher levels of capsule when either full length CpsA (MBP-CpsA-full) or just the DNA-binding domain of CpsA (MBP-CpsA-117) was ectopically expressed. These results support the hypothesis that CpsA is an activator of capsule, and that it does this by binding to the capsule operon promoter, as the DNA-binding domain of CpsA was sufficient for complementation of capsule levels in a ΔcpsA background. However, analyses of these constructs in the context of capsule production during different growth phases revealed that while constitutive expression of MBP-CpsA-full or MBP-CpsA-117 modulates capsule levels, these constructs do so in a growth phase dependent manner. A similar result was observed for the ΔcpsA parent strain, albeit with reduced capsule levels, indicating that although CpsA appears to be a transcriptional activator of capsule, other regulatory components must contribute to actual capsule levels observed at different time points. One possibility would include the CpsB, CpsC, and CpsD phosphor-relay system, which controls polymerization and export of the CPS.

In contrast to results obtained for MBP-CpsA-full and MBP-CpsA-117, the production of CpsA lacking the LytR domain (MBP-CpsA-245) resulted in a decrease in capsule for both the WT and the ΔcpsA parent strains. The presence of the DNA-binding domain was not the cause of decreased capsule, because as mentioned above, expression of this domain alone had an activating effect. This indicated that the DNA_PPF domain was responsible for the decrease in capsule levels, which could be due to a dominant negative function that is adopted by CpsA in

the absence of the LytR domain, perhaps through inappropriate protein-protein interactions. Another possibility is that the DNA_PPF normally represses capsule through the DNA-binding domain by a change in protein conformation, and that the LytR domain controls this repressive mechanism. With either option, because of the extracellular location of the DNA_PPF domain, the repression of capsule is likely facilitated through either a protein-protein interaction or an induced conformational change. While MBP-CpsA-245 led to decreased capsule levels, time course experiments revealed that capsule level still retained a growth phase dependency, again suggesting the contribution of other regulatory components in controlling capsule levels as a function of growth phase.

During initiation of systemic disease, GBS typically disseminates from the genitourinary tract of the mother to the bloodstream of the neonate, an event that causes GBS to switch from a colonizing role to a pathogenic role. This switch likely entails the integration of a number of environmental signals that trigger virulence pathways enabling survival in the host bloodstream. The genitourinary tract is typically defined as an acidic environment, while the circulatory system consists of a neutral pH, and this change in pH may act to signal the activation of virulence cascades, including production of a polysaccharide capsule. The large extracellular domains of CpsA may contribute to environmental sensing function to adopt a pathogenic profile, so alterations in capsule level were monitored in the WT and Δ*cpsA* GBS strains over a range of pH conditions. We determined that there was a responsiveness of capsule to differing pH values, with acidic pH leading to less capsule and alkaline pH leading to more capsule, but this change was not dependent on CpsA, as differing pH values elicited the same phenotype in the Δ*cpsA* strain.

When GBS transitions to the bloodstream to cause systemic disease, bacteria encounter a number of small molecules that are not present in a colonization setting. GBS has previously demonstrated an increase in capsule in response to human serum (119), so we set out to determine whether this increase was dependent upon the presence of CpsA. An increase in capsule was observed for WT GBS after incubation in human serum, confirming previous reports, and the same increase was also observed for the Δ*cpsA* strain, indicating that the responsiveness of capsule to human serum is independent of CpsA. Changes in capsule with regard to pH value and exposure to human serum appear to be regulated in a manner independent of CpsA, and this may be achieved through the CpsB, CpsC, and CpsD phospho-relay system.

The production of a polysaccharide capsule allows GBS to evade immune clearance upon introduction to the host bloodstream during initiation of infection. Therefore, we incubated the above strains in human whole blood to assess how differing levels of capsule due to manipulation of CpsA affect the ability of the bacteria to survive. Despite the production of less capsule in the Δ*cpsA* background, we observed no major difference in survival when compared to the WT strain. Additionally, production of the full length CpsA (MBP-CpsA-full) or the DNA-binding domain (MBP-CpsA-117) in the WT background did not alter the ability of the WT parent to survive in human blood (data not shown). Surprisingly, when MBP-CpsA-full or MBP-CpsA-117 was produced in the Δ*cpsA* background, a decrease in survival was observed compared to the parent strain alone, despite the presence of more capsule as measured by buoyant density centrifugation. Production of CpsA lacking the LytR domain (MBP-CpsA-245) also led to a decrease in survival in both parent strains. These results demonstrate that survival in human whole blood does not always correlate with levels of capsule, and suggest that there is another CpsA- dependent mechanism responsible for the discrepancy in survival when

comparing the WT and Δ*cpsA* strains with either MBP-CpsA-full or MBP-CpsA-117. Therefore, at this time it is not possible to discern whether the greater attenuation associated with both parent strains expressing the MBP-CpsA-245 form of the protein lacking the LytR domain is due to reduced capsule levels or some other property of the bacteria, perhaps associated with the cell wall, or a combination thereof. Supporting this hypothesis is the observation that the GBS Δ*cpsA* strain was attenuated for virulence in the zebrafish model of infectious disease when compared to the WT strain. Although the Δ*cpsA* strain was not attenuated in human whole blood, a regulatory role for CpsA appears to exist in the context of systemic disease. The discrepancy between these two observations may be due to an amplification of the capsule defect phenotype during systemic disease and its absolute requirement, or it may be due to pleiotropic effects that are not apparent during incubation in blood, but affect the ability of GBS to survive or disseminate within a host organism.

The LytR proteins of streptococci have been associated with regulating cell division, including septum formation, cell size, and chain length (24, 62). The homology of CpsA to LytR proteins led us to investigate if any of these phenotypes existed in either a Δ*cpsA* background, or with the addition of different domains of CpsA. We determined that deletion of *cpsA* resulted in decidedly longer chains of cocci when viewed microscopically and when quantified, compared to the WT parent strain. Production of MBP-CpsA-full or MBP-CpsA-117 did not affect chain length of the WT parent strain, but did partially restore a short chain phenotype to the Δ*cpsA* strain. Similar to what was observed for capsule levels, production of CpsA lacking the LytR domain (MBP-CpsA-245) resulted in a shift to an increased amount of long chains in both the WT and, to a lesser extent, the Δ*cpsA* background. The decreased extent of both the capsule and chain length phenotypes in the Δ*cpsA* background may be reflective of protein-protein

interactions that occur in the WT strain between WT CpsA and MBP-CpsA-245 that enhances the degree to which these phenotypes are exhibited. Interestingly, these results also indicate that capsule level does not necessarily correlate with chain length, at least in GBS, as Δ*cpsA* strains harboring MBP-CpsA-full and MBP-CpsA-117 produce more capsule than WT, but retain a higher proportion of long chains than the WT strain. Additionally, the observation that encapsulation leads to longer chains in *S. pneumoniae* (8, 9) is not consistent with our observation that the Δ*cpsA* strain, as well as both parent strains producing MBP-CpsA-245, produce less capsule than WT with vector alone, yet produce primarily much longer chains than the WT/vector strain.

Interestingly, CpsA appears to exert either a direct or an indirect control over chain length, suggesting a possible dual role for this protein that is separate from the control of capsule expression. Additionally, we observed that the WT strain with MBP-CpsA-245 appeared to produce larger sized cells compared to other strains, however, further characterization of these strains at higher magnification levels using electron microscopy would be required to better discern differences in septum placement and actual cell size and shape. We propose that CpsA-dependent changes in cell wall maintenance are responsible for the observed differences in chain length, as growth in medium with a sub-inhibitory concentration of lysozyme, which selectively cleaves peptidoglycan of the cell wall, results in eradication of long chains for all strains and a switch of nearly all bacteria to single cells or diplococci. A number of cell wall active antimicrobial agents were used to investigate molecular changes that may be associated with the cell surface that contribute to changes in chain length, however, no difference in resistance was observed for either the GBS parent strains or GBS with the various forms of MBP-CpsA.

Testing of these strains with additional cell wall reactive agents may help elucidate how CpsA contributes to regulation of the cell wall.

Taken together, the results of this study suggest that GBS CpsA is a modular protein containing three functionally distinct domains. The N-terminal region of GBS CpsA is able to bind to promoters within the capsule operon upstream of both the *cpsA* and *cpsE* genes, and expression of the N-terminal region alone (MBP-CpsA-117) in both the WT and Δ*cpsA* strains is sufficient to increase capsule levels, and to partially complement the Δ*cpsA* strain chain length distribution. This seems to indicate that despite the reduction in DNA-binding specificity of MBP-CpsA-117 when used alone in EMSAs, a tangible effect can still be incurred *in vivo*, possibly through binding of the capsule operon promoters and potentially other gene targets that regulate chain length. The second module of CpsA, the DNA_PPF domain, may be responsible for facilitating protein-protein interactions, a function that could be regulated by the LytR domain as removal of the LytR domain results in dominant-negative or repressive function for both capsule level and chain length. To date, no mechanistic function has been applied to the LytR domain in either CpsA or LytR proteins, but it appears that the LytR domain may play a similar role in both proteins as similar phenotypes of increased chain length and size are observed when it is removed from CpsA, both of which are seemingly independent of capsule level. This suggests that CpsA may also act as a transcriptional attenuator of autolysin genes, which would give it a unique role at the interface of regulating the two major components of the bacterial cell surface, capsular polysaccharide and the cell wall peptidoglycan.

GENERAL CONCLUSIONS

The primary goal of the work presented here consisted of characterizing the role of the streptococcal CpsA protein in regulation of capsule synthesis and cell wall maintenance, and associated contributions to virulence. This was accomplished through molecular analysis of the CpsA protein with regard to capsule production and cell wall stability, and subsequent analyses of virulence using *in vitro*, *ex vivo*, and *in vivo* models of pathogenesis.

The first chapter focuses on the regulatory role played by CpsA in the aquatic pathogen *Streptococcus iniae*. Previous work in the lab demonstrated the importance of CpsA during *in vivo* infection of the zebrafish model host (79). Other work had already established that CpsA was likely a transcriptional activator of capsule synthesis (27), so the attenuation of a *S. iniae cpsA*-ins mutant in the zebrafish model was hypothesized to be due to a deficiency in capsule synthesis (79). Therefore, we began the work in this chapter with the hypothesis that CpsA adopted a unique membrane topology enabling it to bind directly to the promoter of the capsule operon to influence transcription, and that the conserved domains of CpsA contributed to regulation of this activity. Previous work with the *S. iniae cpsA*-ins mutant also established that this strain produced considerably longer chains of cocci than the wild type strain (79). Subsequently, we hypothesized that CpsA may also exert regulatory control over cell wall related processes as well.

To confirm the membrane topology adopted by CpsA, reporter enzyme fusions were made to each distinct topological portion of the protein and these constructs assayed for enzymatic activity. This was accomplished with in-frame fusion of either β-galactosidase or alkaline phosphatase to different regions of CpsA. In this scheme, protein fusions to β-

galactosidase give enzymatic activity only if the region fused to β-galactosidase remains intracellular, as transport across the membrane disrupts the multimeric structure required for enzymatic activity (83, 85). In contrast, protein fusions to alkaline phosphatase give enzymatic activity only if the region fused to alkaline phosphatase is transported across the membrane to an extracellular location, as transport across the membrane is required for formation of a critical disulfide bond needed for enzymatic activity (84). Using this strategy, we confirmed that the membrane topology of CpsA includes a short cytoplasmic N-terminal tail, a transmembrane domain, a short extracellular loop, a second transmembrane domain, a short cytoplasmic loop, a third transmembrane domain, and a large extracellular C-terminus (54). This determination is consistent with previous reports that a *S. aureus* LytR family protein exhibited a short cytoplasmic N-terminus and large extracellular C-terminus (58). In the context of transcriptional regulation, the membrane topology adopted by CpsA and other LytR family proteins suggests that the large extracellular domains likely influence its regulation in some way, perhaps by binding to small molecule ligands in a regulatory capacity, or mediating protein-protein interactions that contribute to regulation of capsule. This would be similar to what has been observed in *V. cholerae* with the proteins ToxR and ToxS, where protein-protein interactions in the periplasm contribute to transcriptional regulation (35, 116). Additionally, the proximity of the extracellular domains of CpsA to the capsular polysaccharide residues on the cell surface may represent a sensory feedback loop that alters the function of CpsA.

The membrane orientation of CpsA implied that if CpsA were capable of binding to the capsule operon promoter, then this activity would have to be facilitated by the small cytoplasmic N-terminal tail and cytoplasmic loop. To assess the ability of *S. iniae* CpsA to bind directly to the capsule operon promoter, a number of protein constructs were purified, representing the full

length CpsA protein as well as sequential truncations from the C-terminal end that removed cytoplasmic portions of the N-terminus. Using these purified proteins in electromobility shift assays (EMSA), we demonstrated that full length CpsA was capable of binding to the *cpsA* promoter DNA, and that this interaction required the entire intact cytoplasmic portion of the protein (54). These results, in conjunction with previous evidence, strongly suggest that CpsA is a transcriptional activator of capsule synthesis, and that CpsA does this by directly interacting with promoter DNA. As mentioned above, this activity may be modulated through a number of mechanisms that include protein-protein interactions or environmental sensing facilitated by the extracellular domains of CpsA.

To analyze the role of the conserved DNA_PPF and LytR domains, located on the extracellular C-terminus of the CpsA protein, truncations were made that removed the LytR domain (CpsA-ΔLytR), or removed both the LytR and N-terminal domains (CpsA-DNA_PPF). Due to our inability to generate a Δ*cpsA* strain of *S. iniae*, or complement the *S. iniae cpsA*-ins mutant, both of these constructs were ectopically expressed in the WT *S. iniae* background and assessed for changes in capsule level. Surprisingly, we found that production of either CpsA-ΔLytR or CpsA-DNA_PPF in the WT background led to a significant decrease in capsule, to levels comparable to what was observed for the *cpsA*-ins mutant. These results signified that in the absence of the LytR domain, the DNA_PPF domain manifests either a dominant negative or repressive phenotype. This likely reflects a dysregulated protein-protein interaction, as the DNA_PPF and LytR domains are located extracellularly, and this phenotype happens in the presence of WT CpsA. These observations also explain our inability to complement the *cpsA*-ins mutant, as it is effectively producing a truncated form of CpsA similar to the CpsA- ΔLytR construct. These observations are also suggestive of a more nuanced regulatory role for CpsA

than simple activation, and indicate that CpsA may modulate transcription in both activating and repressing roles, depending on input from the LytR domain. In addition to modulation of capsule levels, the *cpsA*-ins mutant and WT with CpsA- ΔLytR both exhibit increased cocci chain length as well. This determination suggests that CpsA also contributes to regulation of cell wall related components as well. Further evidence of this relationship consists of changes in resistance to cell wall active antimicrobial agents for the *cpsA*-ins and WT/CpsA-ΔLytR strains. Both lysozyme and bacitracin resistance were increased for the *cpsA*-ins and WT/CpsA-ΔLytR strains, and β-lactam resistance decreased for both strains. The extent to which lysozyme resistance increased suggests specific modifications to the peptidoglycan backbone, which confer lysozyme resistance (31). Bacitracin resistance may have increased as an indirect result of increased free Undecaprenyl-phosphate (Und-P) carrier associated with decreased usage of Und-P for capsular polysaccharides. The cause for increased susceptibility to β-lactams for both of these strains is unclear, but may be due to the increased levels of autolysis observed for these strains. Taken together, the data suggest that manipulation of CpsA results in changes to the molecular structure of the cell wall, resulting in pleiotropic effects that may be directly, or indirectly, related to regulation by CpsA. The extent to which these phenotypes are influenced by the relative abundance or absence of capsule remains unclear and an isogenic acapsular mutant of *S. iniae* is needed to determine the relative contribution of CpsA or capsule to these phenotypes. However, these observations do support the idea that CpsA contributes significantly to regulation of the cell surface, and that cell surface derived molecules may influence this regulatory capacity.

The second chapter focuses on the conservation of CpsA function between *S. iniae* and *Streptococcus agalactiae* (GBS), by analyzing the molecular properties of GBS CpsA and its

contribution to regulation of capsule and cell wall. Although the *S. iniae* and GBS CpsA proteins demonstrate 70% similarity at the amino acid level, the cytoplasmic N-terminal regions of CpsA from these two species share no significant homology. Therefore, it was important to confirm our findings with *S. iniae* in GBS. Additionally, many cell surface related structures and associated regulation can be species specific, again necessitating confirmatory analyses.

Assessment of the ability of GBS CpsA to bind to DNA followed a similar methodology as that utilized for *S. iniae*. However, the GBS capsule operon contains two demonstrated transcriptional start sites (171), upstream of the *cpsA* gene and upstream of the *cpsE* gene. Therefore, our analyses included DNA constituting both putative promoter elements. Using EMSA, we demonstrated that full length GBS CpsA was capable of binding specifically to both promoters, and did not show a preference for either the *cpsA* or *cpsE* promoter to the exclusion of the other. Truncated CpsA constituting just the N-terminal portion of the protein was still able to bind to both promoters, but at slightly reduced specificity. Truncation of CpsA all the way to the N-terminal region of the protein encoding a putative leucine zipper domain did not preclude DNA-binding, but did ablate all semblance of specificity. These results suggest that the entire protein is required for full specificity, but only the putative leucine zipper motif is required for DNA interaction. The presence of a second transcriptional start site in the capsule operon of other streptococcal species has not been verified, but preliminary data suggests that a second promoter element exists in the same relative position within the *S. iniae* capsule operon (B. Hanson and M. Neely, unpublished data). Further analyses of the capsule operon of other streptococcal species is required to verify if a second promoter is species specific, or applies to a broad range of streptococcal species encoding a capsule operon.

The presence of a secondary transcriptional start site preceding the *cpsE* gene indicates that regulation of capsule may be influenced by the genes succeeding each putative promoter element. The *cpsA* promoter contributes to a transcript that includes the entire capsule operon, including the phospho-relay system that has been described in detail (171), suggesting that it serves exclusively as an "on" switch for capsule expression. The *cpsE* gene product catalyzes the initial polymerization event required for capsule production, and thus represents the first committed step in capsule synthesis. Therefore, the *cpsE* promoter may represent an additional layer of regulation, and CpsA may act to enhance or repress activation from this promoter to influence a commitment to capsule production. Another possibility is that post-translational modifications are made to CpsA that influence its relative capacity to regulate transcriptional activation or repression at either of these promoters. Additionally, the presence of a putative leucine zipper domain has mechanistic implications for how CpsA regulates capsule expression, and needs to be verified through targeted mutational analysis. The presence of a functional leucine zipper domain might explain the ability of a small portion of the CpsA protein to bind to DNA, but would also suggest that CpsA can form hetero- or homo-dimers to modulate DNA-binding activity and subsequent expression at either the *cpsA* or *cpsE* promoters. Co-immunoprecipitation experiments are currently in progress to identify any protein-protein interactions that occur with full length CpsA.

Functional analysis of the GBS CpsA protein utilized truncated forms of CpsA that were ectopically expressed in either a WT or Δ*cpsA* GBS background. Using this method, we determined that ectopic expression of full length GBS CpsA (MBP-CpsA-full) resulted in increased capsule levels in both parent strains. Expression of just the N-terminal DNA-binding region of the protein in both parent strains (MBP-CpsA-117) was also sufficient for activating

capsule synthesis. As seen with *S. iniae*, removal of the LytR domain (MBP-CpsA-245) resulted in a dominant negative decrease in capsule levels, indicating repressive function for the DNA_PPF domain, or a dysregulated protein-protein interaction. Analysis of these constructs in the context of growth phase dependent regulation of capsule revealed that CpsA is not the primary activator of capsule synthesis, as growth phase dependency was unaltered, but rather seems to be a "dimmer-switch" for capsule, with ectopic expression of activating regions generally leading to slightly more capsule over time, and ectopic expression of the dominant negative form generally leading to less capsule over time. This indicates that CpsA may act to dynamically control capsule during a transition from commensalism to pathogenesis by selectively enhancing capsule synthesis, and may also modulate the switch from pathogen to commensal by selectively decreasing capsule synthesis.

The modulation of capsule by CpsA may in part require environmental signals within the host, but these signals remain undefined. We hypothesize that the modulatory capacity of the LytR domain may be dependent on sensing environmental signals within the host, however, we have demonstrated that changes in capsule due to growth phase, pH value, and human serum are all independent of CpsA. These observations do not rule out an environmental sensing function for the extracellular domains of CpsA, but do indicate that CpsA is not responsible for modulating capsule in response to the major host derived signals that are encountered during a switch from commensalism to pathogenesis. Therefore, it may be that the membrane localization of CpsA is indicative of an auto-sensory function for these domains, and the close proximity of the cell surface macromolecular structure and its modifications may serve as a signal to the extracellular domains of CpsA to modulate transcription of the capsule locus. Future studies to assess this consideration could utilize purified peptidoglyan-capsule complexes

to discern whether exposure to different components of the macromolecular cell surface induce changes to capsule level, and whether this is a CpsA dependent event. Additionally, quorum sensing mechanisms may contribute to regulation of capsule through CpsA, and an viable method to test this is to expose bacteria to supernatants from terminally grown cells and assess changes in capsule level and dependency on CpsA.

In addition to reduced capsule levels, parent strains of GBS ectopically expressing MBP-CpsA-245 also demonstrated a decreased ability to survive in human whole blood. Surprisingly, despite a reduction in capsule for the parent Δ*cpsA* strain, no reduction in survival was observed. However, infection of zebrafish with either the WT or Δ*cpsA* GBS strains revealed that the Δ*cpsA* strain was attenuated for virulence *in vivo*, indicating that the detrimental effects associated with disruption of CpsA may be more apparent in the context of systemic disease. Furthermore, these results also suggest that CpsA could be targeted with a small peptide or molecule in a therapeutic fashion, enhancing clearance by the host immune system. Plans are underway to identify small peptides that selectively inhibit the function of CpsA, either by interfering with the control exerted by the LytR domain, or by recapitulating the dominant negative effect observed with ectopic expression of CpsA-245 in the presence of WT CpsA.

As seen with *S. iniae*, disruption of *cpsA* in GBS resulted in a long chain phenotype, indicating that CpsA contributes to regulation of cell wall related components in GBS as well. However, probing this phenotype with a variety of cell wall active agents did not reveal any differences in resistance between the WT and Δ*cpsA* strains, or any derivative strains ectopically expressing various forms of CpsA. This is in contrast to what was observed for *S. iniae*, where lysozyme, baciatracin, and β-lactam resistances were altered. This could mean that there are species-specific differences with regard to the macromolecular structure of the cell surface, or

could signify that CpsA functions differently between these two species. Evidence from these studies favors the former conclusion, as CpsA appears to function almost identically for both *S. iniae* and GBS. Future studies will include a full assessment of the macromolecular structure of both species, which may shed light on relative differences in antimicrobial sensitivity as a function of disrupting CpsA.

In conclusion, the function of CpsA appears to be highly conserved amongst closely related streptococcal species, and includes DNA-binding and regulation of capsule and cell wall constituents that are required for full virulence. These studies suggest that CpsA effects transcriptional changes by directly binding to DNA, and that these changes may not be limited to transcriptional activation, but rather appear to be composed of nuanced changes that appropriately regulate increases and decreases in capsule level as the bacteria deem appropriate. Additionally, CpsA may act to coordinately regulate not only capsule, but the cell wall as well, and the relative contribution of cell wall changes that occur in the absence or disruption of CpsA to systemic disease is a primary target of future studies. The results presented here also demonstrate that CpsA can be targeted in a way that is detrimental to the pathogen, as ectopic expression of CpsA lacking the LytR domain results in less capsule and attenuation in human whole blood. This demonstrates that CpsA may be a viable target of future antimicrobial therapy.

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ABSTRACT

ANALYSIS OF THE STREPTOCOCCAL CPSA PROTEIN IN DNA-BINDING AND REGULATION OF CAPSULE AND CELL WALL MAINTENANCE

by

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Major: Immunology & Microbiology

Degree: Doctor of Philosophy

Streptococcus agalactiae (GBS) and *Streptococcus pneumoniae* remain a significant threat to human health worldwide. The ability of these organisms to cause systemic disease is compounded by the production of a polysaccharide capsule that provides immune evasion function. The production of the polysaccharide capsule in pathogenic streptococci is controlled in part by the membrane bound protein CpsA. These studies analyze the contribution of CpsA to regulation of capsule level in the model aquatic pathogen *Streptococcus iniae* and human pathogen GBS, and how this regulation affects virulence in *in-vitro*, *ex-vivo*, and *in-vivo* models of pathogenesis. We have determined that the membrane topology of the CpsA protein consists of a small cytoplasmic N-terminus, and a large extracellular C-terminus that contains the conserved DNA_PPF and LytR protein domains. The cytoplasmic N-terminal region in its entirety is capable of binding specifically to the capsule operon *cpsA* promoter in *S. iniae*, and to two putative promoter elements in GBS which include the capsule operon *cpsA* promoter and the internal *cpsE* promoter. Additionally, CpsA is a modular protein, with the cytoplasmic N-terminus as a capsule-activating domain, the DNA_PPF region as a capsule-repressing domain,

and the LytR region as a control domain that regulates the activities of the other two domains. CpsA also appears to regulate cell wall maintenance, as truncation or removal of CpsA results in longer chains of cocci in both *S. iniae* and *S. agalactiae*, a phenotype that is associated with altered antimicrobial resistance and autolysis activity in *S. iniae*. Taken together, CpsA contributes to the complex regulatory scheme controlling capsule and cell wall, the two major constituents of bacterial cell surface macromolecular structure, and does so in a way that influences pathogenesis during systemic disease. The insights gained through these studies indicates that CpsA can be targeted in a way that is detrimental to bacterial survival in the context of systemic disease, suggesting CpsA may be an important future target of antimicrobial therapy.

AUTOBIOGRAPHICAL STATEMENT

BRETT RICHARD HANSON

I was born in Boulder, Colorado, but grew up in Franklin, New York. I attended Rochester Institute of Technology in Rochester, New York, where I obtained a Bachelors degree in Biotechnology. During this time, I participated in undergraduate research under the guidance of Dr. Paul Craig, with work focused on the development of bioinformatic tools capable of predicting enzymatic function based on protein structure alone. This opportunity instilled in me a passion for research, and led me to pursue a graduate degree in Immunology & Microbiology at Wayne State University School of Medicine.

After spending approximately three and a half years in the lab of Dr. Melody Neely, I have successfully published a first author manuscript in Journal of Bacteriology. Additionally, I have submitted a review article to Current Opinion in Microbiology, and submitted a second first author manuscript to Journal of Bacteriology. My experience here has culminated in the reception of my doctorate in November 2011.

Prior to receiving my doctorate, I secured a postdoctoral fellowship in the lab of Dr. Ming Tan, studying transcriptional regulation in *Chlamydia trachomatis* at the University of California Irvine. I look forward to the opportunity to continue working on microbial pathogenesis, and expect to remain in the field.

While working on my graduate degree I married Nadean Leverock, and we adopted three cats. We look forward to moving to California, but will miss the Union Street Restaurant.