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Cell Envelope Modifications By Lcp Family Proteins Of Group B Streptococcus And Virulence Singaling By Peptides Of The Salivaricin Locus Of Streptococcus Pyogenes

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**CELL ENVELOPE MODIFICATIONS BY LCP FAMILY PROTEINS OF GROUP B
STREPTOCOCCUS AND VIRULENCE SIGNALING BY PEPTIDES OF THE
SALIVARICIN LOCUS OF STREPTOCOCCUS PYOGENES**

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

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Advisor

Date

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CHAPTER 1: GENERAL INTRODUCTION

Group B *Streptococcus*:

Group B *Streptococcus* (GBS), *Streptococcus agalactiae*, is a zoonotic pathogen. Initially identified as the causative agent of bovine mastitis, in the mid twentieth century (1) GBS became the leading cause of neonatal sepsis and meningitis in the developed world (2-6). GBS is commensal in 15-30% of healthy adults, normally as part of the vaginal and rectal microflora(7-9). Neonatal disease can occur via several routes. First, the bacteria can ascend to the uterus and placenta during pregnancy causing chorioamnitis (10, 11) and intrauterine infection of the fetus, which can lead to premature labor and delivery (12) (13) and stillbirth (14) (13) or the neonate being born septic (15). Additionally the infant can aspirate bacteria in the vaginal secretions during delivery, leading to sepsis and meningitis. Also during vaginal delivery the infant can become colonized by the bacteria (4, 7), which can lead to disease, often later onset than the infections resulting from aspiration during delivery. Breast milk has also been implicated in transmission of GBS (3, 4, 16).

Neonatal GBS disease is divided into those that onset before the seventh day of life, called the early onset disease, and those that onset between the seventh day and third month of life, called late onset disease. Late onset disease more often manifests as meningitis and can lead to long-term complications including seizures, learning disabilities, and deafness.

Early onset GBS disease can be prevented by treating women during labor and delivery with intravenous antibiotics—Intrapartum antibiotic prophylaxis (IAP). In the United States the decision to administer IAP is based on a culture based screening

done in the late third trimester (17-19). European countries administer IAP based on a risk based assessment (20, 21). Both of these strategies lead to inappropriate treatment of uncolonized women, and inappropriate lack of treatment of colonized women (21). Colonization is often transient and a culture at 35-37 weeks does not always correlate with colonization at delivery (8). Additionally a culture can be taken or handled inappropriately leading to a false culture result. Further, IAP does not reduce the incidence of late onset disease nor of chorioamniosis and pre-labor onset of disease (17) (6) (4).

GBS is not just a neonatal pathogen, it causes disease in immunocompromised and older adults as well. (22) (23) (24).

Capsule production:

Production of a polysaccharide capsule is an important virulence factor of GBS (25, 26). The capsule protects the bacteria from complement deposition and opsonophagocytic killing. GBS has 10 capsule serotypes (Ia, Ib and II-IX) resulting in polysaccharides of different sugar composition and chain length (27, 28). The gene products encoded by the *cps* locus determine the serotypes. The first four genes of the locus *cpsA-D* are highly conserved across all serotypes. CpsA is a transcriptional activator, CpsB dephosphorylates CpsD, CpsC phosphorylates CpsD and the CpsD phosphorylation state controls polysaccharide chain length. The other genes of the locus encode serotype specific glycosyltransferases.

Capsule assembly is similar to assembly of peptidoglycan of all bacterial species and the O-polysaccharide of Gram-negative lipopolysaccharide (LPS) (29-31). Polysaccharide subunit assembly begins on the cytoplasmic face of the bacterial

plasma membrane by a series of glycosyltransferases. The growing polysaccharide is flipped to the outer face of the bacterial membrane by a Wzx family flippase (32). Polysaccharide subunits are linked to each other by a Wzy family polymerase. In Gram-negative organisms O-antigen is ligated to the core polysaccharide and lipid A components of LPS by WaaL (30). Capsule is ligated to the cell wall by an as of yet unknown enzyme. During this whole process the polysaccharide precursors are linked to undecaprenyl- pyrophosphate as a lipid carrier. Undecaprenyl-PP is dephosphorylated and then moves back to the inner leaflet of the membrane for recycling. Breakdowns in this process can lead to growth arrest, cell death and cell wall abnormalities (33).

LCP Proteins:

The LCP (LytR-Psr-CpsA) family of proteins is found in multiple Gram-positive bacterial species (34). These proteins are important for proper cell envelope carbohydrate expression and cell wall integrity (35). Control of cell envelope carbohydrate (capsule and teichoic acids (36)) expression is key for virulence and cell wall integrity is key for bacterial survival and proliferation in the host environment.

These proteins are all integral membrane proteins with one or more transmembrane domains. They share a common extracellular domain called the LCP domain (34). This domain has been implicated for a role in attachment of cell envelope polysaccharides to the peptidoglycan cell wall (35).

CpsA:

CpsA is a member of the LCP family of proteins. A unique feature of CpsA is that, in addition to the extracellular LCP domain, it also contains an additional

extracellular domain, the accessory domain, not found in any of the other LCP family members. Previous work had identified this domain by its homology to the DNA polymerase processivity factor (DNA_ppf) of phage and eukaryotic DNA polymerases. (34, 37, 38). However, the limited homology and extracellular localization of the accessory domain indicate that it does not serve a role in DNA replication and instead serves another function in the CpsA protein. The accessory domain of the *Streptococcus pneumoniae* Cps2A protein folds independently from the LCP domain (35). The high homology between the pneumococcal and GBS CpsA (50% identical and 69% similarity at the amino acid level) supports that in GBS as well the accessory domain is an independently folding and functioning domain. However, homology between the pneumococcal and GBS proteins is least similar in the accessory domain, indicating that the accessory domain could serve different functions in the two different species of bacteria.

The CpsA protein is widespread across many streptococcal species. There is a high degree of conservation between the CpsA proteins of the pathogenic *S. pneumoniae*, *S. iniae*, *S. agalactiae* and the non-pathogenic *S. thermophilus*.

CpsA of *S. pneumoniae* has been implicated in regulation of expression of *cps* locus genes and in virulence. Deletion of *cps2A* leads to a reduction in the amount of capsule expressed by the bacteria, and reduction in the proportion of phosphorylated CpsD (39, 40), which is important for full activity of CpsD in export of capsule precursor subunits to the extracellular surface of the bacteria (41). *S. pneumoniae* strains that are more invasive have higher expression of *cpsA* in *in vitro* growth, indicating that *cpsA* has a role in virulence in invasive infections. (42). Additionally, expression of *cps2A* is

four fold upregulated 12 and 24 hours post infection in mouse models of disease compared to growth in broth culture (43). Growth in serum containing media or whole mouse blood also leads to an upregulation of *cps2A* production compared to growth in Todd-Hewitt Yeast (THY) broth (43), indicating a virulence function of *cps2A*. Proper regulation of *cps* gene expression is also critical for invasive infection by *S.*

pneumoniae. Ectopic expression of the *cps* locus from a constitutively strong or weak promoter caused attenuation of colonization and virulence in animal models (44).

CpsA of the zoonotic fish pathogen *Streptococcus iniae* has roles in capsule expression, virulence and transcription of the *cps* locus genes (37, 45-47). A virulence screen for *S. iniae* mutants attenuated in a zebrafish model of disease identified several genes in the *cps* locus (45). A *S. iniae* strain with an insertion mutation in *cpsA* had reduced capsule expression and reduced lethality and ability to disseminate to major organs in a zebrafish model of systemic disease (46). This mutant also had reduced lethality in a zebrafish larvae infection model (47). Further, this mutant had reduced promoter activity from the *cpsA* promoter in a reporter assay (unpublished data, Neely lab). Full length purified CpsA of *S. iniae* was shown *in vitro* to be capable of specific binding to the *cpsA* promoter, while C-terminally truncated purified CpsA was capable of binding the *cpsA* promoter, but with no specificity (37). This supports the role of CpsA as a transcriptional activator responsible for capsule expression and virulence.

CpsA of GBS has been implicated in transcriptional activation of the *cps* locus (48). Deletion of *cpsA* leads to a reduced amount of capsule production by GBS, and to decreased transcription of *cps* locus genes (48). Later analysis of GBS CpsA (38) demonstrated a bifunctional role for CpsA in capsule expression and cell wall stability.

Deletion of *cpsA* led to a reduction in capsule expression and to the bacteria forming unusually long chains of cocci. Additionally, episomal expression of a truncated CpsA missing the most C-terminal extracellular LCP domain caused a dominant negative effect when expressed in the wild type strain, resulting in decreased capsule expression and increased chain length (38). This indicated that the extracellular domains of CpsA were key to both the capsule expression and cell wall maintenance functions of CpsA. Analysis of full length and truncated CpsA binding to the two promoters of the *cps* locus showed that full length CpsA is capable of specific binding to the promoters, whereas truncated CpsA missing the extracellular domain is capable of binding, but with reduced specificity. This suggested that the extracellular domain is serving a role in transcriptional regulation of this locus, through promoting proper conformation of CpsA to lead to specific DNA binding (38).

Streptococcus pyogenes:

Streptococcus pyogenes is a human pathogen. Infections caused by *S. pyogenes* range from superficial and self-limiting impetigo and pharyngitis to systemic and life threatening manifestations such as scarlet fever and necrotizing fasciitis. Immune responses to *S. pyogenes* can lead to long term sequelae such as rheumatic heart disease and glomerulonephritis.

Rare among human pathogens, *S. pyogenes* is as sensitive today to beta-lactam antibiotics as when penicillin was first introduced. However, resistance to macrolides is emerging. Macrolides are the antibiotics of choice when a patient is allergic to penicillin, or in a mixed bacterial infection, when other species are, or are likely to be, beta-lactam resistant. Further, the most severe manifestations of *S. pyogenes* infection, necrotizing

fasciitis and streptococcal toxic shock syndrome, respond only to the highest doses of antibiotics, if at all.

Virulence of *S. pyogenes* is a dynamic process, with the bacteria modifying its transcriptome to survive and proliferate in a host while evading the host immune system. During necrotizing fasciitis, an influx of phagocytes to the site of infection is somehow prevented by the bacteria (49). In a mouse model of soft-tissue infection the most highly expressed transcripts are involved in stress adaptation and cellular remodeling, including known virulence determinants (50). Two-component signaling systems (TCSS) are involved in transcriptional regulation in bacteria, and are potential drug targets against pathogenic bacteria (51).

The *salivaricin* locus of *S. pyogenes*:

Previous work in our lab identified an operon important for development of systemic disease by *S. pyogenes* (52, 53). This locus had homology to the lantibiotic production locus of the oral commensal *Streptococcus salivarius* (54). In *S. salivarius* the gene products of this locus are responsible for the production and export of and immunity against a bacteriocin-like inhibitory substance, salivaricin A (55). This peptide contains unique intrapeptide bridges resulting from the dehydration of amino acids (56-58). These lanthionine bridges give this class of bacteriocins the name lantibiotics. Against other Gram-positive bacteria, salivaricin A acts as a pore-forming toxin (59-61). This is important for establishment of a niche in the competitive environment of the oral microflora. Most strains of *S. pyogenes* do not produce an active lantibiotic from this locus (54, 60). However, all sequenced strains of *S. pyogenes* do maintain this locus. Due to the flexibility of the *S. pyogenes* genome, maintenance of this locus across all

strains indicates that this locus has been repurposed for a critical role in *S. pyogenes*. Our data showing that mutations in this locus cause attenuation of virulence suggests that the role of the salivaricin locus in *S. pyogenes* is in virulence (52, 53).

The salivaricin locus contains a two component signaling system SalKR and two peptides at either end of the locus SalA and SalZ. Because *S. pyogenes* does not have alternative sigma factors it relies on two-component signaling systems, 13 of which have been identified in the genome, to cause global transcription changes. Two-component signaling systems have a sensor kinase (in this case SalK) which autophosphorylates in response to environmental signals. SalK then phosphorylates the response regulator (in this case SalR) which functions as a transcriptional regulator to cause changes in expression of target genes. Peptides serve as important signaling molecules in many species of bacteria (62). SalA serves as the lantibiotic in *S. salivarius* causing killing of bacteria that do not produce SalA, but it also serves as a quorum-sensing molecule and is sensed by SalK to upregulate transcription of the *sal* locus genes (60). This causes a positive feedback loop to produce more SalA peptide, but also serves as a way to induce production of the immunity genes.

SalA of most strains of *S. pyogenes* is not an active lantibiotic (54). This is due to mutation of the modification and export genes, *salM* and *salT* respectively, leading to premature stop codons and truncated non-functional proteins being produced from these genes. So, SalA is not modified into a lanthionine containing peptide, but still could be serving a role in peptide signaling. SalZ is a 66 amino acid peptide of unknown function found at the 3' end of the operon. Peptides serve an important signaling role in many species of bacteria. In many Gram-positive species ribosomally

synthesized and non-ribosomally synthesized peptides serve as quorum sensing molecules (63-65). Bacterial peptides can also serve as a role in transcription as co-repressors (66). Peptide signalling serves a key role in sporulation of *Bacillus* species (67, 68). This supports a role for the peptides of the *sal* locus to be serving a signaling role in virulence control of *S. pyogenes* disease.

CHAPTER 2: EFFECTS OF EXTRACELLULAR DOMAINS OF CPSA ON GBS CELL ENVELOPE

Introduction:

Group B *Streptococcus* (GBS), *Streptococcus agalactiae*, is a broad host range pathogen capable of causing severe sepsis and meningoenzephalitis in fish (69-74), bovine mastitis (75-77), human neonatal sepsis and meningitis (1-6) and has been recently implicated in chorioamnitis (10, 11) causing premature birth in humans (12, 13) and stillbirth (13, 14). GBS also can cause a range of invasive infections in immunocompromised and older adults (22-24). However, GBS is also capable of existing as a commensal of the human vaginal and rectal mucosa (9, 78, 79).

The current strategy to prevent GBS infection of neonates is intrapartum antibiotic prophylaxis (IAP) (17), intravenous antibiotics given to a woman during labor and delivery. IAP is administered either to women following positive GBS culture during prenatal screening (17, 19), or if risk based criteria are met (20, 21). This has led to a reduction in incidence of GBS disease in the first week of life (early onset disease) but has not changed the incidence of GBS disease in the first three months of life (late onset disease) (4, 6, 17).

One mechanism allowing bacteria to successfully function as both a commensal and a pathogen is regulation of polysaccharide capsule expression. This is an essential event for GBS immunoevasion and survival in the bloodstream (25), but not essential for colonization, as a GBS isolate with a complete deletion of the *cps* locus is capable of colonizing the vaginal mucosa (80). GBS has ten serotypes defined by the genes of the *cps* locus, leading to different polysaccharide compositions (27). The first regulatory

gene of this locus, *cpsA*, is conserved across all serotypes and plays a role in transcriptional activation of this locus (48).

CpsA is a member of the LCP (LytR-CpsA-Psr) protein family. These are found in a broad range of Gram-positive bacterial species (34) and are important for linkage of cell wall carbohydrates to the cell wall, cell wall maintenance and cell division (35). Cell wall stability and proper control of cell division are important for bacterial survival and proliferation in the stressful environment of the host. LCP family proteins have one or more transmembrane domains and a large extracellular domain containing the conserved LCP domain (34), which is hypothesized to have a catalytic function (35). The CpsA protein, however, is unique among the family of LCP proteins as in addition to the extracellular LCP domain; it possesses an additional extracellular domain, called the accessory domain.

Analysis of the GBS CpsA protein (38) demonstrated a bifunctional role for CpsA in capsule expression and cell wall stability. Deletion of the *cpsA* gene led to a reduction in capsule production and to the bacteria forming unusually long chains of cocci. Additionally, episomal expression of a truncated CpsA protein missing the most C-terminal (LCP) extracellular domain caused a dominant negative effect when expressed in the wild type strain, resulting in decreased capsule production and increased chain length (38). This indicated that the extracellular domains of the CpsA protein were important for both the capsule expression and cell wall maintenance functions. Analysis of full length and truncated CpsA proteins binding to the two promoters of the *cps* locus showed that full length CpsA is capable of specific binding to the promoters, whereas truncated CpsA missing the extracellular domain is still capable

of DNA binding, but with reduced specificity. This suggested that the extracellular domain is serving a role in transcriptional regulation of this locus, through promoting proper protein conformation of CpsA for specific DNA binding (38).

Previous analysis of GBS CpsA protein function revealed that episomal expression of the CpsA-Full protein in an isogenic *cpsA* deletion strain could complement the *cpsA* deletion for wild type capsule expression (38). Surprisingly, episomal expression of a truncated CpsA with the entire extracellular domain removed (CpsA-117, figure 1), was also able to complement *in vitro* to wild type capsule levels. However, expression of a truncated form of CpsA in which only the extracellular accessory domain remained (CpsA-245, figure 1) could not complement for capsule production in the *cpsA* deletion strain (38). Therefore, while the extracellular domain of CpsA is not needed for capsule production *in vitro*, expressing only the extracellular accessory domain in the absence of the LCP domain results in an inability to complement for capsule production. Episomal expression of these same protein constructs in the wild type GBS strain had quite different results. While the CpsA-Full and the CpsA-117 proteins had no effect on wild type capsule expression, episomal expression of the CpsA-245 protein caused a dominant negative effect on capsule production, effectively inhibiting the ability of the wild type strain to produce capsule even in the presence of the wild type CpsA protein (38).

In this study we have identified the regions of the extracellular accessory domain of the GBS CpsA protein that are required for the dominant negative effect. Furthermore, we determined that the dominant negative phenotype could be induced by extracellular addition of the truncated CpsA protein or a short peptide of CpsA.

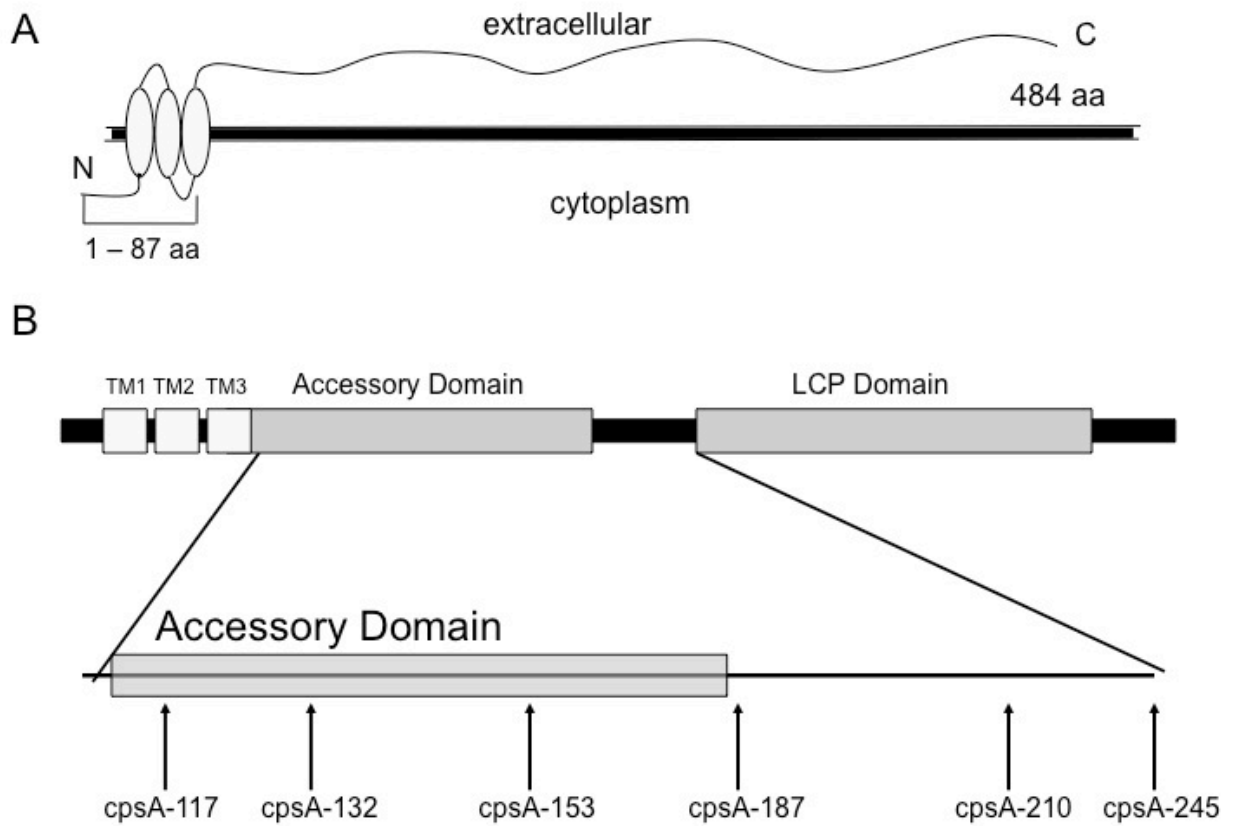


Figure 1: The GBS CpsA protein: A) Membrane topology of CpsA. B) Domains of CpsA and location of truncations

Visualization of peptide binding revealed that this region of CpsA is in contact with the midcell septal region of the bacterial cell wall. Additional cell wall integrity defects were demonstrated in strains with a deletion of *cpsA* and a strain expressing a truncated form of the CpsA protein. Lastly, we provide evidence that deletion of the *cpsA* gene or expression of a truncated CpsA protein leads to attenuation of virulence.

Materials and Methods:

Bacterial strains and growth conditions:

Escherichia coli strains TOP10 and Electromax DH5 α (Invitrogen) were used for construction and maintenance of plasmids. They were grown in aerobic conditions with shaking in liquid LB (Acumedia) or aerobically on LB agarose plates: LB supplemented with 1.5% technical agar (Acumedia) and supplemented with antibiotics at concentrations of: Ampicillin 100 $\mu\text{g}/\text{mL}$, Chloramphenicol 20 $\mu\text{g}/\text{mL}$ or Erythromycin 750 $\mu\text{g}/\text{mL}$.

Streptococcus agalactiae strain 515 and the isogenic *cpsA* mutant (48) were grown statically in closed tubes in Todd Hewitt media (Alpha Biosciences) + 0.2% yeast extract (Acumedia) (THY) with the addition of 1.4% bacteriological agar (Acumedia) for solid media, and grown anaerobically or in 5% CO₂, and supplemented with antibiotics at concentrations of: Chloramphenicol 3 $\mu\text{g}/\text{mL}$ or Erythromycin 2 $\mu\text{g}/\text{mL}$. Cultures grown in the presence of purified protein or synthetic peptide were grown in media supplemented with HALT Protease Inhibitor cocktail (ThermoFisher).

The CpsA-insert strain was constructed using primers 5' GBS *cpsA* insert PstI and 3' GBS *cpsA* insert XmaI (see table1) by amplifying a 454 bp fragment using GBS 515 genomic DNA as template. Purified fragment was cloned into the PstI and XmaI

sites of vector pUC19-Erm (45). Plasmid was transformed into GBS strain 515 and single crossover recombinants selected by plating on THY Erm 2 μ g/mL agarose plates.

CpsA truncations were made using a template encoding an MBP-cpsA (plasmid pGBS *cpsA*-full (38)) using primers 5' MBP RBS BamHI (see table1) and 3' primer depending on truncation (see table1), and cloned into vector pLZ12-rofA for constitutive expression. Plasmids were transformed into GBS strain 515 and its isogenic Δ *cpsA* derivative as previously described (38).

Percoll buoyant density centrifugation:

Buoyant density was determined using linear Percoll (GE Healthcare) gradients as previously described (38). Briefly, 2mL Percoll, supplemented with 0.15M NaCl, diluted to low (1.085 g/cm³) density was carefully overlaid onto 2mL Percoll diluted to high (1.120 g/cm³) density in a 5mL tube and placed at a 15° angle overnight to allow formation of a continuous linear gradient. Gradients were placed vertically and allowed to settle at least 30 minutes prior to use. Overnight bacterial cultures were normalized to an OD₆₀₀=0.6 and concentrated to a volume of 50 μ L in phosphate buffered saline. Culture was carefully added to top of gradient and gradients were centrifuged 30 min at room temperature and 5,000 rpm in a swinging bucket (Eppendorf Centrifuge 5403 with Rotor 16A4-44). Distance traveled in the gradient was measured from the bottom of the meniscus to the center of the cell band, and density was determined by calculation of a linear curve from distance traveled by beads of known density (GE Healthcare).

Protein purification:

Proteins were purified using the pMal-c2x system (NEB) as previously described (38). Elution fractions containing protein were detected by the BCA assay (Pierce) and

Table 1: Primers used in Chapter 2

| Primer name | Sequence (restriction site underlined) |
|-------------------------|--|
| 5' GBS cpsA-insert PstI | AAA <u>ACTGCAGCG</u> TTGCTACTACTTTATATGG |
| 3' GBS cpsA-insert XmaI | TCCCCCGGGGTATCAATACCGCTAAATAG |
| 5' MBP RBS BamHI | CGCGGATCCGCGGATAACAATTTCACACAGG |
| 3' GBS cpsA full PstI | AAA <u>ACTGCAG</u> TTATTCCTCCATTGTGTTC |
| 3' GBS cpsA 245 PstI | AAA <u>ACTGCAG</u> TTATGTTGATATAGAGCCAAAAG |
| 3' GBS cpsA 210 PstI | AAA <u>ACTGCAG</u> TTAAATCGTTTTTATCTGCG |
| 3' GBS cpsA 187 PstI | AAA <u>ACTGCAG</u> TCATAAAACCATAGCCTGACTATC |
| 3' GBS cpsA 153 PstI #2 | AAA <u>ACTGCAG</u> CATTCTATCAAATCAGTAATATTTTC AG |
| 3' GBS cpsA 132 PstI | AAA <u>ACTGCAG</u> TCAAGCTTCTATATTGG |
| 3' GBS cpsA 117 PstI | AAA <u>ACTGCAG</u> TTACTCAATTTTCAGAGTATGAAGC |

were examined by SDS-PAGE to determine protein purity and integrity. Fractions were concentrated as necessary with a 50 kDa centrifugal concentrator (Millipore) and stored at -80 °C in 50% glycerol.

CpsA peptide generation and labeling:

CpsA peptide: INRKNTNHKEGVFNIYISGITDTF was synthesized (GenScript) and resuspended in sterile double distilled water at a concentration of 2 mg/mL. Peptide was labeled with Fluorescein isothiocyanate (FITC) To 192 µL of peptide (2mg/mL) in 568 µL carbonate-bicarbonate buffer (0.1 M, pH 9.0), 40 µL of freshly prepared fluorescein-5-isothiocyanate stock solution (16 mM in DMF) was added and the reaction was incubated in a 37 °C rotary shaker for 3 h. The reaction mixture was loaded onto a 5 mL Sephadex G-25 column and the column was washed with deionized water while collecting fractions. Each fraction was monitored at 280 nm and 495 nm and fractions containing both absorbance values were combined and lyophilized. The resulting orange powder was re-dissolved in water and the concentration of labeled peptide was calculated using equation $C = (A_{280} - A_{495} \times 0.35)/128$ where C is the peptide concentration (M), A_{280} and A_{495} are the absorbance at 280 nm and 495 nm, respectively.

Microscopy:

Phase contrast microscopy was performed on overnight cultures of wild type bacteria grown in the presence or absence of 200 pmol/mL unlabeled CpsA peptide. Five microliters of overnight culture was mounted with a coverslip and observed using a Zeiss Axioscope 40 and imaged with Axiovision 4.7.

Cultures were grown in the presence of FITC-labeled peptide overnight at concentration indicated and normalized to an OD₆₀₀ of 1.0, followed by three washes in phosphate buffered saline (PBS). Five microliters of washed bacteria were observed as a wet mount using a Zeiss Axioscope 40 and imaged with Axiovision 4.7. For competitive binding analysis cultures were grown overnight in the presence of 2.5 µg/mL FITC-labeled peptide with or without a 25 fold molar excess of unlabeled peptide. Cultures were normalized to an OD₆₀₀ of 1.0, followed by three washes in phosphate buffered saline (PBS). Fluorescence of a 100 µL sample in triplicate was read in a Tecan Spectrafluor Plus Fluorometer with Magellan6 software using the fluorescein default settings.

Fluorescent vancomycin staining was performed on midlog cultures. Bacteria were grown overnight and then subcultured 1:50. At midlog (OD₆₀₀=0.3) cultures were concentrated to OD₆₀₀=1.0. Ten microliters of culture and a final concentration of 1µg/µL BODIPY_FL vancomycin (Life Technologies) were incubated 10 minutes at 37°C to allow incorporation of stain into newly formed cell wall. Cultures were then washed 3 times in PBS and then were resuspended in 3µL PBS and observed as a wet mount using a Zeiss Axioscope 40 and imaged with Axiovision 4.7.

Virulence Assays:

Human whole blood infections were performed as described previously (38) Briefly, blood was freshly isolated from healthy volunteers and collected in heparinized vacuum tubes (BD). Bacterial cultures were grown to midlog and washed in phosphate buffered saline. Bacteria were inoculated at a dose of 2×10^5 cfu per mL blood in triplicate in 1.5 mL tubes (Dot Scientific) with end over end rotation for 3 hours at 37°C.

Bacteria were plated on THY agarose plates in duplicate and viable cfu enumerated. Log killed was determined from plating the inocula and subtracting the log of the product cfu from the log of the inoculum cfu.

Zebrafish infections were performed as described previously (81). Briefly, adult zebrafish were injected intramuscularly with midlog bacterial cultures at an infectious dose of 10^6 cfu per fish. For dissemination analysis, fish were sacrificed at 24 hours and viable cfu in the brain, heart and spleen were determined by plating on CNA selective agar (Acumedia).

Results:

CpsA-insert mutant:

To further investigate the function of the extracellular accessory domain of the GBS CpsA protein, a non-polar *cpsA* insertion mutation on the chromosome was constructed and tested for the ability to produce capsule. This mutant strain expresses a truncated form of the CpsA protein, missing the C-terminal extracellular LCP domain (similar to the CpsA-245 truncation, figure 1). Buoyant density analysis revealed that the CpsA-insert strain produces capsule levels even further reduced than those of the *cpsA* deletion strain (figure 2). These results, coupled with our previous data using the episomally expressed CpsA-245 protein in the wild type GBS strain as described above, suggest that the CpsA extracellular accessory domain, when in the absence of the LCP domain, functionally inhibits capsule production.

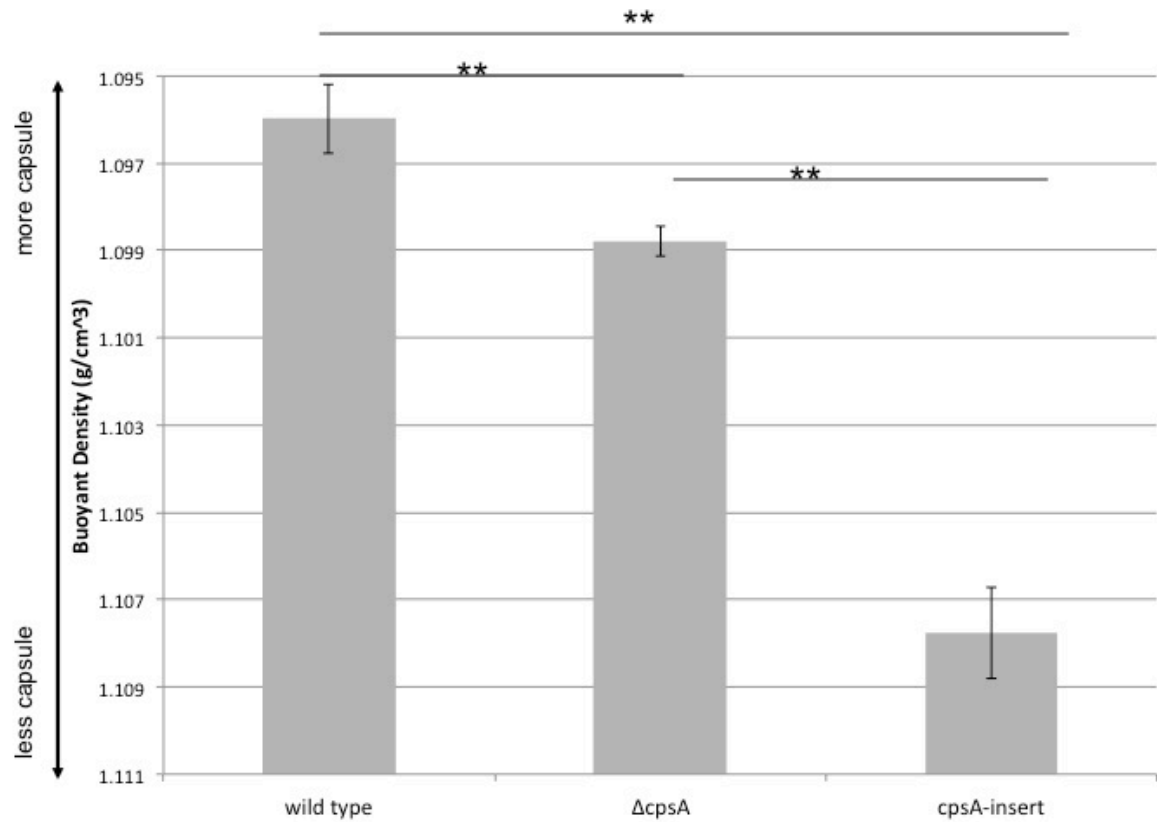


Figure 2: Capsule production. Measurement of capsule production of the GBS wild type, the $\Delta cpsA$ and the $cpsA$ -insert strains as measured by Percoll buoyant density centrifugation. ** p < 0.01.

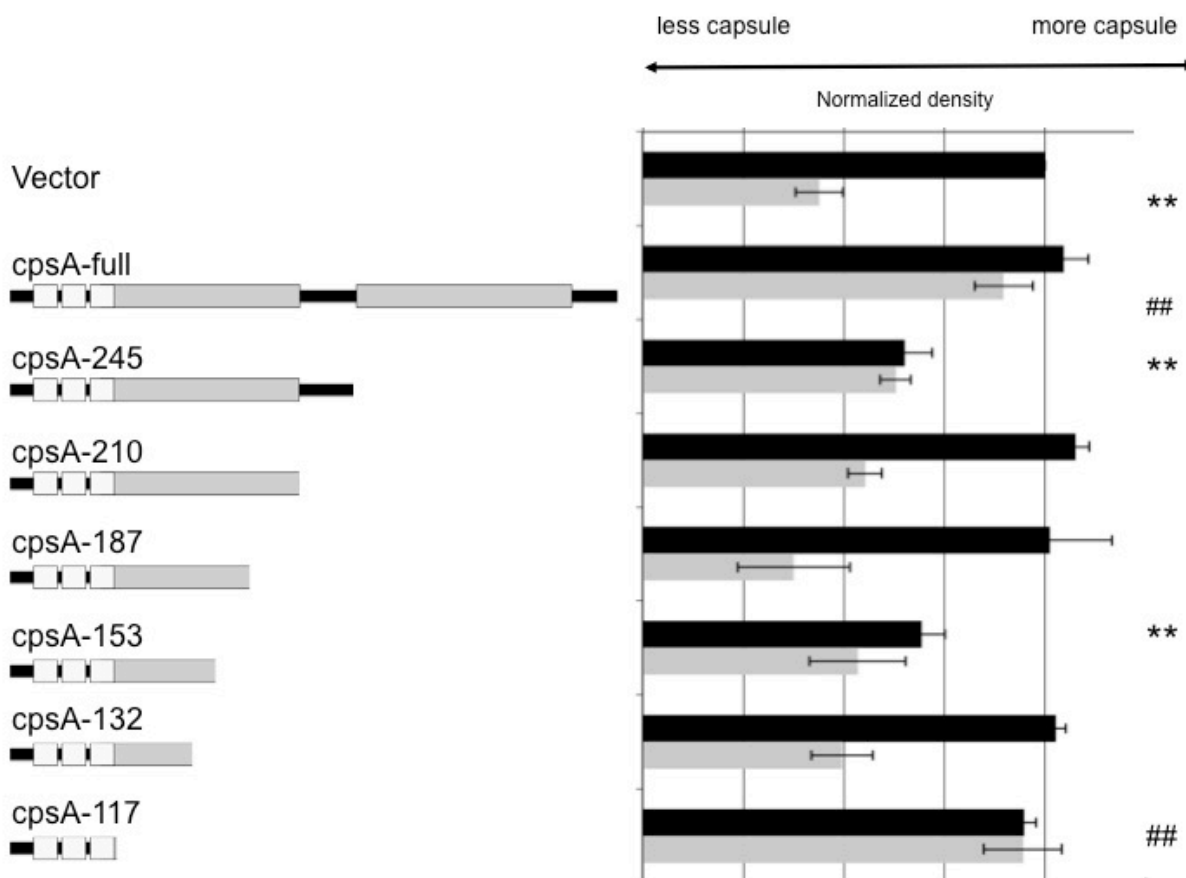


Figure 3: Capsule production by strains with episomally expressed CpsA truncations. Capsule production measured by Percoll buoyant density centrifugation. Notations on left indicate CpsA version expressed from a plasmid in the indicated strain. Black columns represent the wild type strains expressing the indicated construct. Grey columns represent the $\Delta cpsA$ strains expressing the indicated construct. Densities were normalized to wild type with empty vector. ** significantly ($p < 0.01$) different from wild type capsule levels. ## significantly ($p < 0.01$) different from $\Delta cpsA$ capsule levels.

Extracellular domain truncation:

To narrow down the region of the accessory domain responsible for the dominant negative effect, serial truncations of the extracellular domain of the CpsA-245 protein were made (see figure 1). These constructs were expressed episomally in the wild type strain and the $\Delta cpsA$ strain (figure 3). Only the CpsA-117 and CpsA-full were able to complement the *cpsA* deletion strain. The CpsA-210, CpsA-187 and CpsA-132 when expressed episomally in wild type had wild type capsule levels, and the CpsA-153 had reduced capsule level, commensurate with the *cpsA* deletion and the episomally expressed CpsA-245 (figure 3). These results indicate that the dominant negative effect is accomplished by an aberrant function or interaction occurring with the regions of CpsA from amino acids 153-187 and 210-245 with itself and/or other binding partners.

Addition of purified protein:

We next questioned if the dominant negative phenotype caused by expression of the CpsA-245 protein in the presence of the wild type CpsA protein (wild type strain) could exert its inhibitory effect if added exogenously or if it needed to be produced by the bacteria. Therefore, purified proteins of various CpsA truncations were added to a culture of wild type bacteria and assessed for capsule production by buoyant density centrifugation (figure 4). Addition of purified CpsA-117 protein and the CpsA-full protein, which had no effect on capsule levels when expressed episomally in the wild type strain (figure 3), also had no effect on capsule levels when supplied as purified proteins to the cultures (figure 4). However, addition of purified CpsA-245 protein, which causes a dominant negative effect on capsule levels when expressed episomally in the wild type strain, caused a similar reduction in capsule levels when added exogenously to a

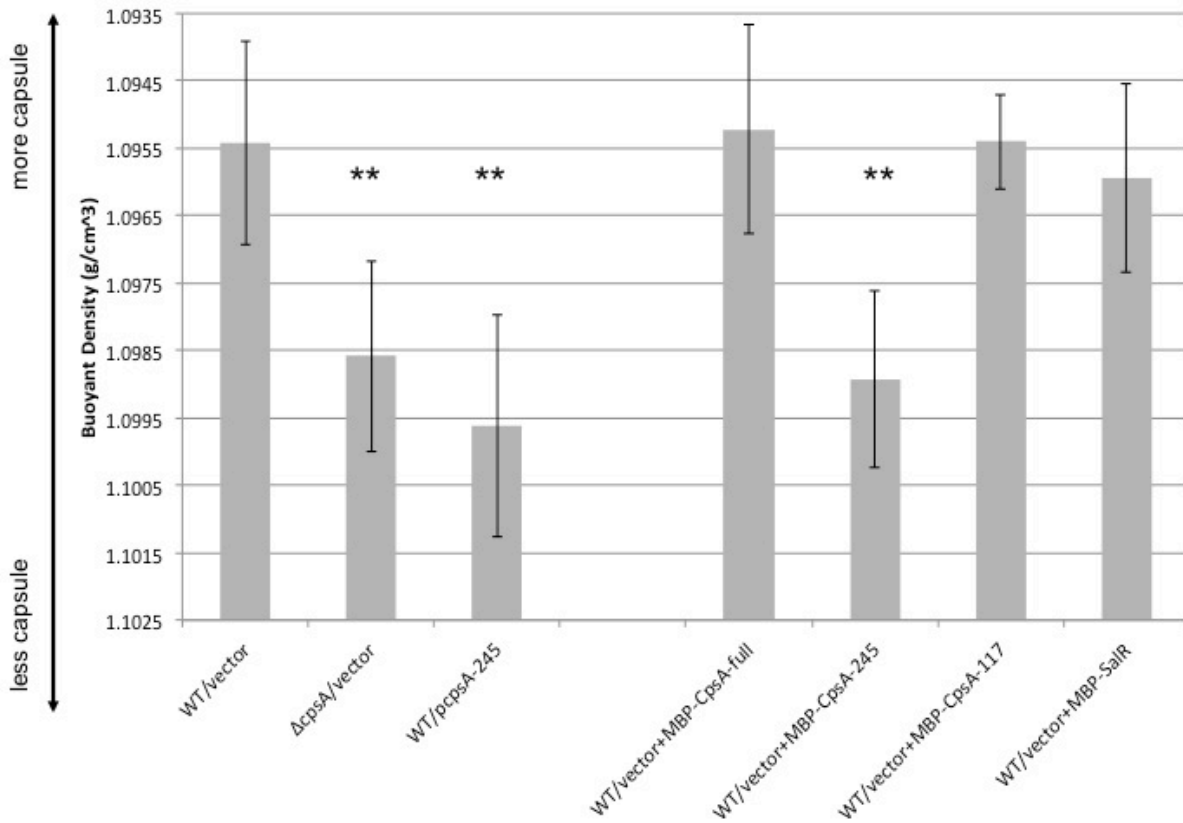


Figure 4: Capsule production in the presence of purified protein. Capsule production measured by Percoll buoyant density centrifugation. Cultures were grown with the indicated protein at a concentration of 20 pmol/mL, in the presence of protease inhibitors. ** $p < 0.01$

culture of wild type bacteria. As a negative control, cultures were grown with the purified SalR protein, a *Streptococcus pyogenes* protein constructed and purified by the same process and had no effect on capsule expression. Therefore, the dominant negative phenotype caused by expression of the CpsA-245 protein is capable of causing the same result when added exogenously.

Addition of CpsA peptide:

The serial truncations of the extracellular domains determined that the most C-terminal end of the accessory domain was important for the dominant negative effect. Therefore, the CpsA peptide, a synthetic peptide corresponding to amino acids 218-240 was constructed. When wild type cultures were grown in the presence of the CpsA peptide, capsule production was significantly reduced (figure 5). However, addition of the unrelated *Streptococcus pyogenes* SalA peptide, of similar size and synthesized by the same process, had no effect on capsule production. These results further confirm the ability of the dominant negative factor to function from the extracellular environment. Moreover, these results demonstrate that the N-terminal DNA binding and transmembrane domains of the CpsA protein are not required for the dominant negative effect to occur.

Previous analysis of *cpsA* mutations or episomal expression of truncated forms of the CpsA protein revealed that in addition to changes in capsule production there were also changes in cell wall architecture as evidenced by the formation of unusually long chains of cocci in these strains (38). Addition of the CpsA peptide to wild type cultures changed the normal chain length from 2-4 cocci per chain (figure 6A) to most

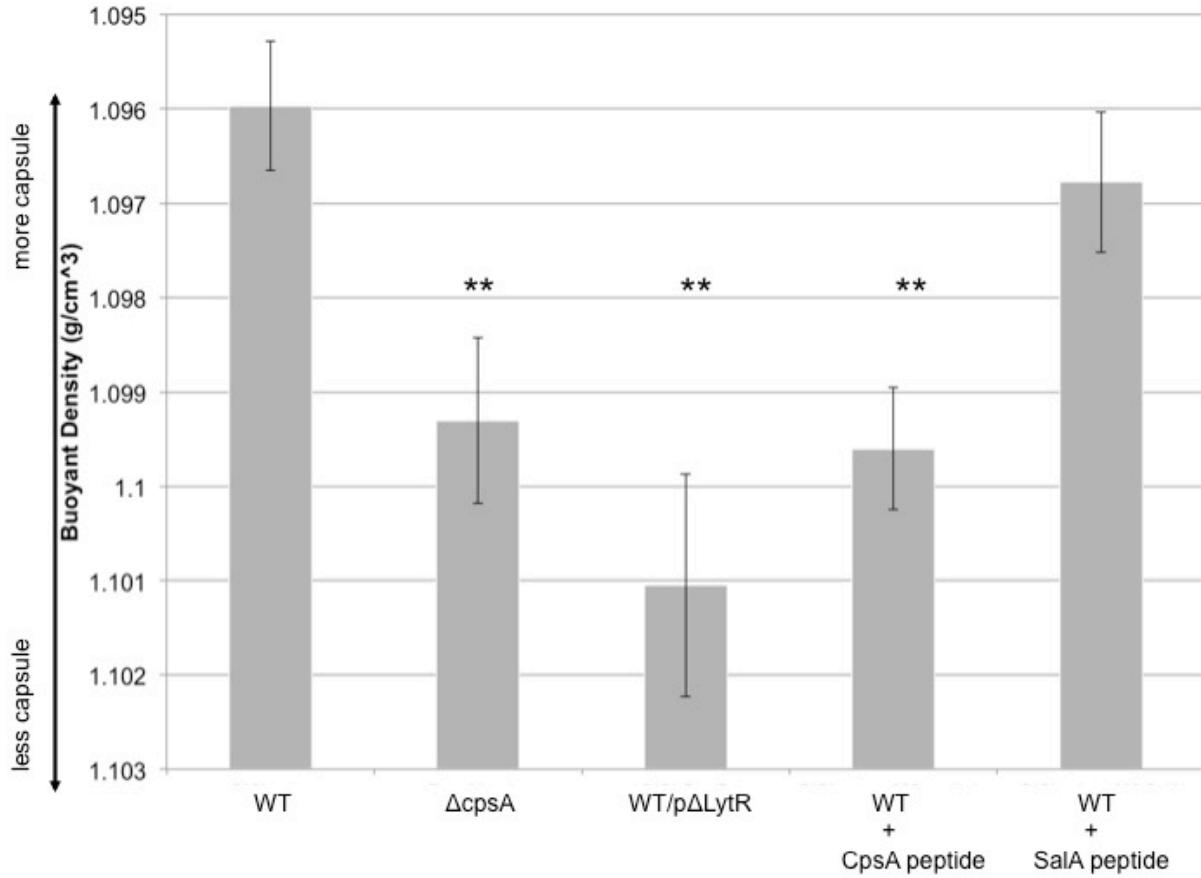


Figure 5: Capsule production in the presence of CpsA peptide. Capsule production measured by Percoll buoyant density centrifugation. Cultures were grown with the indicated peptide at a concentration of 200 pmol/mL of in the presence of protease inhibitors. * $p < 0.05$ ** $p < 0.01$.

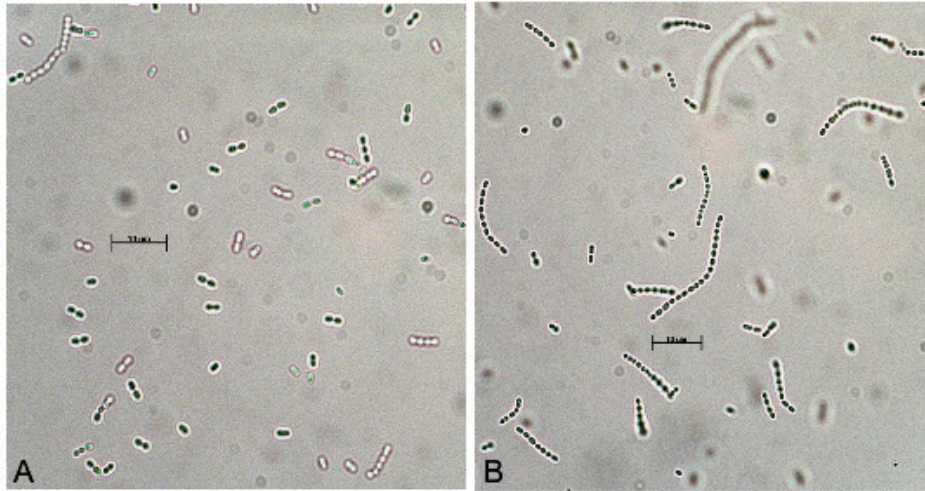


Figure 6: Effect of peptide on chain length. Phase contrast microscopy of overnight cultures of wild type bacteria grown in the presence of protease inhibitors (A) in the absence of CpsA peptide or (B) with 200 pmol/mL CpsA peptide. Scale bar indicates 10 μm .

chains being greater than 10 cocci per chain (Figure 6B). To visualize the interaction of the peptide with the bacteria and any cell wall changes that may be occurring, the peptide was labeled with FITC and examined by fluorescence microscopy (figure 7). When GBS cultures were grown in the presence of the FITC-labeled peptide, nearly identical staining patterns were apparent in both the wild type (figure 7A) and $\Delta cpsA$ (figure 7B) cultures indicating that the peptide is binding to a factor other than the CpsA protein. Both wild type and *cpsA* deletion strain cultures exhibited a long chain phenotype, indicating that the peptide is able to exert a dominant negative effect on the cell wall, similar to the episomal expression of CpsA-245 leading to the bacteria forming long chains of cocci (38). Staining was most intense at the midcell of the bacteria and the region of the division septae suggesting that peptide interactions are located at the division septum, potentially as part of the cell division machinery. When FITC-peptide was incubated with cells in the absence of growth no binding was visualized (data not shown) indicating actively growing cells are required for peptide binding.

To determine if the binding of FITC-peptide to the bacteria was specific, competition experiments were performed. Bacteria were grown in the presence of the FITC-labeled peptide, or labeled peptide plus an excess of unlabeled peptide. Relative fluorescence units (RLU) were determined using a 96-well fluorometer and compared between the two conditions. The wild type culture and the *cpsA*-deletion culture with both labeled peptide and a 25-fold excess of unlabeled peptide exhibited 5.25 ± 1.4 and 6.12 ± 1.21 fold less fluorescence, respectively, when compared to the fluorescence of the cultures grown with only the labeled peptide.

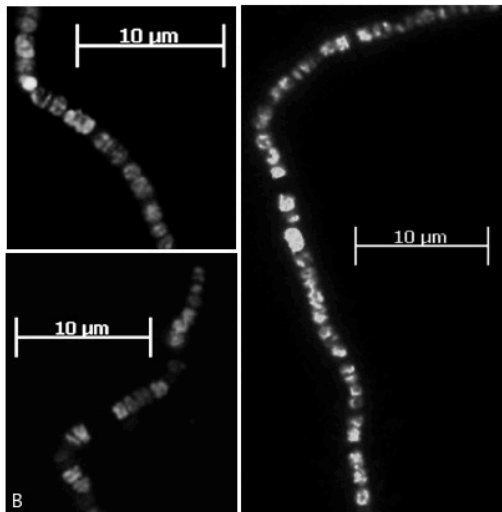
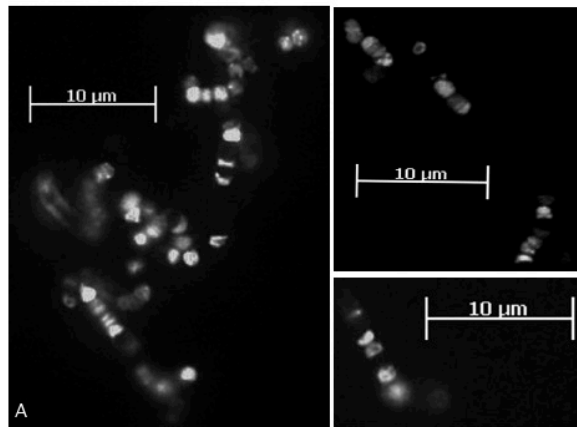


Figure 7: Localization of FITC-labeled CpsA peptide. Fluorescent micrographs of (A) wild type and (B) $\Delta cpsA$ bacteria grown overnight in the presence of FITC-labeled peptide. Scale bar indicates 10 μm .

Fluorescent Vancomycin staining:

In an effort to examine the cell wall effects caused by CpsA protein truncation, actively growing bacteria were pulse-labeled with BODIPY-FL vancomycin (figure 8). The mechanism of action of this compound is the staining of newly synthesized, but not yet cross-linked cell wall to show areas of active cell wall assembly. Wild type (figure 8A) and *cpsA* deletion (figure 8B) strains exhibited clear rings of cell wall around each individual coccus, with some increased thickness at the division septae. CpsA-insert bacteria (figure 8C) exhibited cocci of uneven size, and unequal staining intensity, indicating differences in cell wall thickness in different regions of the bacteria. Additionally, some cocci appear to have multiple division septae forming, indicating an aberrant cell division event. Wild type bacteria episomally expressing the CpsA-245 protein exhibited unequal cell wall staining and evidence of multiple division septae forming per cell (figure 8D). The *cpsA* deletion strain episomally expressing the CpsA-245 protein (figure 8E) appears similar to the CpsA-insert strain, with cocci of uneven size and uneven cell wall thickness, and multiple division septae per cell. These results demonstrate that expression of the truncated CpsA protein leads to changes in cell wall morphology and cell division, perhaps through aberrant protein interactions. In the case of the wild type strain expressing the CpsA-245 protein truncation, which expresses both the wild type CpsA and the truncated CpsA, inhibition of wild type protein function occurs.

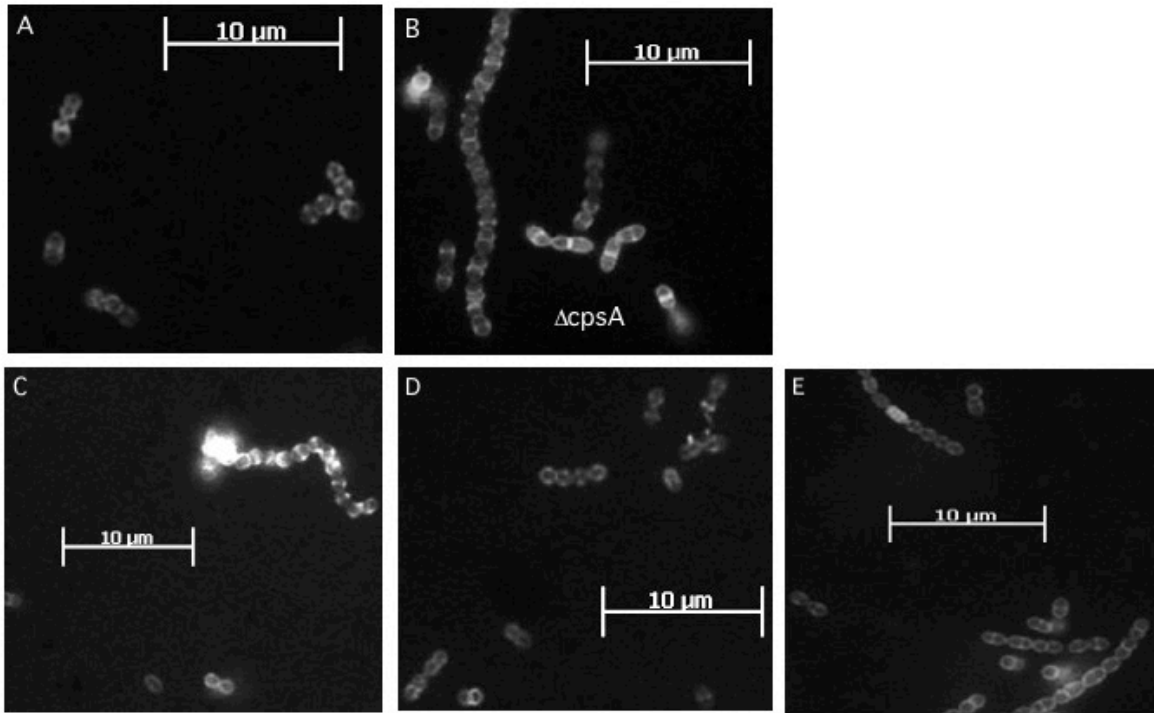


Figure 8: Fluorescent Vancomycin staining of newly formed cell wall. (A) WT strain, (B) Δ cpsA strain, (C) cpsA-insert, (D) WT/pCpsA-245 and (E) Δ cpsA/pCpsA-245. Scale bar indicates 10 μ m.

Virulence Analysis:

The importance of the CpsA protein, particularly the extracellular domains, in promoting bacterial survival in human whole blood was analyzed using the various CpsA mutated strains. While the wild type and *cpsA* deletion strains were killed to similar levels, the CpsA-insert strain exhibited a half log less survival after three hours in blood (figure 9). This indicates that the capsule and cell wall deficits caused by the aberrant function of the accessory domain have virulence consequences in preventing bacterial survival in human blood.

The role of the CpsA protein in virulence in a zebrafish sepsis-meningoencephalitis model was also examined. Fish were injected intramuscularly and assayed for bacterial dissemination to the heart, spleen and brain 24 hours post infection (figure 10). The *cpsA* deletion strain was attenuated in the ability to disseminate to the heart and brain, while the *cpsA*-insert strain was attenuated in the ability to disseminate to all three major organs. Moreover, the ability of the *cpsA*-insert strain to disseminate to the heart and brain was significantly reduced from that shown for the *cpsA* deletion, further supporting the key detrimental effect of the aberrant function of the accessory domain of CpsA in virulence of GBS allowing dissemination away from the initial site of infection into the bloodstream and crossing the blood-brain barrier.

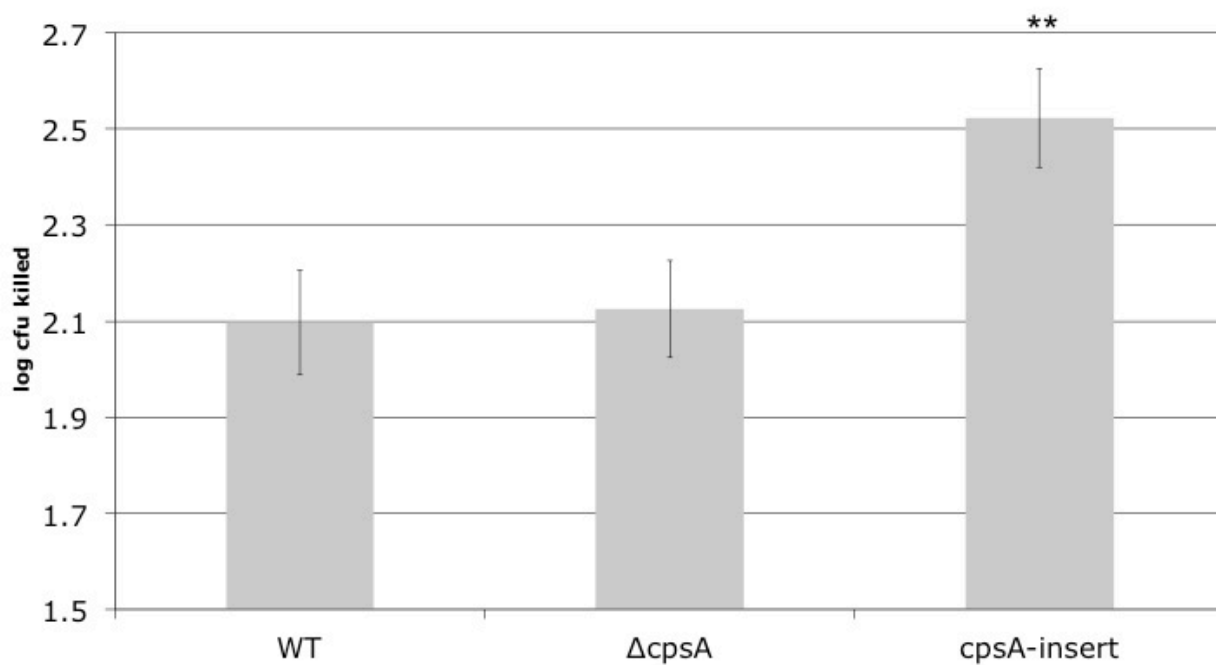


Figure 9: Human whole blood bactericidal assay. Log bacteria killed in three hours in freshly isolated heparinized human whole blood. Trend representative of four independent experiments. Error bars are standard deviation of three technical replicates. ** $p < 0.01$

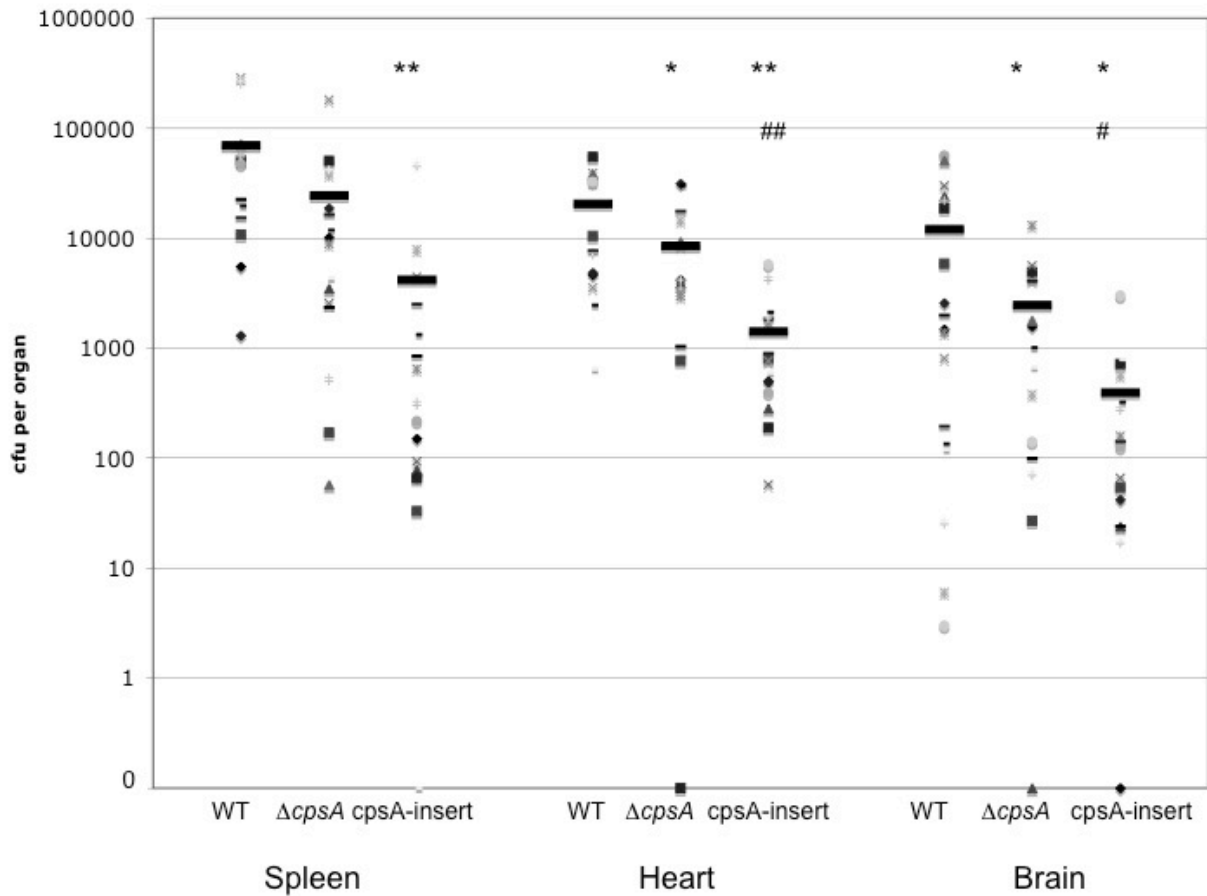


Figure 10: GBS dissemination to zebrafish major organs. Measurement of viable cfu in spleen, heart and brain of individual zebrafish 24 hours post 10^6 cfu intramuscular injection. Each point is one fish, bar indicates mean. * $p < 0.05$, ** $p < 0.01$ different from wild type, # $p < 0.05$ ## $p < 0.01$ different from $\Delta cpsA$. $n = 14-18$ fish per strain.

Discussion:

LCP (LytR-CpsA-Psr) family members are found in a variety of Gram-positive bacteria and have been credited with a wide range of functions and phenotypes. However, mechanisms of how most of these proteins function are as yet undetermined. Recently, LCP proteins have been implicated in the attachment of cell wall carbohydrates, capsule and teichoic acids, to the cell wall (35, 82, 83). Cell wall carbohydrate precursors are made processively on the inner and then the outer leaflet of the bacterial membrane. The carbohydrate precursors are anchored to lipid carriers during this process. LCP family members are hypothesized to act enzymatically to transfer the precursors off of the lipid carrier and onto the peptidoglycan cell wall with some substrate specificity, but can compensate for each other singly mutated (35, 82, 83). A *Staphylococcus aureus* triple mutant in three LCP family members has no wall teichoic acids, releasing teichoic acid instead into the culture supernatant (84). A double mutant of *Streptococcus pneumoniae* in two LCP family members releases capsule into the culture supernatant (82). Mutation of multiple LCP family members can be lethal (85) or cause growth deficits unless compensatory mutations of other genes also occur (35, 82, 86, 87).

The CpsA protein is a member of the LCP protein family and is highly conserved in all GBS strains and serotypes. As mentioned above, the CpsA protein contains an additional extracellular domain, called the accessory domain, that is not found in the other LCP family members. Notations in the genomic databases identify this domain by its homology to the DNA polymerase processivity factor (DNA_PPF) of phage and eukaryotic DNA polymerases. However, the homology is quite limited and this domain

is found on the outside of the cell and, therefore, not likely to play a role in DNA replication. Analysis of the crystal structure of the extracellular domains of the *Streptococcus pneumoniae* Cps2A protein reveals that the accessory domain folds independently of the LCP domain (35). There is high homology between the pneumococcal and GBS CpsA proteins (50% identical and 69% similarity at the amino acid level), suggesting conserved topology.

Previous work with ectopic expression of extracellularly truncated GBS CpsA protein identified a dominant negative effect on capsule expression when the CpsA-245 protein, (containing only the accessory domain in the absence of the LCP domain) was expressed in the wild type strain (38). Additionally, ectopic expression of this truncation caused abnormal cell wall phenotypes leading to the production of long chains of cocci. However, episomal expression of the CpsA-full protein or the CpsA-117 protein did not cause a dominant negative effect on capsule or cell wall phenotypes. These results indicate that the accessory domain, in the absence of the LCP domain, resulted in aberrant function with a decrease in capsule production and cell wall integrity. Serial truncations constructed in this study identified two regions of the accessory domain that are key to the dominant negative effect.

A dominant negative effect can occur when a mutant copy of a protein interferes with the function of the wild type protein. This can be accomplished through direct interaction of the mutant copy with the wild type protein, thereby blocking function, or through the interaction of the mutant copy of the protein with the normal binding partners or substrates required for proper function of the wild type protein. In the case of the CpsA protein, we demonstrated that the dominant negative effect occurs through

expression of the protein episomally from within the cell, or exogenously by adding a purified truncated CpsA protein or synthetic CpsA peptide to the extracellular environment. The demonstration of the fluorescent peptide binding to both the wild type and the *cpsA* deletion bacteria indicates that the dominant negative effect is not produced by direct interaction with wild type CpsA protein. The ability of addition of truncated purified protein or synthetic peptide to induce a dominant negative effect when added to culture, without requiring cellular expression of the mutant protein supports a scenario in which the accessory domain/peptide interferes with mechanisms required for the normal cell wall integrity and capsule production.

Disruption of LCP family proteins leads to cell wall and cell division abnormalities in multiple bacterial species. In *Streptococcus mutans* mutation of the LCP family member *brpA* led to abnormal cell division events and atypical cell wall morphology (85, 88). *S. mutans lytR* mutants also have disregulated cell division leading to the formation of long chains of cocci (89). *Staphylococcus aureus* triple *lcp* mutants have unusually thin cell walls and aberrantly located division septae (84, 87) and are hypersensitive to the cell wall targeting antimicrobials bacitracin (86) and oxacillin (90). Mutation of the *S. aureus msrR* gene, encoding an LCP family member, causes cells to be unusually large and have misplaced division septae (91). Double LCP mutants in *Streptococcus pneumoniae* grew more slowly and had cell division septae placed in aberrant locations, leading to cocci of uneven size (82) as did mutations in the pneumococcal *lytR* alone (92). Mutation of the *Enterococcus hirae psr* gene led to increased sensitivity to the cell wall targeting antimicrobial, lysozyme (93). Together

these studies indicate that LCP family members play key roles in stability of the Gram-positive cell wall and in proper cell division.

Our data similarly show disruption of the GBS cell wall and cell division in *cpsA* mutant strains or through binding of the CpsA peptide to the wild type strain causing the formation of long chains of cocci. Visualization of FITC-labeled peptide that contains 24 amino acids from the C-terminal end of the CpsA accessory domain binding to the midcell region of the bacteria, potentially at the division septae, is the first time that CpsA has been shown to interact physically with the cell division process, though LCP family members of *S. pneumoniae* also have been shown to be localized at the midcell septal region (82). This interaction results in wild type strains forming abnormally long chains of cocci. This phenotype of bacteria forming abnormally long chains of cocci, with cell walls of uneven thickness and multiple division septae forming per cell also occurs when the accessory domain is expressed in the absence of the LCP domain, either through episomal expression of truncated CpsA in the *cpsA* deletion strain, or in the case of the *cpsA*-insert strain, which has expression of a chromosomal truncated CpsA. These results suggest that either the LCP domain is required for proper function, or the accessory domain is participating in aberrant interactions preventing proper function of the protein. This provides an explanation as to why the episomally expressed CpsA protein truncations containing all or part of the accessory domain (*cpsA*-245, -210, -187, -153 and -132) cannot complement for capsule expression when expressed in the *cpsA* deletion strain (figure 3). However, episomal expression of the CpsA protein truncation that removes the entire extracellular domain (CpsA-117) is able to complement for capsule expression when expressed in the *cpsA* deletion strain.

Collectively, these results demonstrate that the intracellular and transmembrane domains alone of CpsA are able to complement the deletion *in vitro*, but only in the absence of the accessory domain.

The CpsA protein of *S. pneumoniae* as well as the zoonotic fish pathogen *Streptococcus iniae* has been implicated in regulation of expression of the *cps* locus genes and in virulence (37, 39-41, 45, 47, 81). Proper regulation of *cps* gene expression is also critical for invasive infection by *S. pneumoniae*. Ectopic expression of the *S. pneumoniae cps* locus from a constitutively strong or weak promoter caused attenuation of colonization and virulence in animal models (44). *S. pneumoniae* strains that are more invasive have higher expression of *cpsA* in *in vitro* growth, indicating that *cpsA* has a role in virulence in invasive infections (42). Similarly, an *S. iniae* strain with an insertion in the *cpsA* gene had reduced capsule expression and reduced lethality and ability to disseminate to major organs in a zebrafish model of systemic disease (81).

The GBS *cpsA*-insertion strain analyzed in this work carries a non-polar chromosomal insertion in the *cpsA* gene, resulting in expression of a truncated CpsA containing the accessory domain but missing the LCP domain. As was shown for *S. iniae* and *S. pneumoniae*, GBS CpsA mutants are also attenuated for virulence. We demonstrated that the *cpsA*-insertion strain produces less capsule than the *cpsA* deletion strain and is further attenuated in human blood and in dissemination to major zebrafish organs than the *cpsA* deletion strain, indicating expression of a truncated CpsA is even more detrimental to GBS virulence than the complete absence of CpsA. This suggests that the aberrant function of the CpsA accessory domain, either alone or in the presence of the wild type CpsA protein, plays an important role in virulence. This

could be at the level of capsule dysregulation, or cell wall instability could be causing increased bacterial lysis *in vivo*, or a combination of both.

While this truncation of the CpsA protein is causing a function that we assume is aberrant for the normal full length CpsA, conversely, the accessory domain may have a function *in vivo* of controlling downregulation of capsule production when in particular host environments. Specific conformations of the full-length protein may allow the accessory domain to interact with CpsA binding partners in a negative way, thereby, causing inhibition of capsule production and cell wall synthesis/division. The recent crystal structure of the *S. pneumoniae* Cps2A revealed a region that could not be modeled, containing a disordered loop (35, 82). When comparing this structure to the GBS CpsA, we find that the disordered loop region is in the most C-terminal region of the accessory domain, the region responsible for the dominant negative effect of the CpsA-245 protein. Therefore, this region of the protein may take on multiple conformations in response to sensing of the local environment, allowing positive or negative interactions with components of the capsule production/cell wall machinery.

The ability to produce the dominant negative effect from extracellular addition of the CpsA peptide has obvious implications for treatment and/or prevention of GBS infection. Since GBS treated with the dominant negative factor have less capsule, this would allow clearance by the host immune system. Furthermore, these bacteria also have aberrant cell walls potentially making them more susceptible to lysis *in vivo*. Research is currently in progress to further exploit and optimize these phenotypes. Taken together, this study provides additional information on the roles of the extracellular domains of GBS CpsA in capsule expression, cell wall integrity and in

virulence of GBS. This supports a role of LCP family members as serving key functions in regulation of cell envelope polysaccharide expression and cell wall integrity and as virulence factors of Gram-positive bacteria.

CHAPTER 3: EFFECTS BY LEUCINE ZIPPER MOTIF OF CPSA ON GBS CELL ENVELOPE

Introduction:

The leucine zipper motif is a common protein-protein interaction domain. Leucine zipper motifs are characterized by 5-6 leucines 6 amino acids apart, to allow all to be on the same face of an alpha helix. These motifs participate in homo- or heterodimerization of proteins. Most often this domain stabilizes the interaction of transcriptional regulators with their cognate DNA sequences. (94).

Leucine zipper motifs were first discovered in eukaryotic transcriptional regulation, C/EBP α and Fos-Jun being the earliest and best characterized. Later work showed these motifs were also found in bacteria. The DnaA protein, involved in bacterial chromosome origin of replication recognition, forms a homomultimeric protein complex aided by protein-protein interactions at an N-terminal leucine zipper motif (95-97). Global transcriptional regulatory proteins of bacteria including PrfA of *Listeria monocytogenes* (98), VirB of *Shigella flexneri* (99), and RegA of *Citrobacter rodentium* (100) are leucine rich and contain putative leucine zipper motifs. The LacR repressor of carbohydrate catabolism genes of *Escherichia coli* requires tetramerization for full function, while radiation induced damage to the leucine zipper motif blocks tetramerization (101).

Leucine zipper motifs have other diverse functions in bacterial proteins. A leucine zipper motif is important to stabilize the multimeric complex important for protease function in the essential protease FtsH of *Escherichia coli* (102). A cell surface and secreted protein, Lzp, of *Streptococcus pyogenes* functions as a virulence factor binding

human immunoglobulins for immunoevasion by the pathogen (103). The plant pathogen *Agrobacterium tumefaciens* requires a type IV secretion system for virulence, and a leucine zipper motif is key for formation of the required pilus (104).

Many bacterial cell division proteins contain leucine zipper motifs. The chromosome segregation protein, ParB, of *Pseudomonas aeruginosa* contains a leucine zipper motif important for dimerization and DNA-protein interactions at the *parC* sites and also interaction with its partner ParA for proper nucleoid segregation (105). These motifs are also important for proper assembly of the divisosome complex in *E. coli* (106, 107). In *Bacillus subtilis* and *Staphylococcus aureus* a leucine zipper motif in the YlaN proteins is important for cell shape (108, 109).

We identified a leucine zipper motif in the first transmembrane domain of the CpsA protein of GBS (figure 11). A transmembrane localization of a leucine zipper motif is not unusual (104, 110, 111). We demonstrate that this motif is important for CpsA functions in capsule production and cell wall integrity. Episomal expression of CpsA protein with a mutation in the leucine zipper motif fails to complement a *cpsA* deletion strain for capsule production and causes a dominant negative effect on capsule production when expressed in the wild type strain. Further, episomal expression of leucine zipper mutant CpsA leads to formation of long chains of cocci and cell wall and division abnormalities. Also, purified CpsA protein with the leucine zipper motif mutated is less stable in vitro. *In vivo* multimerization assays, of tagged wild type and leucine zipper mutant CpsA expressed in *E. coli*, showed that this motif is not necessary for multimerization of CpsA.

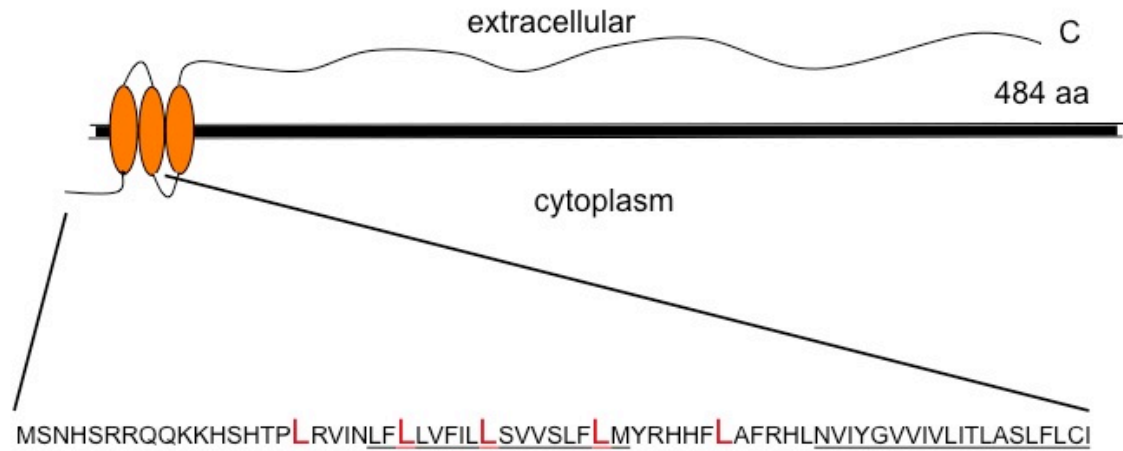


Figure 11: Schematic of CpsA showing topology and sequence of first two N-terminal transmembrane domains: leucine zipper motif in red and larger font, transmembrane domains underlined.

Materials and Methods:

Bacterial strains and growth conditions:

Leucine zipper mutant *cpsA* for overexpression in GBS was made using splicing by overlap extension PCR to change the three internal leucines (L24, L31, L38) of the motif to alanines using primers 5' MBP-BglIII site and 3' *cpsA* L to A, and 5' *cpsA* L to A and 3' *cpsA* BglIII site (see table 2 for sequences) and using a 1500 bp fragment digested with BglIII from pGBS-*cpsA*-full (Hanson, 2012) as a template, then ligated back into pGBS-*cpsA*-full to create pGBS-*cpsA*-leu zip. This allowed the protein to be expressed as a maltose binding protein (MBP) fusion, allowing for identification using anti-MBP antibody in Western Blot analysis. The construct was expressed in pLZ12-*rofA* allowing constitutive expression in GBS.

Extracellularly truncated leucine zipper mutant *cpsA* for overexpression in GBS was made using pGBS-*cpsA*-leu zip as template and primers 5' MBP RBS BamHI and 3' GBS *cpsA* 117-STOP-PstI (see table 2). It was cloned into the BamHI and PstI sites of pLZ12-*rofA* to allow constitutive expression in GBS.

Leucine zipper mutant *cpsA* for MBP purification was made using splicing by overlap extension PCR and primers: 5'GBS-*cpsA*-MBP-SmaI and 3' *cpsA* L to A, and 5' *cpsA* L to A and 3' GBS *cpsA*-full-PstI (see table 2 for sequences) and 515 genomic DNA as a template. It was then cloned into the XmaI and PstI sites of pMal-c2x (NEB) and purified as described previously (Hanson 2012).

Leucine zipper mutant *cpsA* for C-terminal intein-chitin binding protein (CBP) purification and detection with anti-CBP antibody via Western Blot was made using splicing by overlap extension PCR and primers 5'GBS *cpsA* intein NdeI and 3' *cpsA* L to

Table 2: Primers used in chapter 3

| | |
|---------------------------|--|
| 5' MBP-BglII site | CAAAGATCTGCTGCCGAACC |
| 5' cpsA-L to A | GAT TAA TTT ATT TGC TTT GGT GAT TTT TAT TTT GGC AAG TGT AGT CTC ATT ATT TGC TAT GTA TCG TCA CC |
| 3' cpsA-L to A | GGT GAC GAT ACA TAG CAA ATA ATG AGA CTA CAC TTG CCA AAA TAA AAA TCA CCA AAG CAA ATA AAT TAA TC |
| 3' cpsA BglII site | CAT CAG ATC TTG ATA CTG TTG |
| 5' MBP-RBS-BamHI | CGC GGA TCC GCG GAT AAC AAT TTC ACA CAG G |
| 3' GBS-cpsA-117-STOP-PstI | AAA ACT GCA GTT ACT CAA TTT CAG AGT ATC AAG C |
| 5' GBS-cpsA-MBP-SmaI | TCC CCC GGG TCT AAT CAT TCG CGC CGT C |
| 3' GBS-cpsA-Full-PstI | AAA ACT GCA GTT ATT CCT CCA TTG TGT TC |
| 5' GBS cpsA intein NdeI | GGA ATT CCA TAT GTC TAA TCA TTC GGC G |
| 3' GBS cpsA intein SapI | GAG GGC TCT TCC GCA TTC CTC CAT TGT GTT C |
| 5' RBS intein BamHI | CGCGGATCCGAAGGAGATATACATATG |
| 3' intein PstI | TGCACTGCAGCAGGTCATTGAAGCTGCC |
| 5-GBS CpsA-RBS-BamHI | CGCGGATCCGTATTGGAGAATTACAATGTCTAA TC |
| 3-GBS CpsA-FLAG-PstI | AAA ACT GCAGTCACTTGTCTCATCGTCCTTGTA GTCTTCCTCCATTGTG TTCTTAATTTTTGA |

A, and 5' *cpsA* L to A and 3' GBS *cpsA* intein SapI (see table 2 for sequences) and 515 genomic DNA as a template and cloned into the NdeI and SapI sites of pTXB1 (NEB) to make pTXB1-*cpsA*-leu zip. Wild type *cpsA* for C-terminal intein purification was made using primers 5' GBS *cpsA* intein NdeI and 3' GBS *cpsA* intein PstI (see table 2 for sequences) and 515 genomic DNA as a template and cloned into the NdeI and SapI sites of pTXB1 to make pTXB1-*cpsA*. CpsA-intein-CBP fusion proteins for over expression in GBS were made using primers 5' RBS intein BamHI and 3' intein PstI (see table 2 for sequences) and the pTXB1-*cpsA* and pTXB1-*cpsA*-leu zip plasmids as templates, and cloned into the BamHI and PstI sites of pLZ12 to make pGBS-*cpsA*-intein and pGBS-*cpsA*-leu zip-intein respectively.

C terminally FLAG tagged CpsA for FLAG purification and detection with anti-FLAG antibody via Western Blot was made using primers 5' GBS *cpsA* RBS BamHI and 3' GBS *cpsA* FLAG PstI using 515 genomic DNA as template. Leucine zipper mutant CpsA with C-terminal FLAG tag was made using splicing by overlap extension PCR and primers 5' GBS *cpsA* RBS BamHI and 3' *cpsA* L to A, and 5' *cpsA* L to A and 3' GBS *cpsA* FLAG PstI (see table 2 for sequences) and 515 genomic DNA as template. Digested PCR products were cloned into the BamHI and PstI sites of pLZ12 *rofA* to make pGBS *cpsA* FLAG and pGBS *cpsA* leu zip-FLAG respectively.

Percoll buoyant density centrifugation:

Buoyant density was determined using linear Percoll (GE Healthcare) gradients. Percoll solutions of low (1.085 g/mL) and high density (1.120 g/mL) were made and supplemented with a final concentration of 0.15mM NaCl. 2 mL low-density solution was carefully overlaid onto 2 mL high-density solution in a 5 mL Falcon tube (BD).

Tubes were placed at a 15-degree angle and allowed to form continuous gradients overnight. Tubes were placed vertically 30 minutes prior to assay to allow gradients to settle. Bacterial cultures were grown in Todd Hewitt (Alpha Biosciences) + 0.2% yeast extract (Acumedia) supplemented with appropriate selective antibiotics. Overnight cultures were normalized to $OD_{600}=0.6$ per 1 mL and concentrated to 50 μ L in phosphate buffered saline. Culture was carefully added to top of gradient and centrifuged 30 minutes at room temperature and 5000 rpms in a swinging bucket centrifuge (Eppendorf Centrifuge 5403 with Rotor 16A4-44). Distance of migration was measured from the bottom of the meniscus and density calculated from the distance of migration of beads of known density.

Microscopy:

Phase contrast microscopy was performed on overnight cultures of bacteria grown in Todd Hewitt (Alpha Biosciences) supplemented with 0.2% yeast extract (Acumedia) supplemented with appropriate selective antibiotics. Wet mounts were made of 3 mL culture and were photographed (Zeiss Axioscope 40 and imaged with Axiovision 4.7). Images are of representative fields of at least 3 biological replicates.

Fluorescent vancomycin staining was performed on midlog cultures. Bacteria were grown overnight and then subcultured 1:50 in Todd Hewitt (Alpha Biosciences) supplemented with 0.2% yeast extract (Acumedia) with appropriate selective antibiotics. At midlog ($OD_{600}=0.3$) cultures were concentrated to $OD_{600}=1.0$. 10 μ L culture and a final concentration of 1 μ g/ μ L BODIPY_FL vancomycin (Life Technologies) were incubated 10 minutes at 37 degrees to allow incorporation of stain into newly formed cell wall. Cultures were then washed 3 times in phosphate buffered saline and then

were resuspended in 3 μ L phosphate buffered saline and observed as a wet mount (Zeiss Axioscope 40 using a FITC filter and imaged with Axiovision 4.7). Images are of representative fields from 3 biological replicates.

SDS-PAGE analysis:

In vitro protein stability was determined by SDS-PAGE analysis. Equal amounts of CpsA-full-MBP (38) and CpsA-leu zip-MBP were mixed with 6X SDS loading buffer and boiled 15 minutes prior to loading a 12% SDS-PAGE gel. Samples were run at 83V through the stacking gel and voltage increased to 96V for resolving. Dye front was run off the bottom of the gel and stained using the Simply Blue Safe Stain (Invitrogen).

Multimerization analysis:

In vitro multimerization was performed with purified MBP-cpsA full. 5 μ g protein was mixed with 0-1% formaldehyde for 10 minutes at room temperature. Crosslinker was quenched with 0.8 mM glycine and 6X SDS buffer was added. Samples were boiled 5 minutes and separated on a 12% SDS-PAGE gel, and stained using Simply-Blue Safe stain.

In vivo multimerization analysis were performed on *E. coli* cultures expressing pMal-cpsA constructs. Overnight cultures were diluted 1:100 in fresh media and grown 3 hours. They were then induced 2 hours with 0.3 mM IPTG. Cultures were normalized to OD₆₀₀=1.0 and washed three times in Phosphate Buffered Saline (PBS). Samples were crosslinked 30 minutes at room temperature with 2 mM DSP (Thermo Fisher). Crosslinker was quenched by resuspending sample in 20mM Tris, 20% Sucrose, 1mM EDTA buffer supplemented with HALT Protease inhibitor cocktail (Thermo Fisher). Samples were sonicated in a bath sonicator for 5 minutes of 10 second on/ 10 second

off pulses. Control (non crosslinked) and samples for crosslink reversal were mixed with 6x SDS sample buffer and crosslinked samples were mixed with 6x non-reducing SDS sample buffer and boiled 5 minutes. Samples were separated on a 6% SDS-PAGE gel, transferred to a PVDF membrane (SantaCruz), blocked with 4% milk in TBST buffer. Anti-MBP polyclonal serum was diluted 1:10,000 in blocking buffer and membrane incubated overnight at 4°C overnight with agitation. Primary antibody was washed off with three washes in TBST and goat-anti-rabbit conjugated to Alkaline Phosphatase was incubated for 3 hours at room temperature with agitation. Secondary antibody was washed off with three washes in TBST. The membrane was equilibrated 5 minutes in detection buffer, and then detected with BCIP at 0.1 µg/mL in detection buffer with agitation until bands developed.

Virulence Assays:

Zebrafish infections were performed by injecting fish intramuscularly with 10^6 cfu of midlog bacteria. Four hours post injection fish were sacrificed and spleens, hearts, and brains were isolated. Organs were homogenized and plated on CNA agar plates (Acumedia) supplemented with chloramphenicol to ensure that cfu count reflected bacteria that had maintained the plasmid during infection.

Results:

A leucine zipper motif was identified in CpsA, within the first transmembrane domain (figure 11). The three internal leucines of this motif were mutated to alanines using splicing by overlap extension PCR.

Capsule expression by leucine zipper mutant CpsA:

Deletion of *cpsA* leads to a reduction in capsule gene expression by RT-PCR and in capsule levels by ELISA (48) and buoyant density centrifugation (38). To determine the role of the leucine zipper motif in CpsA function, we expressed a leucine zipper mutant CpsA ectopically, utilizing the pGBS-*cpsA*-leu zip plasmid in wild type and Δ *cpsA* bacteria and performed buoyant density centrifugation (Figure 12). Capsule levels of wild type bacteria episomally expressing the leucine zipper mutant CpsA were significantly reduced compared to wild type bacteria with empty vector alone, indicating the leucine zipper mutant protein is exerting a dominant negative effect. Capsule levels of Δ *cpsA* bacteria episomally expressing the leucine zipper mutant CpsA were comparable to the Δ *cpsA* strain with empty vector, while capsule levels of Δ *cpsA* bacteria episomally expressing wild type CpsA were restored to wild type levels. This indicates that episomal expression of the leucine zipper mutant CpsA fails to complement the *cpsA* deletion, whereas episomal expression of the wild type CpsA does. This suggests that the leucine zipper domain plays a role in activation of capsule production. Additionally, since episomal expression of the leucine zipper mutant CpsA causes a decrease in capsule levels of wild type bacteria, this suggests that the leucine zipper motif is facilitating key protein-protein interactions of CpsA essential for capsule production.

Capsule production by extracellularly truncated leucine zipper mutant CpsA:

Truncation of the extracellular domain of the CpsA protein showed this region is key for protein function. An episomally expressed truncated CpsA missing the entire extracellular domain (the CpsA-117 truncation) can complement the *cpsA* deletion,

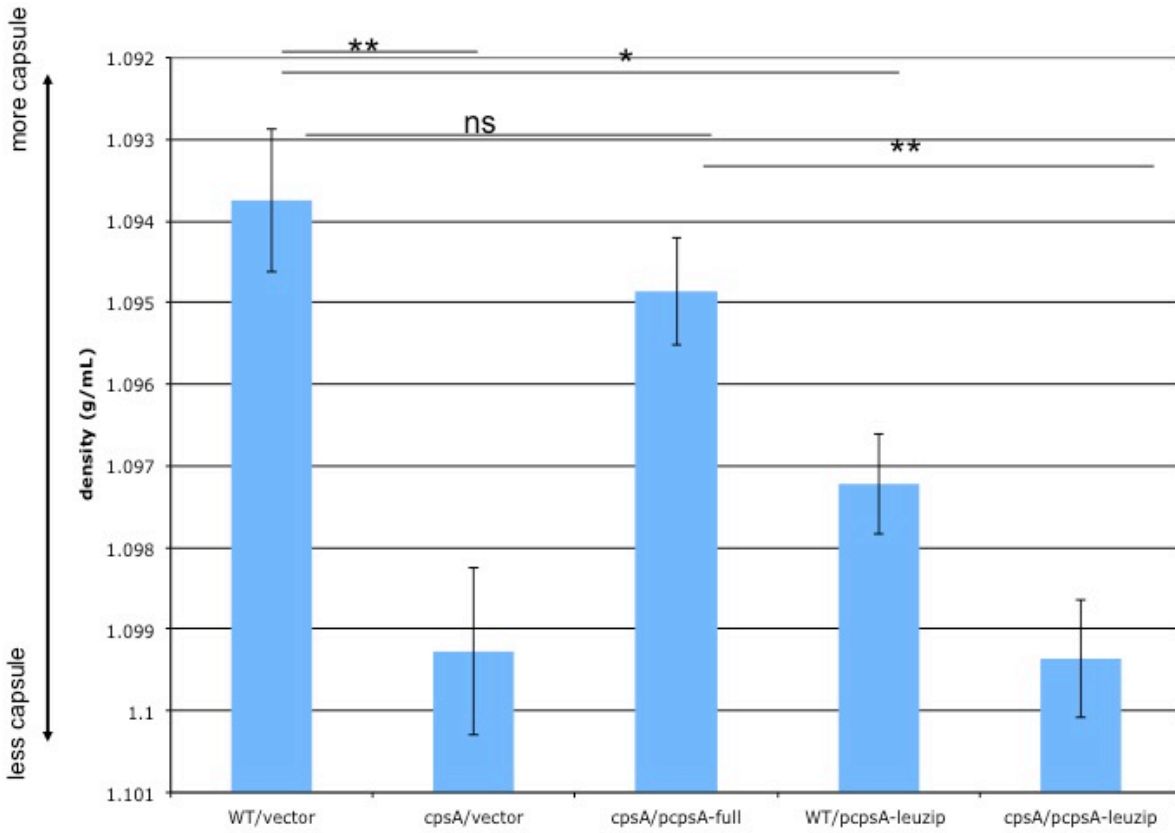


Figure 12: Capsule production with episomal expression of leucine zipper mutant CpsA. Percoll buoyant density centrifugation, ns=not significant, * $p < 0.05$, ** $p < 0.01$

whereas an episomally expressed truncated CpsA missing the most C terminal domain (the LCP domain) is unable to complement the *cpsA* deletion, and further, has a dominant negative effect when expressed in the wild type strain ((38), Chapter 2). Therefore we examined the effects of the leucine zipper motif in the absence of the extracellular domain and constructed an episomal CpsA-117 leucine zipper mutant. This mutant, when expressed in wild type, has a dominant negative effect on capsule production, whereas the episomally expressed wild type CpsA-117, in a wild type strain, has wild type capsule levels. Further, the leucine zipper mutant CpsA-117 cannot complement the *cpsA* deletion, while the wild type CpsA-117 is capable of complementing the deletion (Figure 13). This shows that the leucine zipper motif is important for the function of the CpsA protein, even in the absence of the extracellular domain.

Cell wall phenotypes from episomal expression of leucine zipper mutant CpsA:

Deletion of *cpsA* leads to a long chain phenotype in GBS, as does episomal expression of a truncated form of CpsA missing the C terminal LCP domain (38). This long chain phenotype is indicative of cell wall abnormalities when CpsA is not present or is non-functional, as in the Δ LCP form of CpsA. To determine the effects of the leucine zipper motif on chain length we observed overnight cultures under phase contrast microscopy (Figure 14A). Wild type bacteria expressing the leucine zipper mutant CpsA protein formed longer chains than wild type harboring empty vector alone. Additionally, the cocci were larger and the chains appeared to be kinked. This indicates that in addition to the dominant negative effect on capsule production (Figure 14) the leucine zipper mutant further exerts a dominant negative effect in cell wall maintenance. Δ *cpsA*

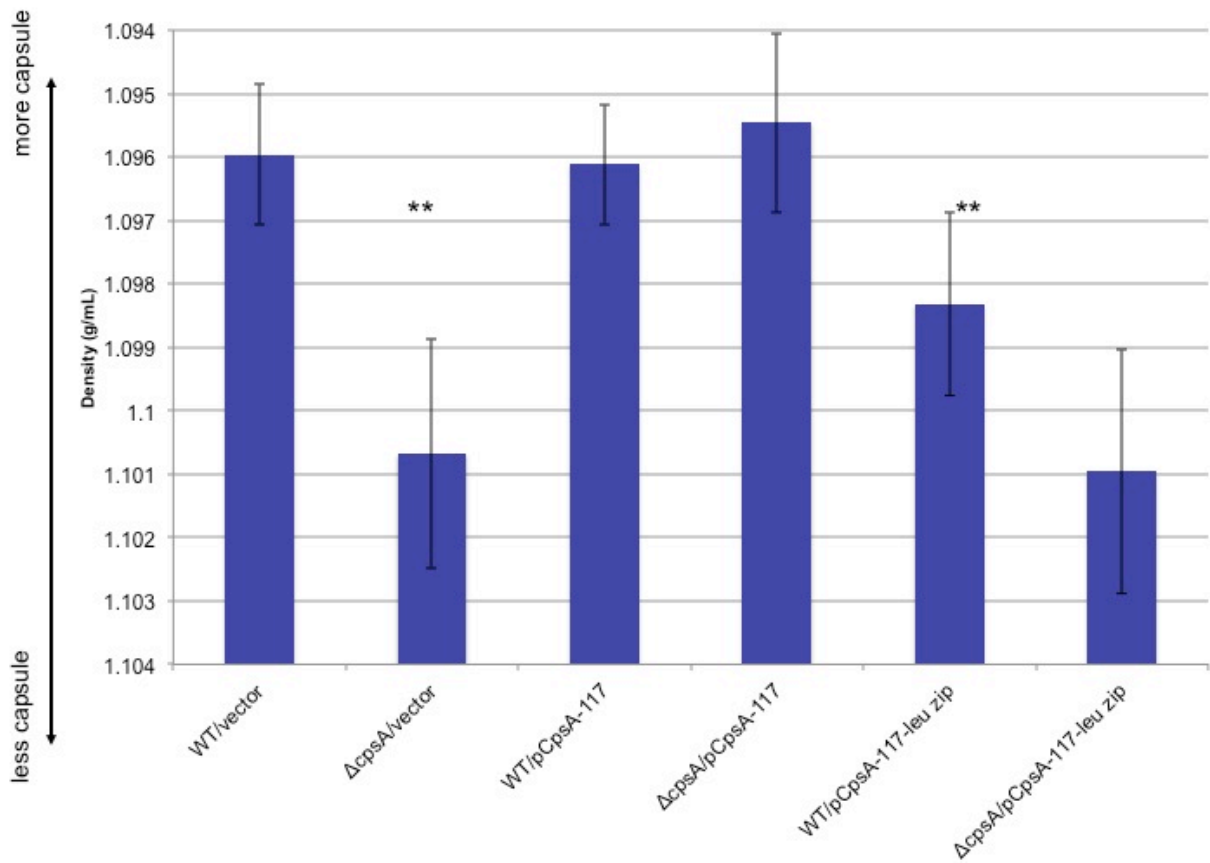


Figure 13: Capsule production with episomally expressed leucine zipper mutant CpsA-117 truncation. Percoll buoyant density centrifugation, **p<0.01.

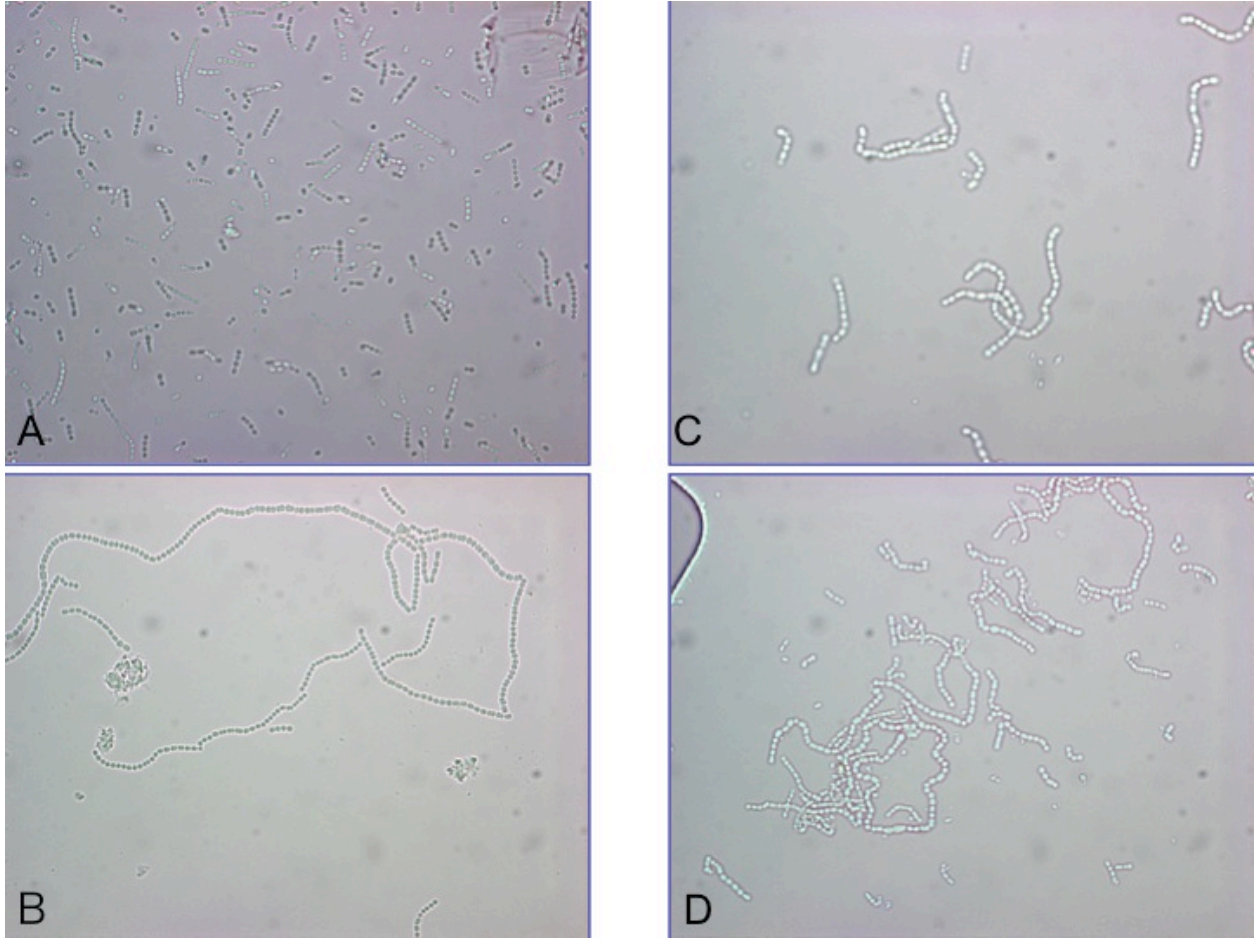


Figure 14: Phase contrast microscopy of episomally expressed leucine zipper mutant CpsA. A) WT/vector, B) $\Delta cpsA$ /vector, C) WT/pCpsA-leuzip, D) $\Delta cpsA$ /pCpsA-leuzip. All at 1000x magnification.

bacteria episomally expressing leucine zipper mutant CpsA formed chains of similar length to $\Delta cpsA$ with empty vector, indicating that the leucine zipper mutant CpsA fails to complement the deletion, as in capsule levels. Furthermore, the chains of the $\Delta cpsA$ bacteria episomally expressing leucine zipper mutant CpsA appeared kinked and had larger cocci. This indicates the cell wall deficits are worse than a mere deletion of *cpsA*. To further examine possible cell wall defects, we performed fluorescent vancomycin staining (Figure 15). The antibacterial action of vancomycin is through the blocking of crosslinking of cell wall muropeptide subunits. Fluorescent vancomycin will therefore label newly formed cell wall by labeling only non-crosslinked muropeptide subunits. This allows visualization of the septae as this is where most cell wall is formed. Wild type bacteria (Figure 15A) with empty vector exhibit an even ring of cell wall around each coccus and a single bright pole per coccus, indicating the forming division septa. Episomal expression of the leucine zipper mutant CpsA (Figure 15C) in the wild type background leads to uneven cell wall thickness around each coccus and no clear pole indicating a forming division septae. Also, as in the phase contrast images, the chains appear kinked. $\Delta cpsA$ strain (Figure 15B) with empty vector formed long chains. Cell wall staining is less even around each coccus than in wild type, exhibiting thick staining at the septa, possibly indicating incomplete separation of cocci following division. All septae are located in the same plane, giving the chains a straight phenotype. $\Delta cpsA$ strain episomally expressing a leucine zipper mutant CpsA (Figure 15D) has uneven cell wall staining around each coccus. Some cocci exhibit multiple bright poles, indicating multiple septation events per cell. Furthermore these septae are not in the same plane, leading to the kinked chain morphology. Taken together, these data

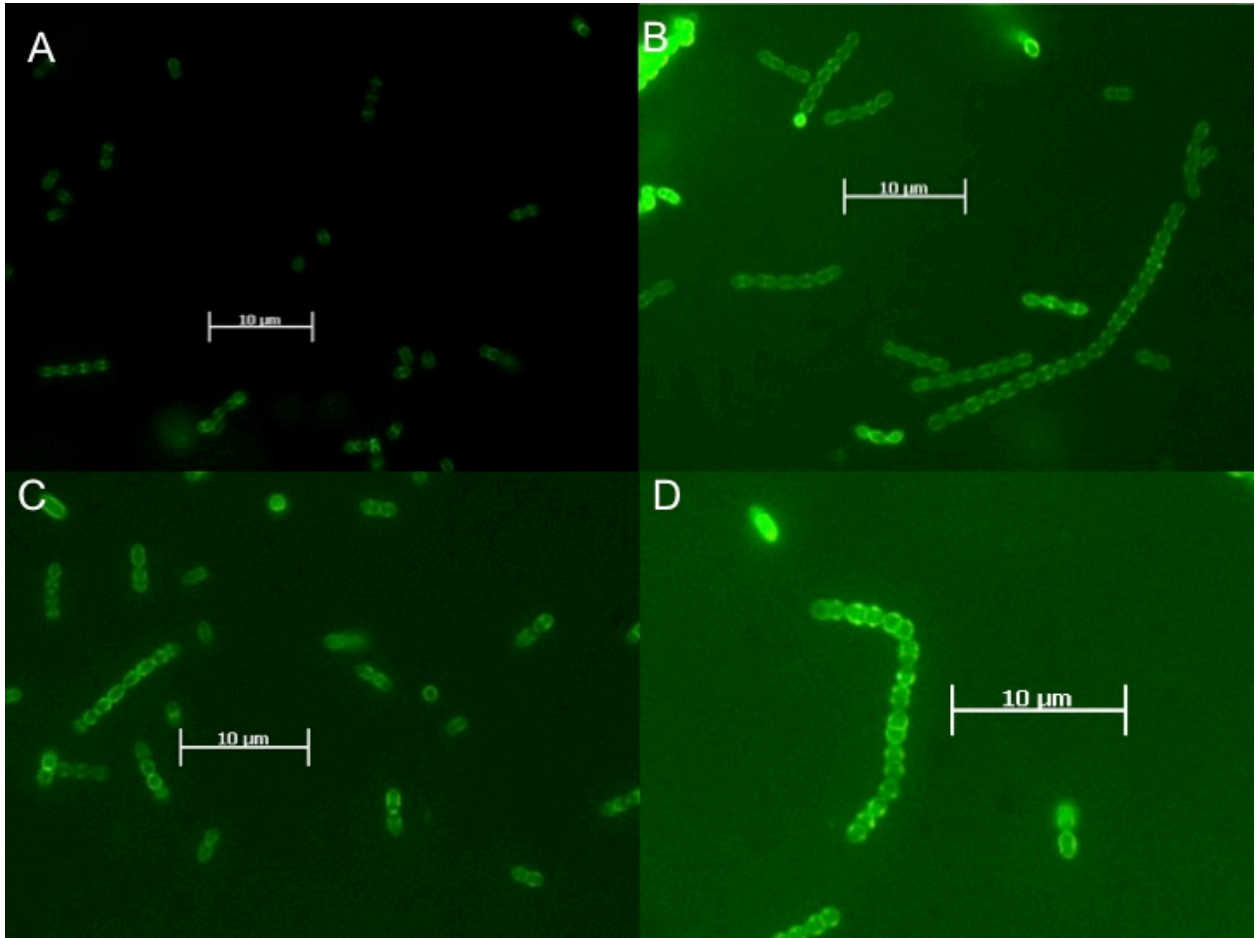


Figure 15: Fluorescent vancomycin staining of episomally expressed leucine zipper mutant CpsA. A) WT/vector, B) $\Delta cpsA$ /vector, C) WT/pCpsA-leuzip, D) $\Delta cpsA$ /pCpsA-leuzip. Scale bar indicates 10 μm.

suggest that CpsA is important for cell wall maintenance and cell division, and that the leucine zipper motif is important in these processes.

Stability of purified CpsA:

Since leucine zipper motifs are important for protein-DNA interactions and GBS CpsA is a DNA binding protein (38) we purified the leucine zipper mutant CpsA as an MBP fusion protein to examine the role of this motif in CpsA promoter binding. SDS-PAGE analysis of purified protein (Figure 16) indicated that this protein is not stable *in vitro*. Purified protein of the wild type sequence is primarily the expected 96 kDa fusion protein. However, the purified leucine zipper mutant protein is primarily the 43 kDa MBP alone degradation product, indicating that the mutation of the leucine zipper motif has made this protein unstable.

Multimerization of CpsA:

Leucine zipper motifs are also important for protein multimerization, therefore we examined the ability of CpsA and leucine zipper mutant CpsA to multimerize. The first experiment examined *in vitro* crosslinking using formaldehyde (figure 17). When adding formaldehyde to purified wild type protein, three multimers were visualized. Since the leucine zipper mutant purified protein was unstable and CpsA is a membrane protein, and membrane localization might be important to proper protein function, we examined multimerization of MBP fusion proteins expressed in *E. coli* (Figure 18). Both wild type and the leucine zipper mutant CpsA proteins when uncrosslinked are primarily the expected 96 kDa fusion protein, therefore *in vivo* we do not observe the protein stability issues that occurred with the purified protein *in vitro*. Reversing the crosslink restores the same species as the uncrosslinked control samples. The crosslinked samples do

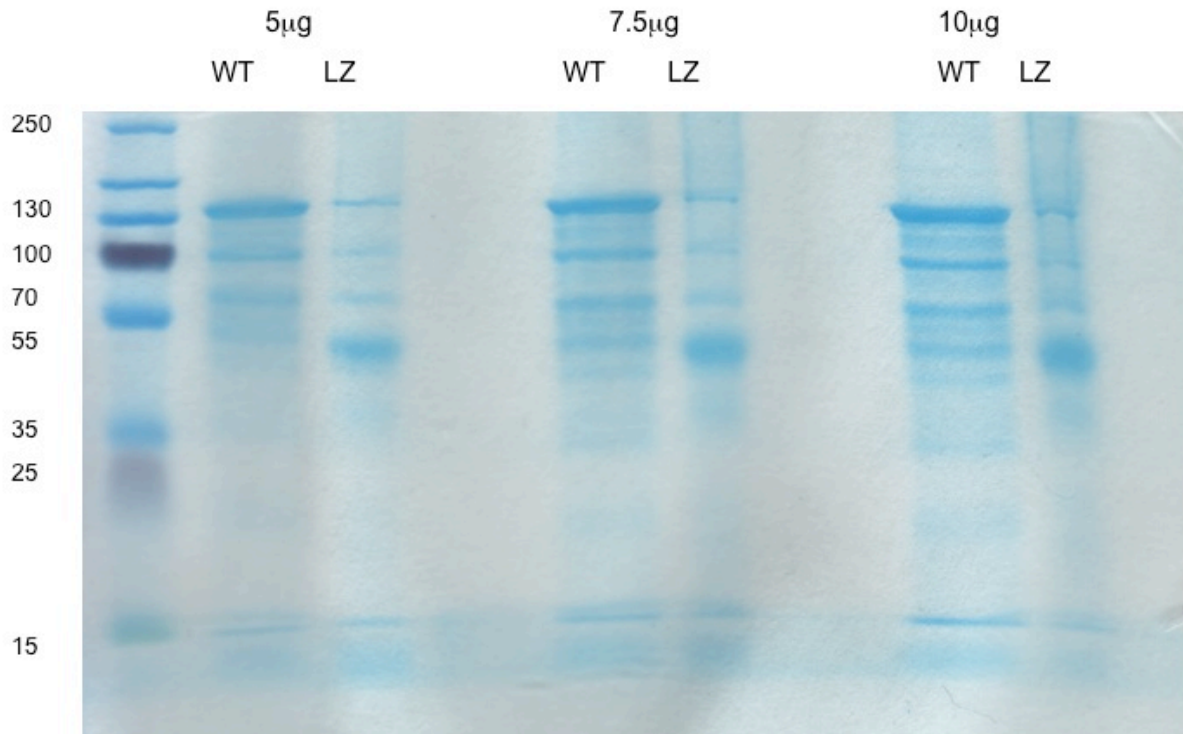


Figure 16: SDS-PAGE analysis of purified MBP-tagged wild type (WT) and leucine zipper mutant (LZ) CpsA. Indicated amount of purified protein was analyzed by SDS-PAGE to determine integrity of purified protein.

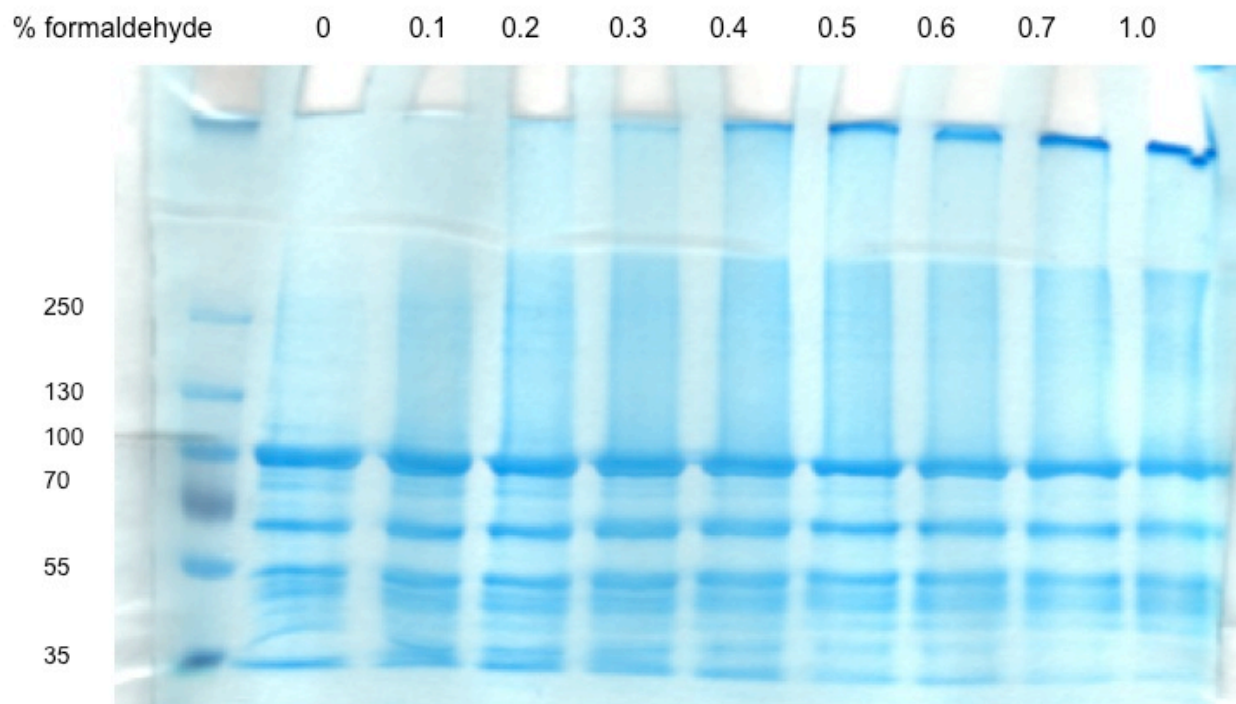


Figure 17: Formaldehyde crosslinking of purified MBP-tagged wild type CpsA. Purified protein was incubated with indicated concentration of formaldehyde and then analyzed for multimerization by SDS-PAGE.

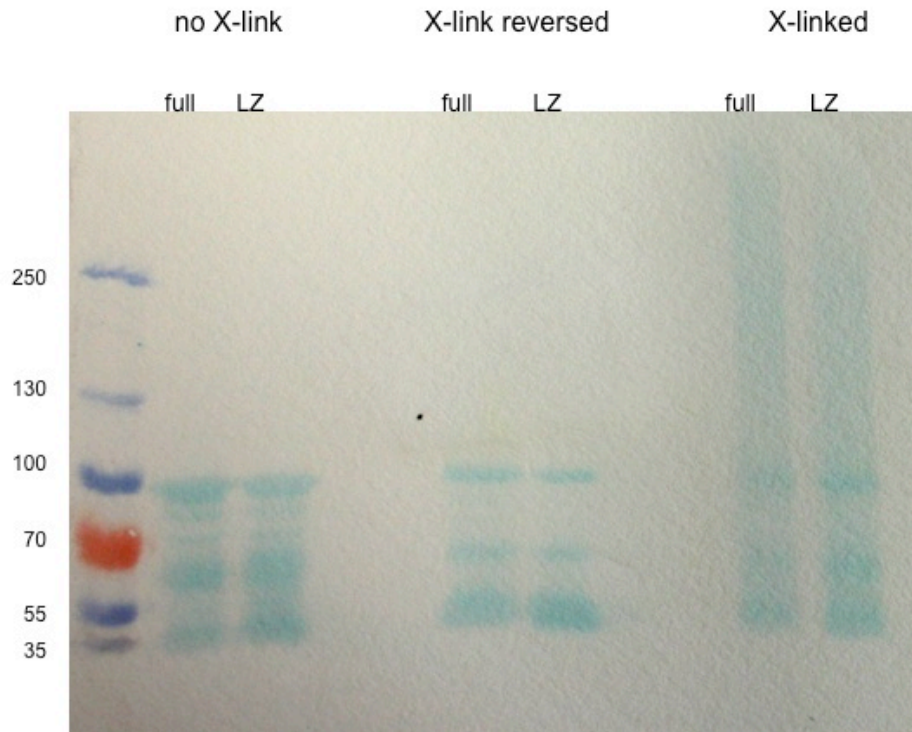


Figure 18: *in vivo* crosslinking of *E. coli* lysates expressing MBP-tagged wild type CpsA (full) or leucine zipper mutant CpsA (LZ). Protein expression was induced and then lysates were crosslinked by DSP, followed by sonication and Western Blot analysis to determine multimerization.

not contain clear bands indicating multimerization of either wild type or leucine zipper mutant CpsA. Therefore at this time we are unable to determine the role of the leucine zipper motif in multimerization of CpsA.

Since the extracellular domain of CpsA is also important for CpsA function ((38) and Chapter 2) and could potentially be participating in homomultimerization, we next examined the multimerization of the CpsA-117 and CpsA-leucine zipper mutant-117 in *E. coli* to determine the importance of the leucine zipper motif in multimerization in the absence of the extracellular domain (Figure 19). Both wild type CpsA-117 and leucine zipper mutant CpsA-117 are similarly stable *in vivo* in the no crosslink control samples, with the primary species being the expected 53 kDa fusion protein. Reversing the crosslink does not restore the same species as the uncrosslinked control indicating that there is an aggregation event unrelated to our crosslinking, so at this time we cannot determine the role of the leucine zipper motif in multimerization of extracellularly truncated CpsA.

C-terminally tagged CpsA:

In order to examine CpsA multimerization further and the role of the leucine zipper mutant in that multimerization other tagged versions of CpsA were generated. N-terminal MBP-tagged CpsA was never able to be shown to be expressed in GBS by western blot (data not shown). The possibility that N-terminal modifications were occurring led us to make C-terminal fusions. Additionally, having multiple tags available would allow for multiple options in coimmunoprecipitation studies. A C-terminal intein-chitin binding protein construct was produced. This construct, when expressed in GBS, was unable to complement the *cpsA* deletion strain for capsule production (Figure 20).

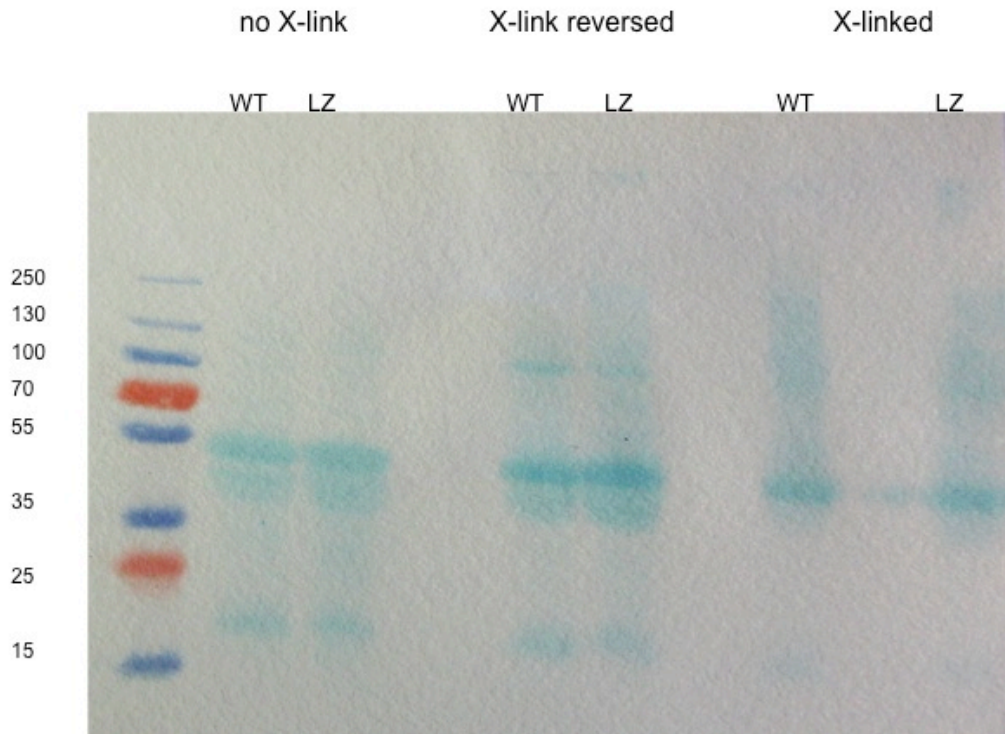


Figure 19: *in vivo* crosslinking of *E. coli* lysates expressing MBP-tagged wild type CpsA-117 (WT) or leucine zipper mutant CpsA-117 (LZ). Protein expression was induced and then lysates were crosslinked by DSP, followed by sonication and Western Blot analysis to determine multimerization.

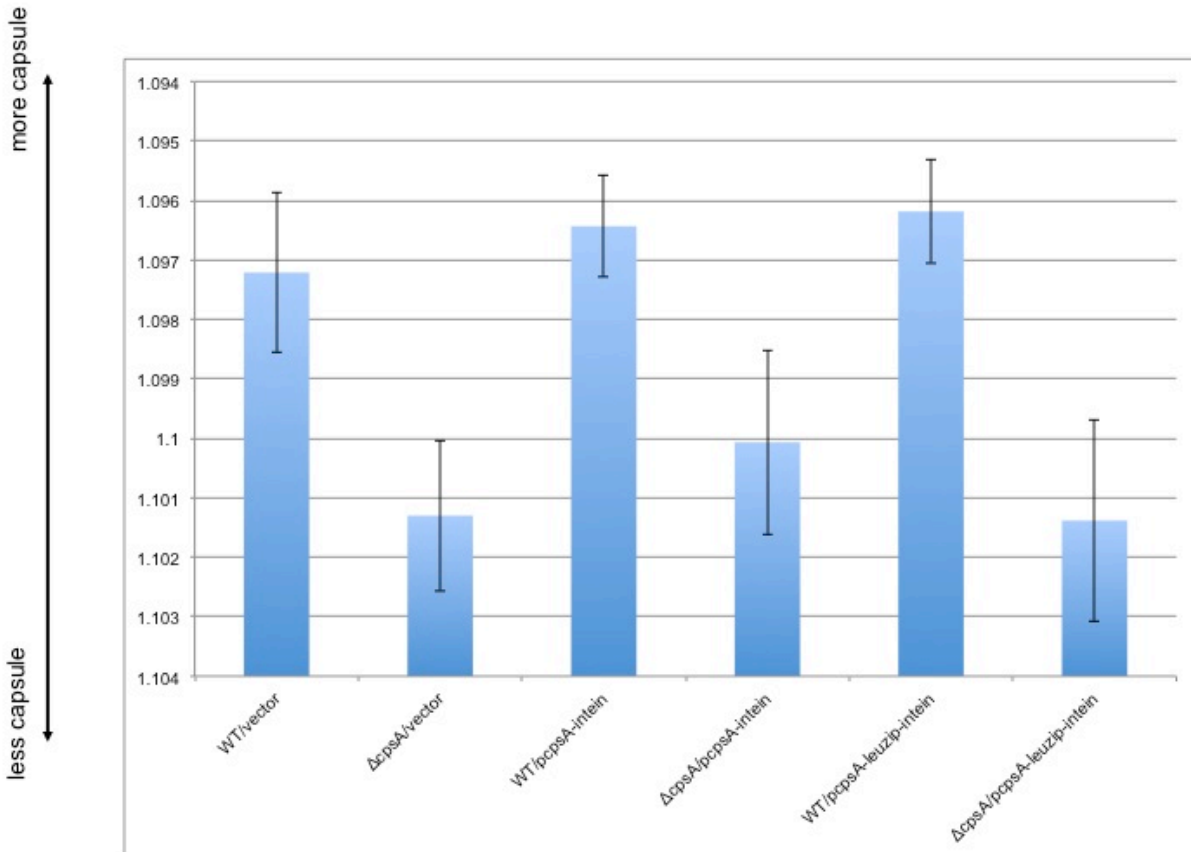


Figure 20: Capsule production by episomally expressed C-terminal intein-tagged CpsA and leucine zipper mutant CpsA. Percoll buoyant density centrifugation.

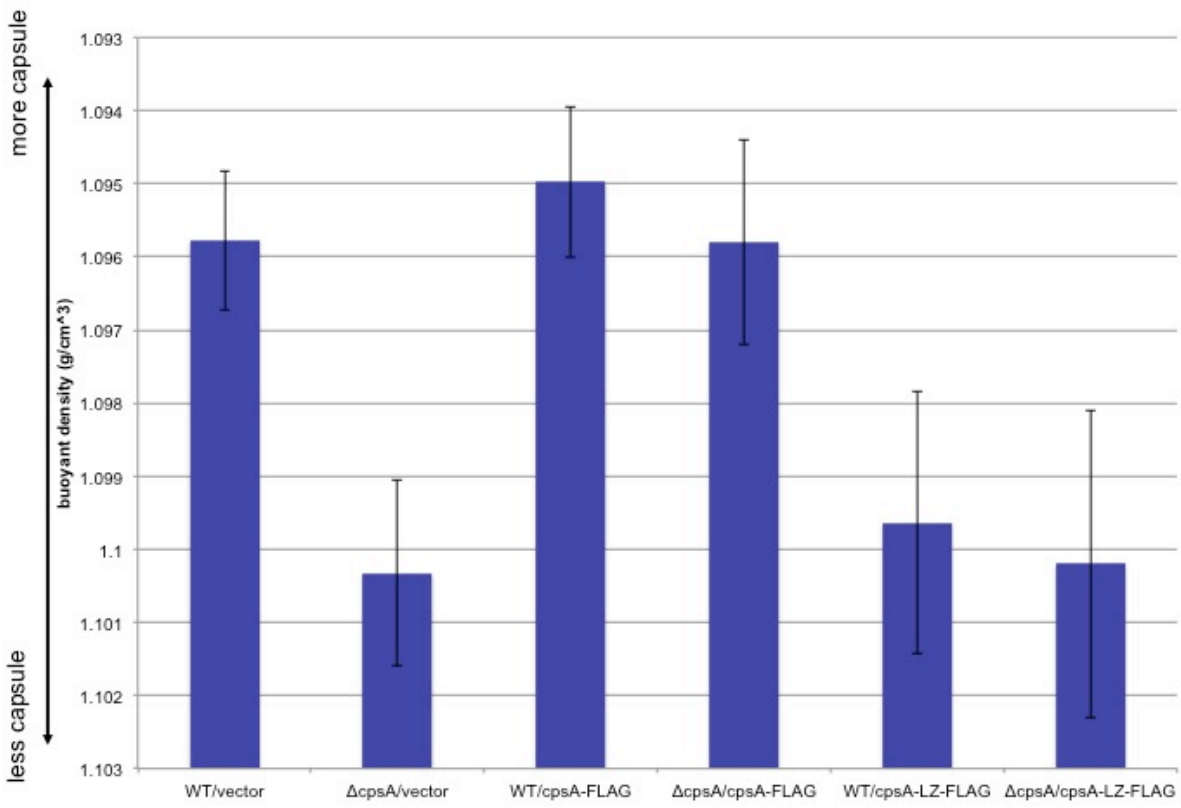


Figure 21: Capsule production by episomally expressed C-terminal FLAG-tagged CpsA and leucine zipper mutant CpsA. Percoll buoyant density centrifugation.

This could be due to the large C-terminal tag inhibiting the proper function of the extracellular domains.

C-terminally FLAG-tagged CpsA and leucine zipper mutant CpsA were also constructed. A FLAG-tagged wild type CpsA episomally expressed in the *cpsA* deletion strain was able to complement capsule production (Figure 21), indicating that the small C-terminal FLAG tag does not interfere with protein function. The FLAG-tagged leucine zipper mutant CpsA had a similar phenotype to the N-terminally MBP-tagged leucine zipper mutant CpsA in that it cannot complement the *cpsA* deletion strain for capsule production and has a dominant negative effect on capsule production when expressed in the wild type strain.

Virulence effects of episomally expressed leucine zipper mutant CpsA:

To examine virulence consequences of the leucine zipper motif, zebrafish were infected with the wild type and *cpsA* deletion strains with empty vector or expressing pGBS-*cpsA*-leu zip, and bacterial dissemination to spleens, hearts and brains was determined by plating for viable cfu four hours post infection (Figure 22). The *cpsA* deletion strain was attenuated in all three organs, and the *cpsA* deletion strain episomally expressing pGBS-*cpsA*-leu zip could not complement the deletion for dissemination to the spleen. The wild type strain, episomally expressing pGBS-*cpsA*-leu zip exhibited similar dissemination to the wild type strain with empty vector. This indicates that at least at early time points post infection, episomal expression of leucine zipper mutant CpsA in a wild type strain does not have a dominant negative effect on virulence. Episomal expression of a leucine zipper mutant *cpsA* in a *cpsA* deletion strain is able to complement the bacterial ability to disseminate to the heart and blood,

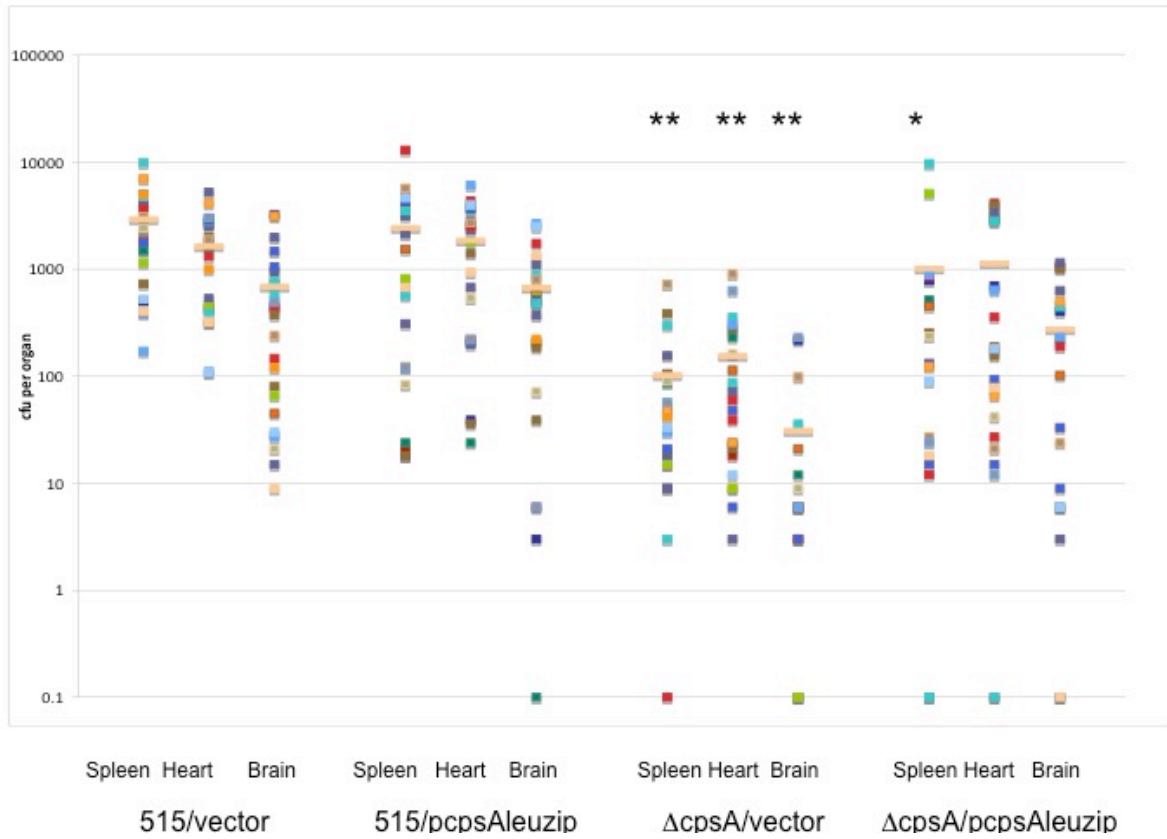


Figure 22: GBS dissemination to zebrafish major organs. 4 hpi cfu/organ following 10^6 cfu per fish intramuscular injection. Each point represents one fish, bar is mean. * $p < 0.05$, ** $p < 0.01$ compared to wild type cfu per organ.

at least at early time points post infection. Further analysis with a chromosomal leucine zipper mutant will be necessary to examine later time points post infection such that plasmid loss is not a factor.

Discussion:

The leucine zipper motif located in the first transmembrane domain of the CpsA protein is important for both the capsule expression and cell wall integrity/cell division functions of CpsA. Expression of a protein with the three internal leucines of this motif mutated to alanines is unable to complement a *cpsA* deletion strain for capsule production or cell wall integrity. Additionally, this mutant protein, when expressed episomally in the wild type strain, causes a dominant negative effect on both capsule expression and cell wall integrity. This suggests that the leucine zipper motif is mediating key interaction between CpsA and itself, as we have seen homomultimerization of wild type CpsA, and/or with its binding partners.

Further work needs to be done to see if the leucine zipper motif is acting to cause specific binding of DNA at the *cpsA* promoter. This will require purification of a stable leucine zipper mutant CpsA protein to perform electrophoretic mobility shift assays (EMSAs). If the FLAG-tagged leucine zipper mutant CpsA is also found to be unstable *in vitro*, purified membrane fraction containing CpsA or leucine zipper mutant CpsA could be used in EMSAs.

Also, further work on the multimerization of CpsA needs to be done. Better cross-linking *in vivo*, either in *E. coli* or ideally in GBS lysates, needs to be performed to see clear multimers of CpsA and determine the role of the leucine zipper motif. Additionally the binding partners of CpsA need to be determined. Co-

immunoprecipitation of both full length and CpsA-117 with and without the leucine zipper motif will identify the binding partners of CpsA that bind the extracellular domain (interact with CpsA full, and CpsA-leu zip-full, but not the CpsA-117 truncations) and that bind the leucine zipper motif (those that interact with CpsA full and CpsA-117, but not the leucine zipper mutants). Additionally, binding partners that interact at the leucine zipper motif could be predicted by bioinformatics identifying transmembrane leucine zipper motifs in other GBS proteins and further analyzed.

CHAPTER 4: ROLE OF LYTR IN CELL ENVELOPE MAINTAINENCE OF GBS

Introduction:

The LytR-CpsA-Psr (LCP) family of proteins is a widespread class of proteins from many Gram-positive bacterial species (34, 35). These proteins are involved in cell envelope regulation and stability. Recently, they have been implicated in the attachment of cell wall sugars, capsule and wall teichoic acids, to the peptidoglycan cell wall (35, 82, 83).

LytR is a transcriptional regulator of autolysin production in many Gram-positive species of bacteria(35, 92, 112-114). Additionally it has been implicated in the proper formation of division spetae in *Streptococcus pneumoniae* (92).

In many species of Gram-positive bacteria LCP family members have been shown to compensate for each other and disruption of multiple LCP proteins has been shown to lead to severe bacterial growth and survival deficits. In *Staphylococcus aureus* triple deletion of all three LCP proteins leads to bacteria without wall teichoic acids, instead releasing teichoic acid into the culture supernatant (115). *Streptococcus pneumoniae* double LCP mutants release capsule into the culture supernatant (82). Multiple deletion of LCP genes can be lethal in *Streptococcus mutans* (85) or cause severe growth deficits in the absence of compensatory gene mutations (35, 82, 86, 116).

In this work, we characterize an insertional mutantion disrupting the *lytR* gene of GBS. We disrupt *lytR* in both a wild type and *cpsA* deletion background to determine the effects of *lytR* individually and in combination with *cpsA* in bacterial growth, capsule production, cell wall integrity and virulence.

Materials and Methods:

Construction of LytR::pUC insert mutants:

The LytR insert was constructed by amplifying a 490 bp region from 515 genomic DNA using primers (5' GBS LytR ins EcoRI-CCGGAATTCGCTAAATCATCATGAAGAGC- and 3' GBS LytR ins PstI-AA~~AA~~ACTGCAGTTAAGCTCCCATCAACAGC) and cloned into the EcoRI and PstI sites of plasmid pUC19-Erm (45). Plasmid was electrotransformed as described previously (38) into GBS strain 515 and its isogenic *cpsA* deletion strain (48), single crossover recombinants were selected by resistance to Erythromycin on THY (Todd Hewitt (Acumedia) supplemented with 0.2% yeast extract (Acumedia) and 1.4% bacteriological agar (Acumedia)) Erm 2 µg/mL plates.

Bacterial growth curves:

Growth curves were done by normalizing overnight bacterial cultures to an $OD_{600}=1.0$ and then diluted 1:50 in fresh THY (Todd Hewitt (Alpha Biosciences) supplemented with 0.2% yeast extract (Acumedia)), and 200 µL of culture was put into a 96 well plate in triplicate and OD_{600} read every hour for 7 hours. Data is representative of three biological replicates.

Percoll gradient buoyant density analysis:

Buoyant density was determined using linear Percoll (GE Healthcare) gradients. Percoll solutions of low (1.085 g/mL) and high density (1.120 g/mL) were made and supplemented with a final concentration of 0.15mM NaCl. 2 mL low-density solution was carefully overlaid onto 2 mL high-density solution in a 5 mL Falcon tube (BD).

Tubes were placed at a 15-degree angle and allowed to form continuous gradients overnight. Tubes were placed vertically 30 minutes prior to assay to allow gradients to settle. Bacterial cultures were grown in Todd Hewitt (Alpha Biosciences) + 0.2% yeast extract (Acumedia). Overnight cultures were normalized to $OD_{600}=0.6$ per 1 mL and concentrated to 50 μ L in phosphate buffered saline (PBS). Culture was carefully added to top of gradient and centrifuged 30 minutes at room temperature and 5000 rpms in a swinging bucket centrifuge (Eppendorf Centrifuge 5403 with Rotor 16A4-44). Distance of migration was measured from the bottom of the meniscus and density calculated from the distance of migration of beads of known density (GE Healthcare). Data is average of four biological replicates.

Microscopy:

Phase contrast images were performed on overnight cultures grown in THY. 5 μ L of culture was placed on a microscope slide and mounted with a cover slip and visualized on a Zeiss Axioscope 40 and imaged with Axiovision 4.7. Images are of representative fields of at least 3 biological replicates.

Fluorescent vancomycin staining was performed on midlog cultures. Bacteria were grown overnight and then subcultured 1:50 in THY. At midlog ($OD_{600}=0.3$) cultures were concentrated to $OD_{600}=1.0$. 10 μ L culture and a final concentration of 1 μ g/ μ L BODIPY_FL vancomycin (Life Technologies) were incubated 10 minutes at 37 degrees to allow incorporation of stain into newly formed cell wall. Cultures were then washed 3 times in PBS and then were resuspended in 3 μ L PBS and observed as a wet mount (Zeiss Axioscope 40 using a FITC filter and imaged with Axiovision 4.7). Images are of representative fields from 3 biological replicates.

Virulence analysis:

Human whole blood infections were performed. Overnight bacterial cultures were diluted 1:50 in fresh THY and grown to midlog. At midlog, cultures were normalized to an $OD_{600}=0.3$, corresponding to 10^8 cfu/mL. Normalized cultures were resuspended in PBS, then diluted 1:10 in PBS. A 20 μ L inoculum (2×10^5 cfu) per 1 mL blood, in triplicate, was incubated 3 hours at 37°C with end over end rotation in 1.7 mL tubes (Dot Scientific). One hundred microliters was plated in duplicate per infection on THY agar and viable cfu enumerated. Log killed was determined by subtracting the log of the product cfu from the log of the inoculum cfu.

Murine macrophage infections were performed. RAW 267.4 murine macrophages were seeded at 10^6 cells per well of a 24 well plate and allowed to adhere overnight. Bacterial cultures were grown to midlog and normalized to $OD_{600}=0.3 = 10^8$ cfu/mL and diluted 1:100 in DMEM +10% fetal calf serum. Macrophages were washed three times in PBS, then infected for one hour. Non-adherent bacteria were removed with five washes in PBS. Extracellular bacteria were killed with 100 μ g/mL gentamycin in DMEM +10% FCS for one hour. Antibiotic was washed off with three PBS washes. One set of wells was lysed in 1 mL ddH₂O and plated for viable count at time =0. At 18 and 24 hours post infection the supernatant was removed to enumerate those bacteria that had emerged from the macrophages and cells were washed 5 times with PBS then lysed in 1 mL ddH₂O to enumerate those bacteria still cell associated.

Results:**Capsule production by LytR-insert mutants:**

The CpsA protein has a role in expression of capsule by GBS. Deletion of the *cpsA* gene or expression of a truncated CpsA protein leads to decreased capsule production (Chapter 2 and (38, 48)). We first examined the capsule production of the *lytR::pUC* mutant strain and the $\Delta cpsA$ *lytR::pUC* double mutant strain to determine the role of the LytR protein alone and in combination with the CpsA protein in capsule expression (Figure 23). Both single mutant strains exhibited a reduction in capsule expression compared to the wild type strain, but the strain with a double mutation had capsule levels further reduced beyond that of each of the single mutant strains. This indicates that the two proteins can partially compensate for each other to allow some capsule production, but in the absence of both proteins, capsule production is greatly reduced.

Growth effects of LytR mutation:

Since other organisms with multiple mutations in genes encoding LCP proteins have growth phenotypes (35, 82, 86, 116), we next examined the growth rates of the wild type strain, compared to the $\Delta cpsA$, *lytR::pUC*, and $\Delta cpsA$ *lytR::pUC* double mutant strains (Figure 24). The wild type strain and the single mutant strains exhibited similar growth kinetics, whereas the double mutant strain exhibited a longer lag phase and slower growth rate in log phase. This could indicate that the disruption of two LCP family members is so detrimental to GBS that it is preventing proper growth.

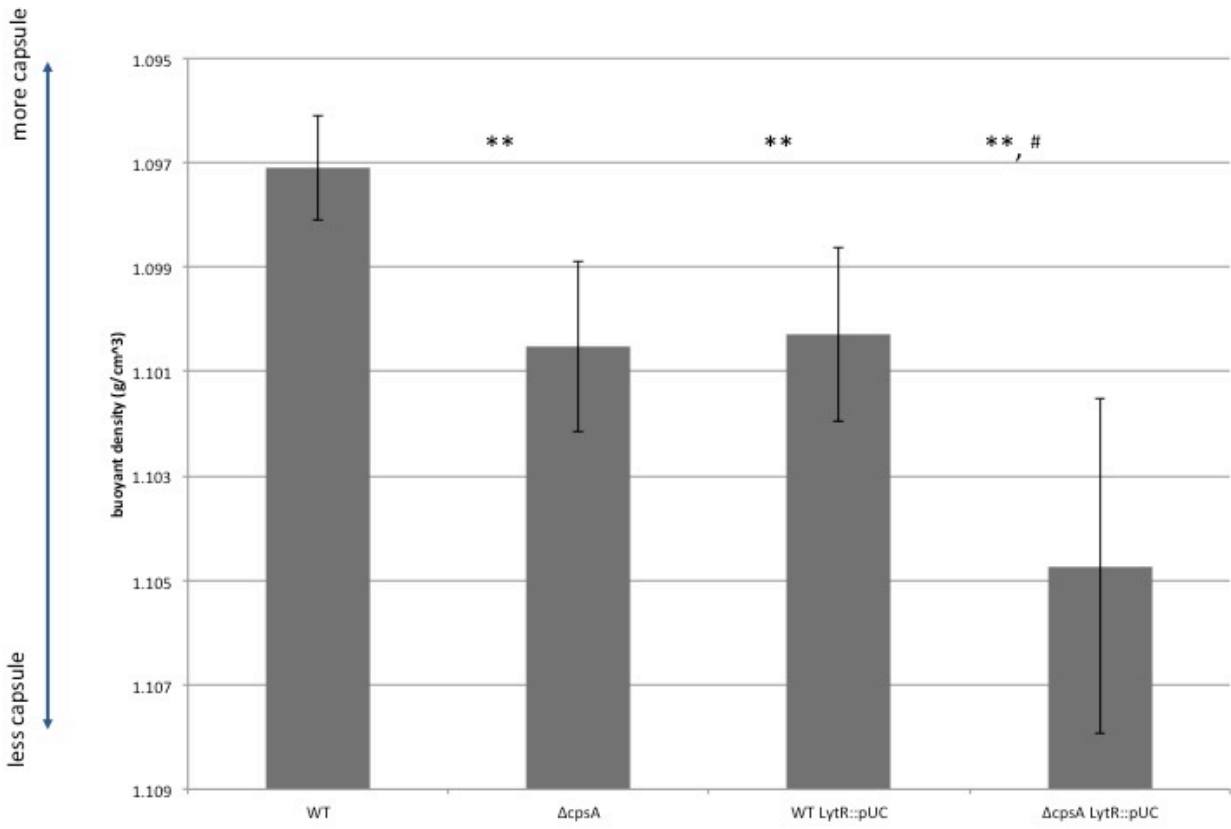


Figure 23: Capsule production by LytR::pUC strains. Percoll buoyant density centrifugation. **p<0.01 compared to wild type, ##p<0.01 compared to single ($\Delta cpsA$ or *lytR::pUC*) mutant strains.

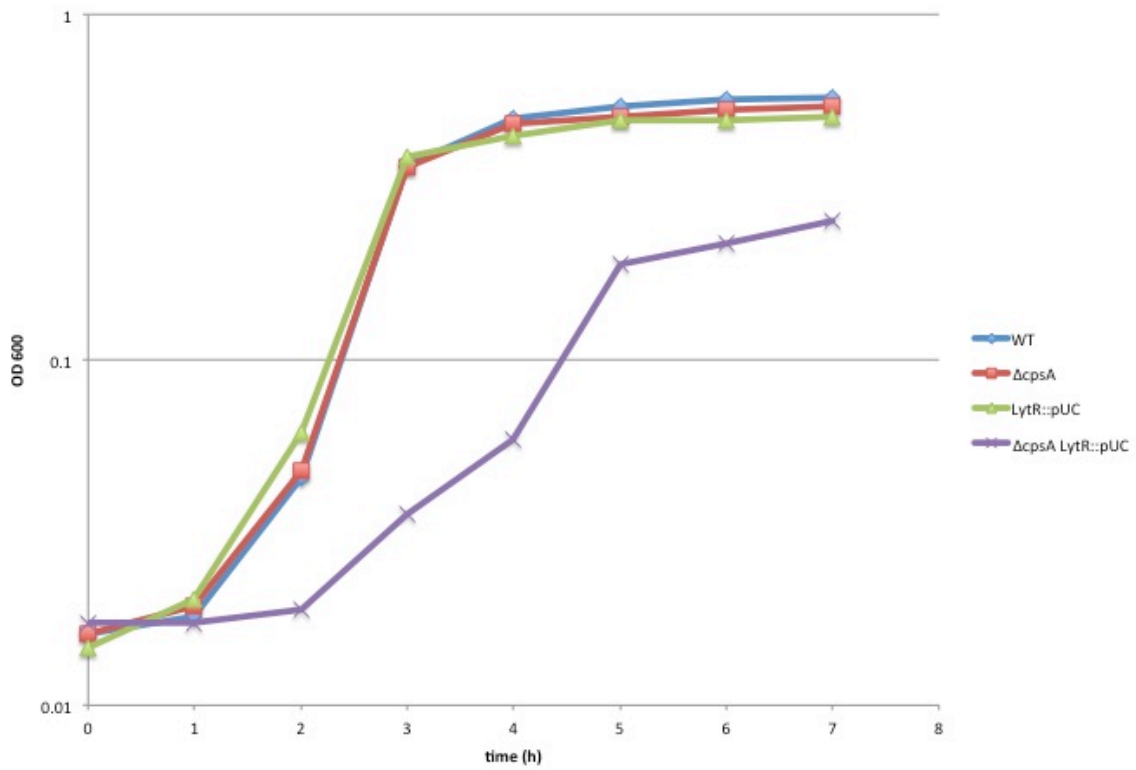


Figure 24: Bacterial growth curve. Growth of bacteria in THY media.

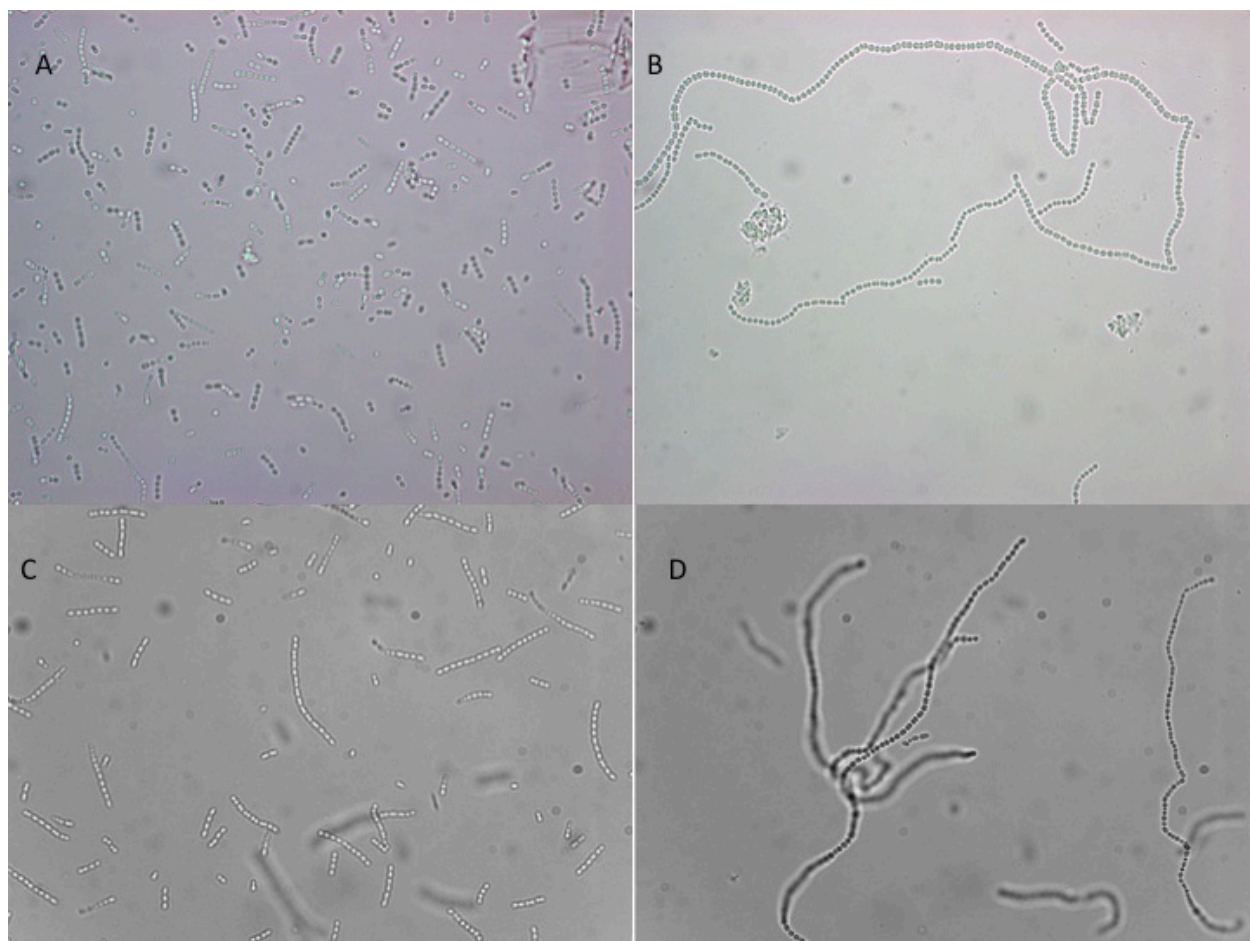


Figure 25: Phase contrast microscopy. A) wild type, B) $\Delta cpsA$, C) *lytR::pUC*, D) $\Delta cpsA$ *lytR::pUC*. All at 1000x magnification.

Cell wall effects of LytR mutation:

Examination of the *lytR::pUC* strain under phase contrast microscopy showed the bacteria form unusually long chains of cocci (Figure 25). Wild type bacteria (Figure 25A) form typical chains of 2-5 cocci in length. The chains of the strains with a single mutation (Figure 25B and 25C) were unusually long, and the double mutant strain (Figure 25D) formed chains even longer than those of the single mutant strain. This again indicates a combinational effect of LytR and CpsA in bacterial growth since chain length irregularities can indicate cell division and cell wall defects.

Cell wall was further examined by fluorescent vancomycin staining (Figure 26). This compound will label newly formed cell wall. Wild type bacteria (Figure 26A) and the *cpsA* deletion (Figure 26B) bacteria exhibit clear rings of cell wall and even coccus size per chain. Examination of the *lytR::pUC* (Figure 26C) and the double mutant, $\Delta cpsA$ *lytR::pUC* (Figure 26D) strains indicated very aberrant cell walls with cocci of uneven size and indications of multiple division septae forming per coccus.

Virulence effects of LytR mutation:

The virulence phenotype of the LytR-insert mutant strains was examined by *ex vivo* human whole blood infections. The wild type, $\Delta cpsA$, and *lytR::pUC* strains were killed similarly in human whole blood, whereas the double mutant strain exhibited killing greater than one log more than that exhibited by the wild type bacteria (Figure 27). This indicates that the LytR and CpsA proteins can compensate for each other to promote survival in blood but loss of both proteins leads to an inability to cause septic infection.

Since capsule is an antiphagocytic molecule, the ability of these mutant strains to survive in cultured murine macrophages was examined (Figure 28). The single and

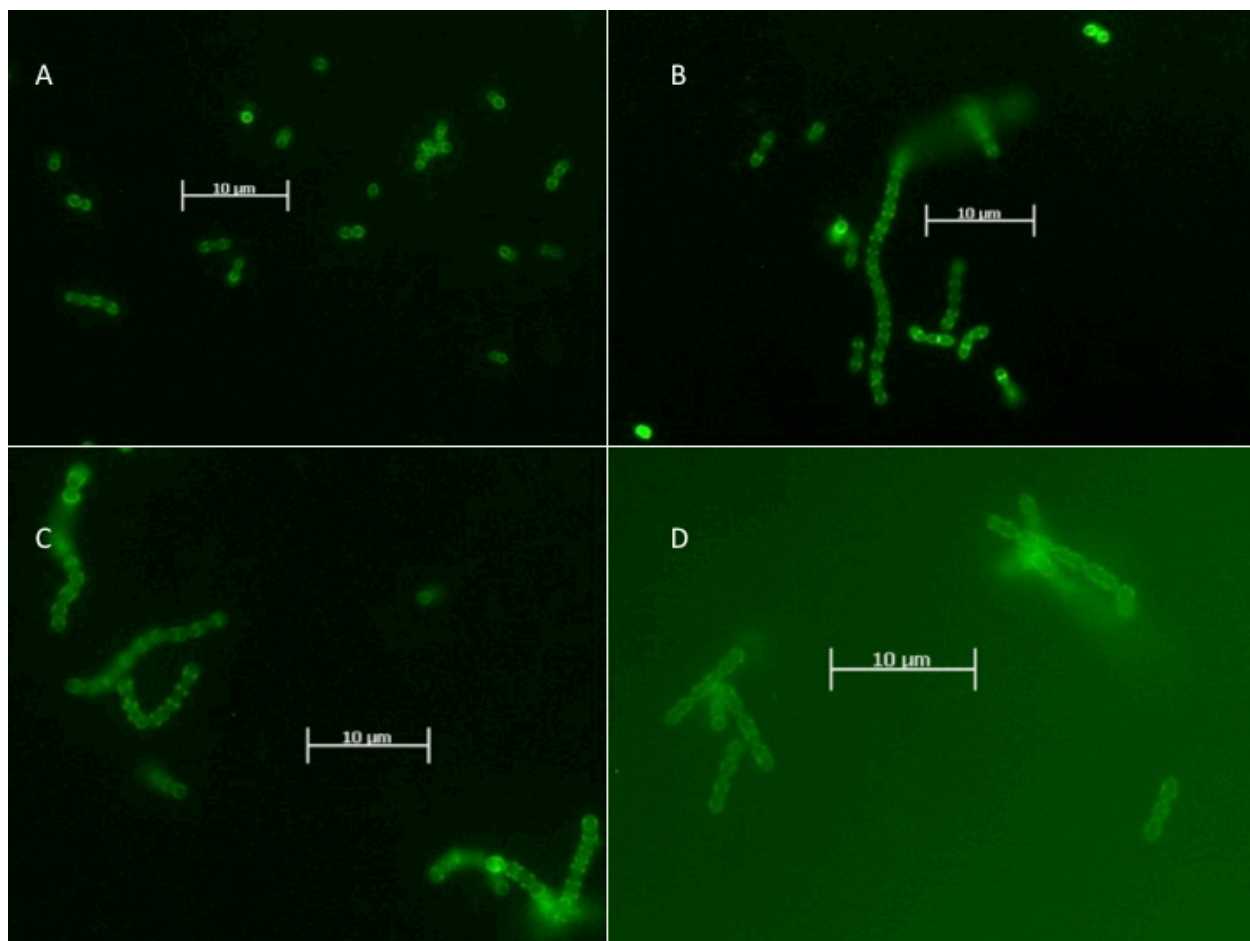


Figure 26: Fluorescent vancomycin staining. A) wild type, B) $\Delta cpsA$, C) *lytR::pUC*, D) $\Delta cpsA$ *lytR::pUC*. Scale bar is 10 μ m.

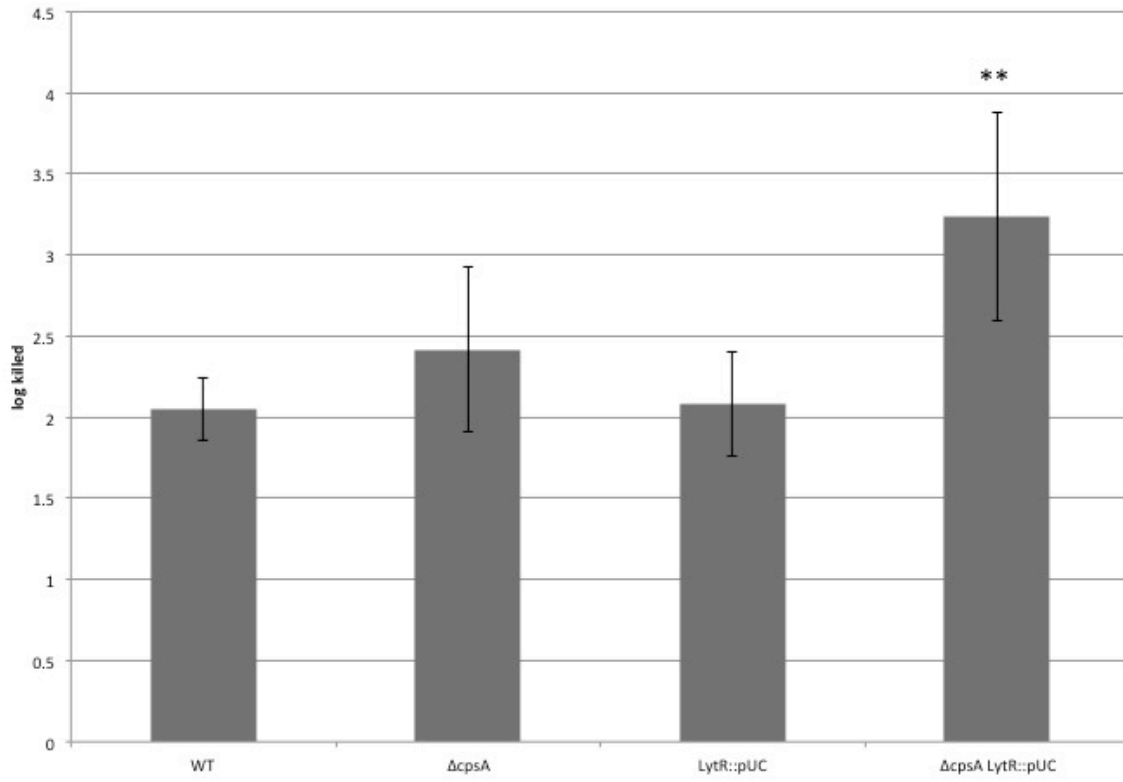


Figure 27: Human whole blood bactericidal assay. Log bacteria killed in three hours in freshly isolated heparinized human whole blood. Trend representative of three independent experiments. Error bars are standard deviation of three technical replicates. ** $p < 0.01$ compared to wild type.

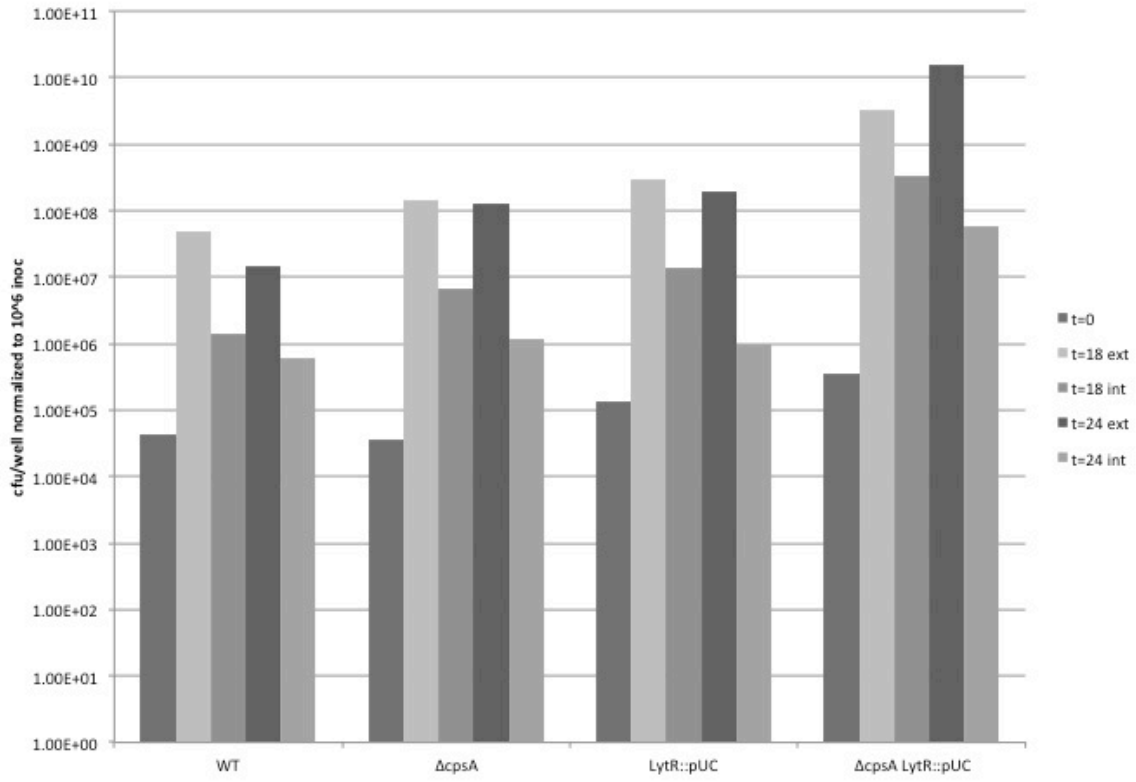


Figure 28: Murine macrophage infection. Average cfu per well at indicated time point either in supernatant (ext) or cell associated (int). Representative of two independent experiments.

double mutant strains were more highly internalized at the zero time point, which is to be expected since they are known to be expressing less of an antiphagocytic molecule. After 18 and 24 hours the mutant strains have replicated to a higher concentration within the macrophages indicating that strains carrying these mutations are more able to survive within the macrophages. Further analysis needs to be done to determine the mechanism for this phenotype. One possibility is that the mutant strains may be less able to activate the macrophages and are therefore better able to survive intracellularly. To address this possibility, analysis of infection with pre-activated macrophages should be performed. Additionally, the mutant strains may not be able to escape the macrophages and therefore are reproducing to a higher number intracellularly. Though we do have extracellular counts at 18 and 24 hours showing similar extracellular colony forming units (CFUs), those reflect extended growth in rich media. Therefore extracellular CFUs at earlier time points post infection should be determined. Lastly, the mutant strains may not be able to kill the macrophages to the same extent as the wild type strain, allowing for better survival within the macrophages. To examine this effect, measurement of lactate dehydrogenase release from the macrophages, indicating membrane damage, could be performed, as well as measuring levels of apoptosis of the macrophages.

Discussion:

Taken together, the capsule, cell wall and virulence data suggest that the GBS CpsA and LytR proteins can partially compensate for each other functionally. However, in the absence of both proteins the bacteria are less able to produce capsule, are less able to grow in culture and to survive in human blood, indicating an inability to cause

septic infection. Further virulence assays using a whole animal model, in our case zebrafish, should be performed to determine the role of the LytR protein alone and in combination with the CpsA protein in their ability to cause lethal infection and ability to promote bacterial dissemination to major organs.

This work is still preliminary, but there are several interesting avenues to pursue. Both the CpsA and LytR proteins contain the conserved LCP domain. If this domain is the functional enzymatic part of the protein it would be interesting to see how the CpsA protein truncations perform in the *lytR::pUC* and Δ *cpsA lytR::pUC* strain backgrounds. An unexplained finding from our work with the strain expressing the CpsA-117 truncation is that episomal expression of this protein, which has the entire extracellular domain of CpsA removed, is able to complement the *cpsA* deletion strain for capsule expression, but it is unclear by what mechanism. Perhaps the LCP domain of the LytR protein is capable of functionally compensating for the missing LCP domain of the CpsA-117 protein truncation. Additionally, we were unable to explain the mechanism of the dominant negative effect of episomal expression of CpsA truncations containing all or part of the accessory domain in the absence of the LCP domain. Expression of these truncations in the *lytR::pUC* and Δ *cpsA lytR::pUC* strains could further elucidate this mechanism since there would not be the confounding LCP domain of the LytR protein present.

The synthetic CpsA peptide should also be examined with the *lytR::pUC* and Δ *cpsA lytR::pUC* strains. This could determine if the synthetic peptide is interacting with a common substrate/binding partner of both of the LytR and CpsA proteins or if it is interacting with a unique substrate/binding partner of CpsA. If found to have

promiscuous activity against multiple LCP family members of the same species, this peptide could even have therapeutic implications against multiple Gram-positive bacterial pathogens.

Lastly, a strain with a triple mutation in the genes encoding all three LCP family members of *S. agalactiae* should be produced. This could further delineate the contributions of each member of the LCP family in GBS and give insight into its cell envelope biosynthesis, and cell division regulation.

CHAPTER 5: VIRULENCE SIGNALING OF THE *STREPTOCOCCUS PYOGENES* *SALIVARICIN* LOCUS

Introduction:

Previous work in our lab identified a highly conserved genetic locus in *Streptococcus pyogenes* as being important for causing systemic disease (52, 53). This locus had homology to the Salivaricin producing locus of the oral commensal bacteria *Streptococcus salivarius* (54). In *S. salivarius* the gene products of this locus are responsible for the production of a lantibiotic peptide, Salivaricin A (55). Salivaricin A acts as a pore-forming toxin against many species of Gram-positive bacteria also found in the oral microenvironment. (59-61). Only the M4 serotype of *S. pyogenes* is capable of producing an active lantibiotic from this locus (54, 60), yet all sequenced strains of *S. pyogenes* maintain this locus. This suggests that the gene products of this locus have been repurposed in *S. pyogenes* for a role other than bacteriocin production. Our data showing that mutants in this locus are attenuated in virulence suggests that the role of the salivaricin locus in *S. pyogenes* is in virulence (52, 53).

The *sal* locus of *S. pyogenes* (Figure 29) is bracketed by genes encoding two peptides, SalA and SalZ, and contains genes encoding a two-component signaling system, SalKR. Transcription in this locus is from two promoters, one upstream of *salA* and one upstream of *salKR*. (52). Differential transcription from the *salKR* promoter is observed when deletions are made in the *salA* and *salKR* genes, indicating a role for SalA and SalKR in transcription of this locus (52).

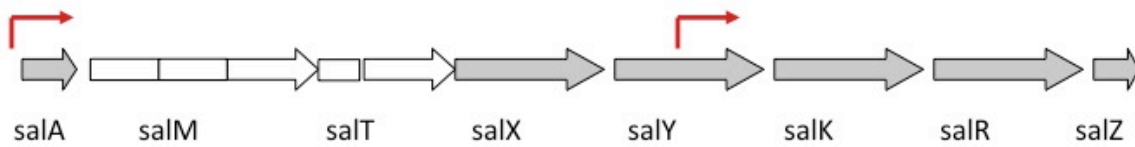


Figure 29: Genomic organization of the *S. pyogenes sal* locus. White arrows indicated open reading frames (ORFs) interrupted by premature stop codons, Grey arrows indicate intact ORFs. Small arrows above indicate promoters.

The work described here details an examination of the *in vitro* conditions required for specific DNA binding of the SalR protein to the *salKR* promoter and the roles of the peptides, SalA and SalZ, in virulence and transcription of the locus.

Materials and Methods:

Bacterial strains, media, and growth conditions:

Plasmids were maintained in either *Escherichia coli* DH5 α cells or *E. coli* TOP10 cells (Invitrogen) and cultured aerobically in Luria-Bertani medium (Acumedia) supplemented with 100 μ g/mL ampicillin (Amp), 25 μ g/ml kanamycin (Kan) or 20 μ g/mL chloramphenicol (Cam) at 37°C. *Streptococcus pyogenes* strain HSC5 (117-119) and isogenic *salA*, *salKR*, and *salA-salKR* deletions (52) were cultured anaerobically in Todd-Hewitt medium (Alpha Biosciences) supplemented with 0.2% yeast extract (Acumedia) (THY). When appropriate, *S. pyogenes* cultures were supplemented with selective antibiotics, Cam (3 μ g/mL), or Kan (500 μ g/mL). For solid media, THY was supplemented with 1.4% bacteriological agar.

Construction of SalZ insert strain:

To examine the role of SalZ in virulence and transcription a strain with an insertion mutation in the *salZ* gene was generated. This plasmid insert upstream of the *salZ* gene inserted transcriptional terminators preventing the transcription of *salZ*. Using primers 5'-SalZ-insert-AatII—CAGCGACGTCGAAAATGGTAAGGAGATAGC and 3'-SalZ-insert-PstI--AAA ACT GCA GCA TAT CAG GCA TTT CCC TAC, a 700 bp fragment was amplified from HSC5 genomic DNA and cloned into the AatII and PstI sites of pBR Ω KM2. The plasmid was electrotransformed into wild type *S. pyogenes* as

described previously (117) and single crossover recombinants were selected by plating on THY-Kan agar plates.

Electrophoretic mobility shift assay (EMSA):

The SalR protein was purified as an MBP fusion protein using the pMal-c2x system (NEB). Using primers 5-SalR-SmaI—
TCCCCCGGGATGAAGATTTTATTAATTGATGACC and 3-SalR-PstI—
AAAAACTGCAGGTCGTTTGATTATCTGCAACTCAG and HSC5 genomic DNA as a template *salR* was amplified and cloned into the PstI and XmnI sites of pMal-c2X. SalR-D53E and SalR-D53N were generated by splicing by overlap extension PCR and primers 3` salR D to E rev—GTAAATTAATTTCAAGAAATATAATATCG and 5` salR D to E fwd—CGATATTATATTGCTTGAAATTAATTTAAC, 5` salR D to N fwd—CGATATTATATTGCTTAATATTAATTTAAC and 3` salR D to N rev—GTAAATTAATATTAAGCAAATATAATATCG. Proteins were purified as described previously (38) and stored at -80°C in 50% glycerol.

The probe was amplified from HSC5 using primers 5-salYK-pro del1A—CGCGGATCCCAGCTAATGAGATTATAGAGTC and 3-salY pro#4—GCATAGAGCATTACCATC and digoxigenin-labeled (DIG) using a Roche second generation DIG labeling kit according to manufacturer's instructions.

EMSAs were conducted by mixing the specified amount of protein with a constant amount of probe in a binding buffer containing 100 mM HEPES (pH 7.2), 1 mM EDTA, 50 mM KCl, 50 mM MgCl₂, 1mM dithiothreitol, and 30% (vol/vol) glycerol for 30 min at room temperature. For reactions with competitor DNA, an excess of unlabeled probe was used as a specific competitor, and salmon sperm DNA was used as a

nonspecific competitor. The samples were loaded onto a 6% polyacrylamide native gel consisting of 6% (vol/vol) polyacrylamide, 44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA, pre-run at 4°C and 150V. Electrophoresis was performed at 4°C at 180V for 10 minutes and 300V for 25 minutes. The gel was then transferred to a nylon membrane (Santa Cruz) using a semidry transfer apparatus (BioRad). The membrane was UV crosslinked for 15 minutes followed by blocking in 4% milk and 0.1 M maleic acid buffer for 30 minutes. Alkaline phosphatase conjugated anti-DIG antibody was diluted 1:10,000 in 4% milk 0.1M maleic acid buffer and incubated with the membrane for 30 minutes. The membrane was washed three times in 0.1M maleic acid supplemented with 0.03% Tween-20. Chemiluminescent detection was performed with CDP-Star (Roche) according to the manufacturer's instructions, followed by exposure to X-ray film.

***Ex vivo* human whole blood infections:**

Blood was isolated from healthy volunteers using the vacutainer system (BD) and collected in heparinized tubes (BD). *S. pyogenes* overnight cultures were diluted 1:50 in fresh THY and grown to midlog phase. Cultures were normalized to an absorbance of $OD_{600}=0.3=10^8$ cfu per mL in phosphate buffered saline (PBS) and serially diluted in PBS to 10^4 cfu/mL. Ten microliters (100 cfu) of culture was added to 1mL of blood in a 1.7 mL tube in triplicate. Tubes were incubated at 37 degrees C for 3 hours with end over end rotation. Following incubation, the cfu/mL was determined by serial dilution on THY agar plates.

Human polymorphonuclear (PMN) cell infections:

Human PMNs were isolated and infected as described previously (120). Blood was isolated from healthy volunteers using the vacutainer system (BD) and heparinized tubes (BD) and mixed in equal volumes with 3% dextran (MP Biomedicals) in saline using 50-ml conical tubes to sediment red blood cells. The remaining cells were centrifuged at 800 x g at 4°C. Pellets were resuspended in 35 ml of 0.9% NaCl, and a 10-ml underlay of Ficoll-Paque (GE Healthcare) was applied. This was centrifuged for 30 min at 410 x g in a swinging bucket rotor at room temperature to separate out the granulocyte cell fraction. The supernatant was gently aspirated. Remaining red blood cells were lysed by resuspension of the pellet with 10 ml sterile H₂O for 28 seconds, and isotonicity was quickly restored by adding 10 ml 1.8% NaCl. Purified PMNs were centrifuged at 500 x g and resuspended in 3 ml DMEM. For infections, PMNs were diluted to a final concentration of 1×10^6 cells per mL in DMEM with 50% human serum. Overnight cultures of *S. pyogenes* were diluted 1:50 in fresh THY and grown to midlog phase. Cultures were normalized to an absorbance of $OD_{600}=0.3=10^8$ /mL in PBS. PMNs were infected at an multiplicity of infection (MOI) of 1 and control tubes of 50% human serum 50% DMEM were inoculated with an equal concentration of bacteria and were incubated 3 hours with end over end rotation at 37 degrees. Following incubation, cfu/mL was determined by serial dilution on THY plates. Percent survival was calculated by comparing cfu in cultures containing PMNs to cfu in cultures in 50% serum 50% DMEM with no PMNs.

Zebrafish infections:

Overnight *S. pyogenes* cultures were diluted 1:50 and grown to midlog phase in fresh THY. Cultures were normalized to an absorbance of $OD_{600}=0.3=10^8$ /mL in THY

and diluted 1:10 in THY. Ten microliters of bacteria (10^5 cfu) was injected into the dorsal muscle of anesthetized adult zebrafish. Six fish were infected per strain and either survival monitored over 6 days post infection, or 24 hours post infection, fish were sacrificed and spleens aseptically removed and homogenized in PBS. Homogenate was serially diluted and plated for viable cfu on CNA plates (Acumedia).

Alkaline phosphatase reporter assay:

S. pyogenes strains harboring plasmids pMNN1-*salKR* pro or pMNN1-*salKR del2 short* pro (52) were grown overnight in THY supplemented with selective antibiotics, and overnight cultures diluted 1:50 in THY supplemented with selective antibiotics. Cultures were grown to midlog phase (OD_{600} approximately 0.3) and concentrated to $OD_{600}=0.75$. Fifty microliters of culture was added to 200 μ L 1-mg/ml p-nitrophenylphosphate (Sigma) dissolved in 1 M Tris (pH 8) in triplicate in a 96 well plate. Reaction was incubated in the dark one hour at room temperature. Following reaction, three optical densities (OD_{405} , OD_{550} , and OD_{600}) were measured using a spectrophotometric plate reader (VersaMax). Alkaline phosphatase activity was determined using the following formula: $[OD_{405} \times (1.75 - OD_{550})] / (\text{volume} \times \text{time} \times OD_{600}) \times 1,000$.

Results:

SalR binding to *salKR* promoter:

Previous data showed that transcription from the *salKR* promoter was increased in a *salKR* deletion strain, suggesting a role of this two-component system in repression of this promoter (52). In order to further examine this effect, we first tried to determine the conditions necessary for specific SalR protein binding by EMSA. Previous analyses

had failed to find conditions necessary for specific binding. Typically, two-component signaling systems activate transcription when the sensor kinase phosphorylates the response regulator. We examined in vitro binding of the purified SalR protein to the *salKR* promoter probe after pre-incubation with the small molecule phosphate donor acetyl phosphate. With both SalR and SalR pre-incubated with acetyl phosphate the non-specific unlabeled DNA is effectively able to compete the labeled probe out of the protein-DNA complex (Data not shown). These results indicate that the addition of phosphorylated SalR protein does not stimulate a specific binding event.

Response regulators are phosphorylated by the sensor kinase on a specific aspartic acid residue, in the case of SalR, Asp-53. In other two-component systems, mutation of this residue to an asparagine makes the protein unable to be phosphorylated, while mutation of this residue to a glutamate (phosphomimic) makes this protein act as if it is constitutively phosphorylated. To determine if SalR requires phosphorylation to specifically bind DNA, these mutations were constructed in the Asp-53 residue. Comparison of the binding potential of these three purified proteins by EMSA indicated better apparent binding of the SalR-D53E phosphomimic compared to the wild type or SalR-D53N phosphomutant (Data not shown). However, neither of these mutant proteins was capable of specific binding of the probe (Data not shown). Therefore, specific binding conditions of SalR are still unknown.

Role(s) of SalA in septic infection:

The 42 amino acid *S. pyogenes* SalA peptide is the homolog of the *S. salivarius* Salivaricin A lantibiotic. Disruptions of the *salM* and *salT* genes lead to no active lantibiotic production by *S. pyogenes*. However, previous data (52) showed that

transcription from the *salKR* promoter is increased in a *salA* deletion strain, and even further increased in a *salA salKR* double deletion strain, indicating that the SalA peptide serves a role in transcription and may function cooperatively with the SalKR proteins. To examine the role of the SalA peptide, alone and in combination with SalKR, in virulence we performed *ex vivo* human whole blood infections (Figure 30). Wild type *S. pyogenes* is able to grow over two logs in three hours in human blood. The growth of the *salA* deletion strain and the *salKR* deletion strain is approximately one log less than the growth of the wild type strain. This attenuation is significant and indicates that the SalA peptide and the SalKR two component signaling system are key for growth during a septic infection. The *salA salKR* double deletion strain not only cannot grow in human blood, it is in fact killed. To determine the extent of the attenuation of the double mutant strain, further *ex vivo* blood infections were performed with an inoculum two logs higher (Figure 31). With the higher inoculum the *salA salKR* double mutant strain was seen to have a two log reduction in cfu in human blood. These data indicate that SalA acts synergistically with SalKR in promoting bacterial survival in a septic infection.

To further examine the role of SalA and SalKR alone and in combination in septic infection we examined the ability of these mutant strains to survive in a human PMN infection model (Figure 32). The *salA* and *salKR* single deletion strains are significantly attenuated compared to the wild type strain in ability to survive in human PMNs in fifty percent serum, compared to growth in fifty percent human serum alone. The *salA salKR* double mutant strain is also significantly attenuated compared to the wild type strain, but exhibited no difference in survival when compared to the single mutant strains. This

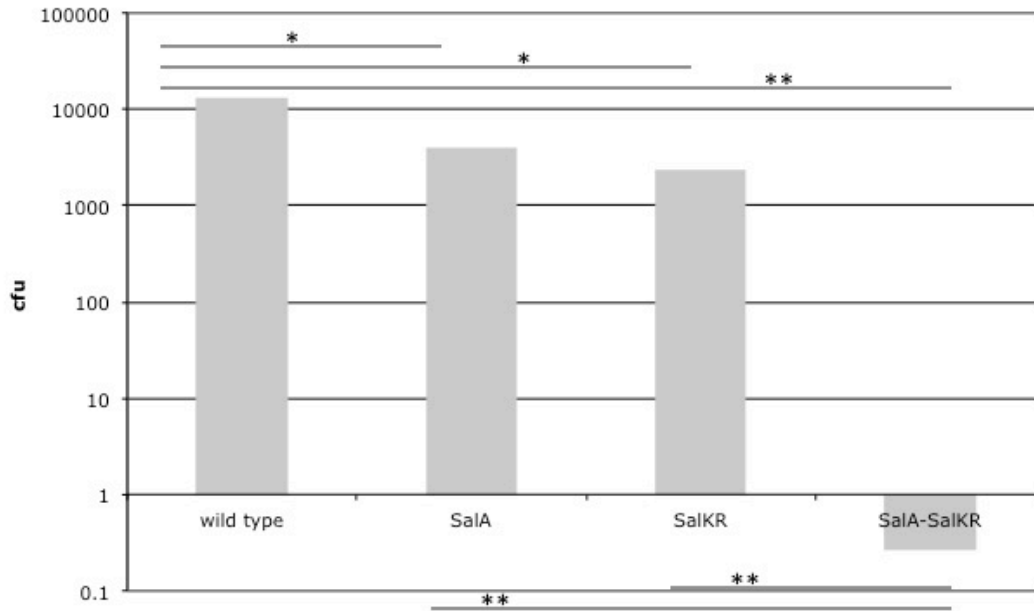


Figure 30: Human whole blood bactericidal assay. Bacterial growth in heparinized human whole blood over 3 hours. Average of 4 biological replicates, each having 3 technical replicates. 100 cfu inoculum. * $p < 0.05$, ** $p < 0.01$

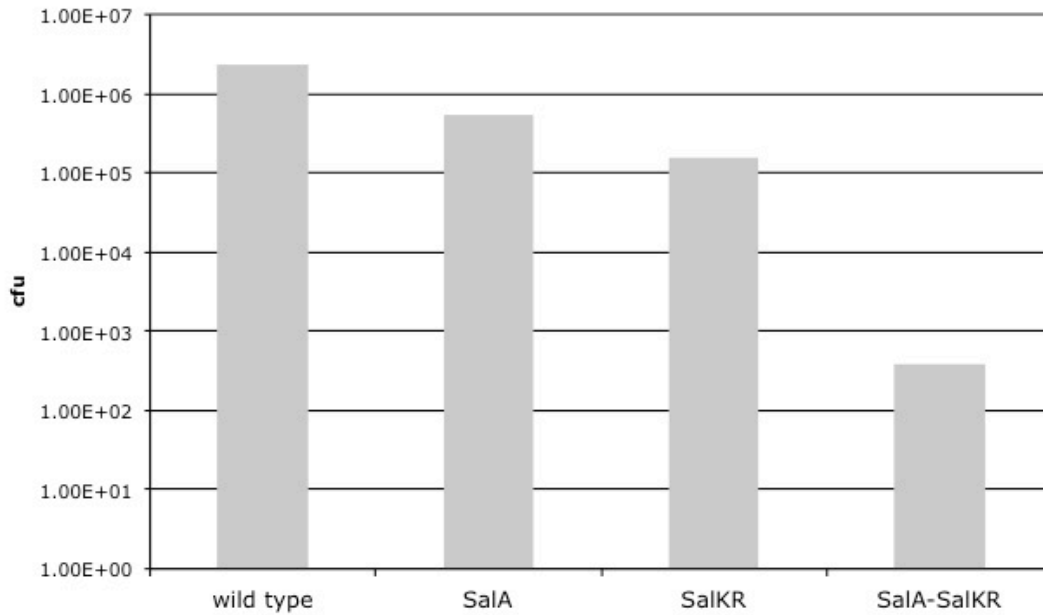


Figure 31: Human whole blood bactericidal assay. Bacterial growth in heparinized human whole blood over 3 hours. Trend representative of 3 biological replicates, each having 3 technical replicates. 10^4 cfu inoculum. * $p < 0.05$, ** $p < 0.01$

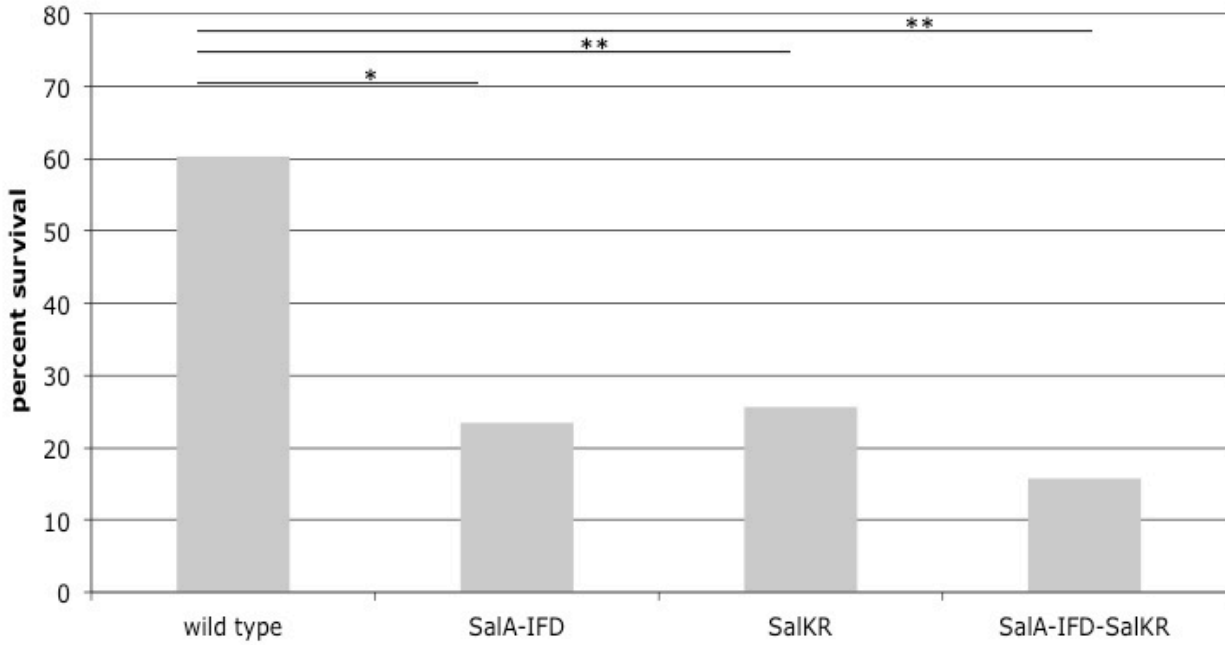


Figure 32: Human PMN infection. Bacterial survival over 3 hours in 50% human serum with 10^6 PMNs per mL compared to survival in 50% human serum alone. Average of 4 biological replicates each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$.

indicates that the cooperative action of SalA and SalKR promoting survival in human whole blood is not evident in a PMN infection. The factor present in whole blood that is missing in PMNs in fifty percent human serum that causes this effect is yet to be determined.

Role(s) of SalZ in virulence and regulation of the *sal* locus:

SalZ is a 66 amino acid peptide of unknown function encoded at the 3' end of the *sal* locus. To explore the functions of SalZ in transcription and virulence we generated a *salZ*-insert strain. First we examined the ability of this mutant strain to survive in an *ex vivo* human whole blood infection (Figure 33). The *salZ*-insert strain is about a half log attenuated in ability to grow in this assay, indicating an important role for SalZ in bacterial growth in a septic infection.

Next we examined the ability of the *salZ*-insert strain to cause a lethal infection in a whole animal model of disease. Following intramuscular infection of zebrafish with the wild type or *salZ*-insert strain, fish were monitored for survival for six days (Figure 34). Fish infected with wild type *S. pyogenes* succumb rapidly to infection and exhibit only thirty percent survival six days post infection. Fish infected with the *salZ*-insert strain exhibited significantly attenuated survival on days one, two, four, five, and six post infection, with eighty percent of fish surviving six days post infection. This data indicates that SalZ is important for the ability for *S. pyogenes* to cause lethal infection in a zebrafish model of myonecrosis.

To further assess the role of SalZ in the ability to cause disease in the zebrafish model we examined bacterial dissemination to the zebrafish spleen at twenty-four hours post infection (Figure 35). The *salZ*-insert strain exhibited over one log fewer cfu per

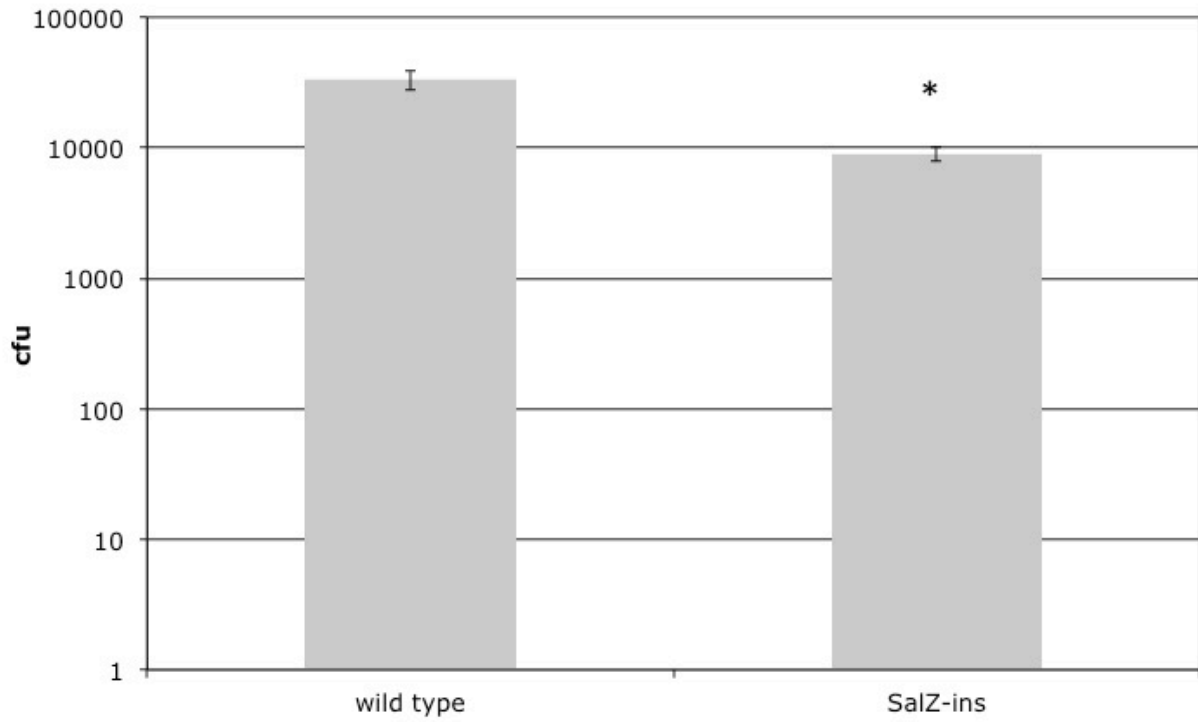


Figure 33: Human whole blood bactericidal assay. Bacterial growth in heparinized human whole blood over 3 hours. Average of 4 biological replicates, each having three technical replicates. 100 cfu inoculum. * $p=0.012$

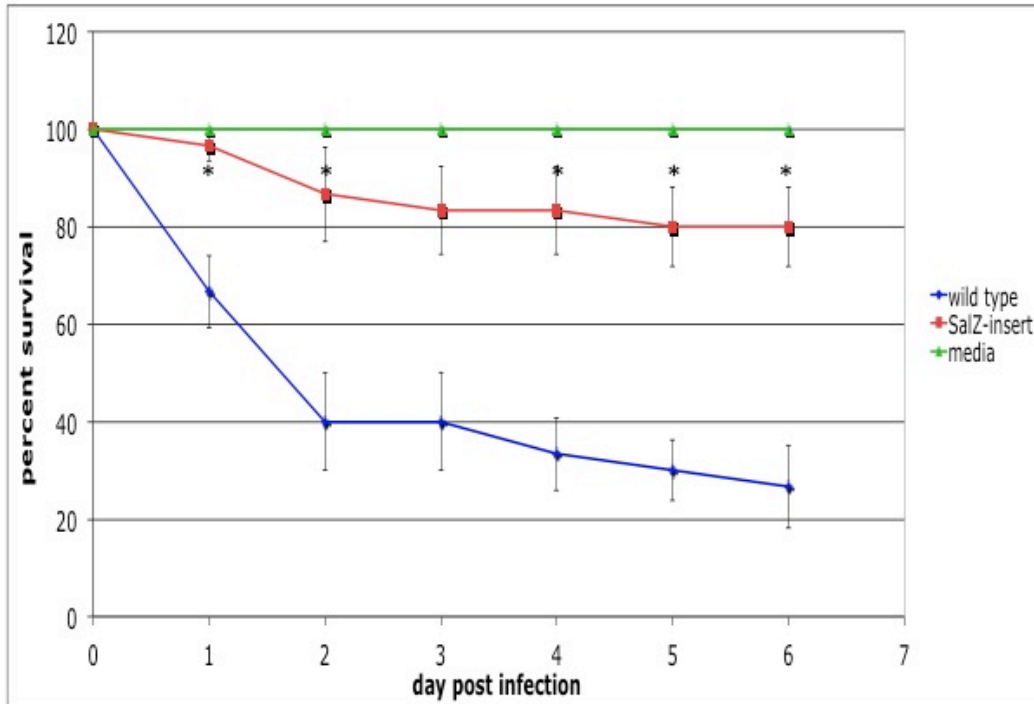


Figure 34: Zebrafish survival. Percent zebrafish survival following 10^5 cfu intramuscular injection. Average of three biological replicates, each with 6 fish per strain. * $p < 0.05$.

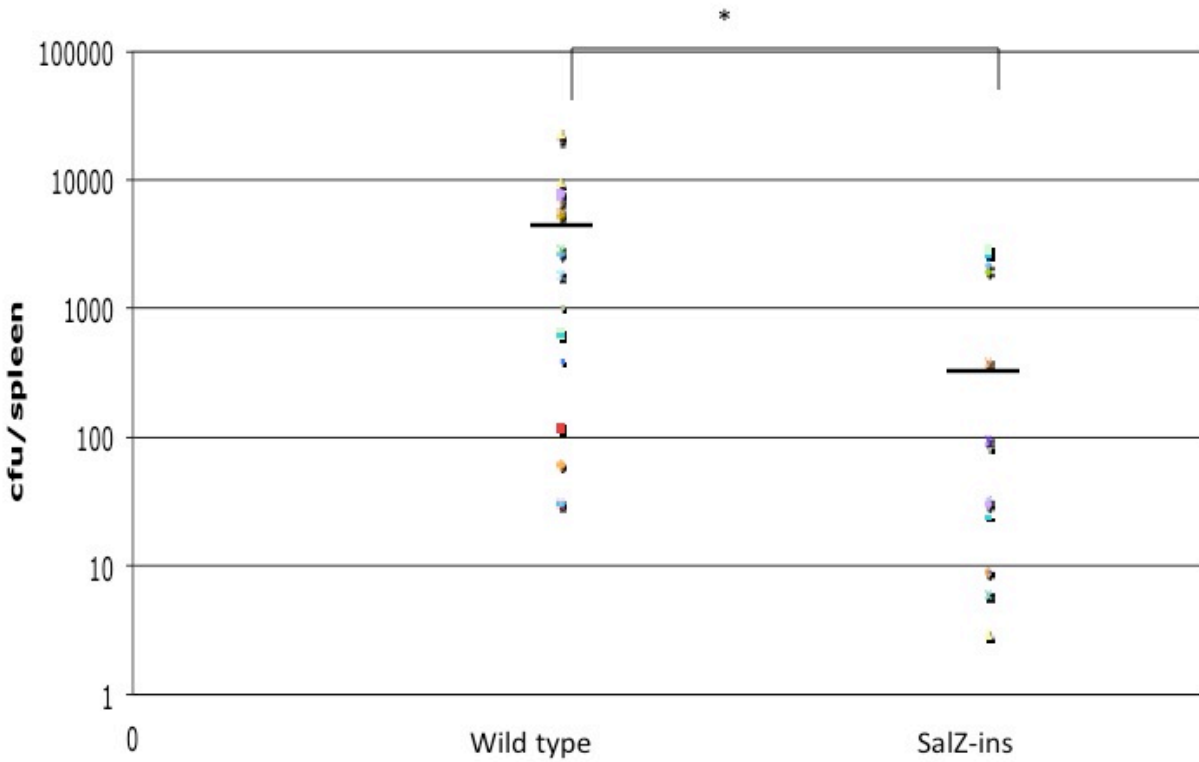


Figure 35: Dissemination to zebrafish spleen. 24 hours post 10^5 cfu intramuscular injection, viable cfu in the spleen. Each dot represents one fish and the bar represents the mean. N=24 fish per strain. * $p=0.014$

spleen compared to the wild type, further supporting a key role for this peptide in *S. pyogenes* systemic infection.

Lastly, we explored the role of SalZ in transcription of the *salKR* promoter, using an alkaline phosphatase reporter assay comparing transcription in the *salZ*-insert strain with transcription in the wild type and *salKR* deletion strains (Figure 36). We examined both the full-length *salKR full* promoter and the *salKR del2 short* promoter fusions (52). The *salKR pro del2 short* was previously identified as not having any SalKR repression sites (52). The *salKR full* promoter showed higher activity in the *salKR* deletion strain than in the wild type, yet in the *salZ*-insert strain, transcription of the reporter is significantly reduced. This supports a role for SalZ in transcriptional activation of this promoter. The *salKR del2 short* promoter also showed lower activity in the *salZ*-insert strain when compared to transcription in the wild type or *salKR* deletion strains, which as shown previously are not significantly different from each other. Transcription of the *salKR del2 short* promoter is significantly higher than that of the *salKR full* promoter in the *salZ*-insert strain. This suggests that the *salKR del2 short* promoter does have regulatory sites that are SalZ responsive, that are separate from the SalKR responsive sites. However, we have not ruled out the fact that all transcription is attenuated in the *salZ*-insert strain, or the alkaline phosphatase enzyme is less stable in the *salZ*-insert strain.

Discussion:

The work described here shows that phosphorylation of SalR does increase apparent binding, but does not lead to specific DNA binding in EMSAs. Further work needs to be done to identify the conditions necessary for specific binding. One

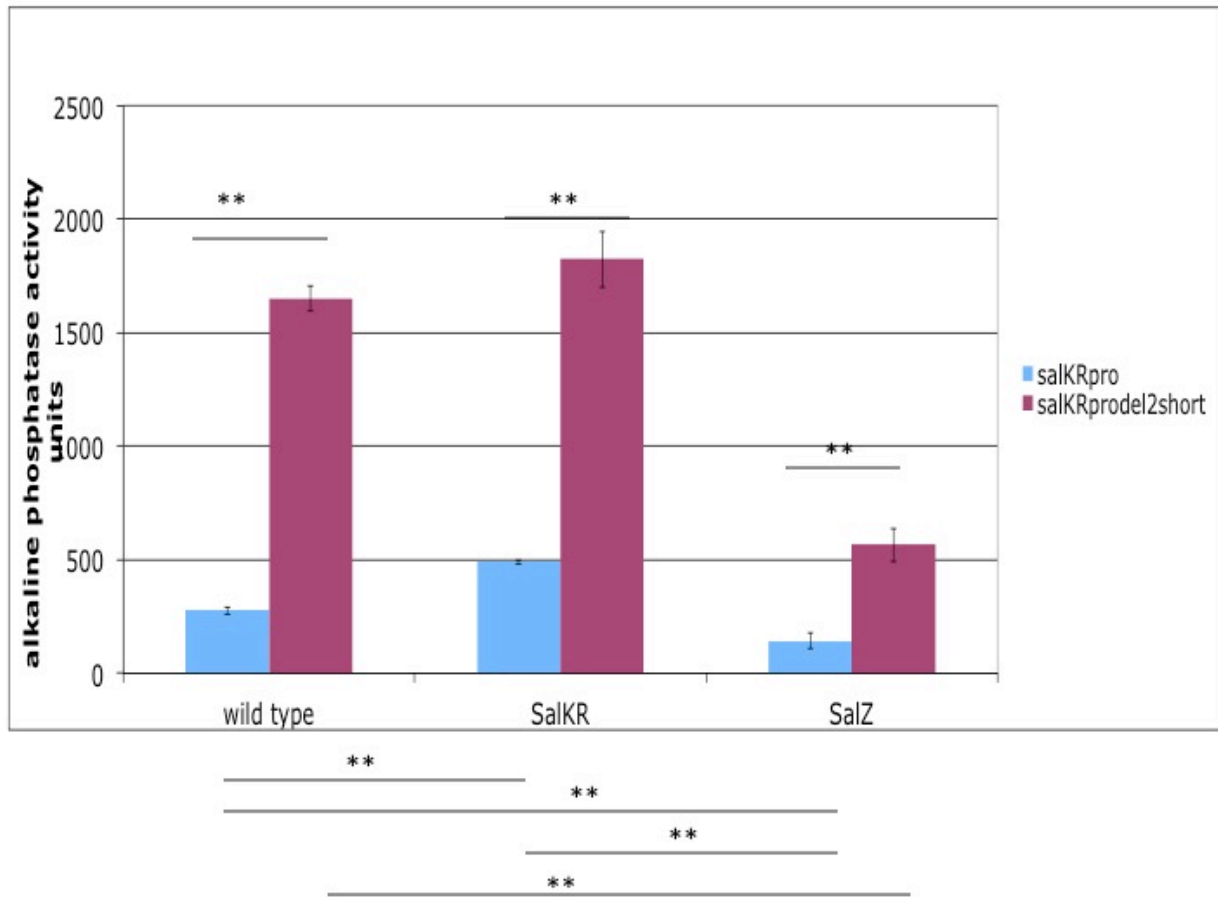


Figure 36: Promoter activity. Promoter activity by alkaline phosphatase assay of the *salKR* full and *salKRdel2* short promoters in the wild type, Δ *salKR*, and *salZ*-insert strains. Averages of three biological replicates, each with three technical replicates. **p<0.05.

possibility is that the peptides of the *sal* locus, SalA and/or SalZ, could act as cofactors. The combinational effects of SalA and SalKR in transcription (52) and in virulence (this work) suggest an interaction between these proteins. Alternatively, SalA could be sensed through SalK and signal to SalR as occurs in *S. salivarius* (60). However, due to the interruption of the *salT* gene in *S. pyogenes*, SalA may not be exported and may be accessible to SalR to act as a cofactor in DNA binding and transcription. The role of SalZ is still unknown, and it could serve as a transcriptional cofactor as well. The fact that transcription from the *salKR* promoter increases in a *salA* deletion strain and decreases in a *salZ*-insert strain, suggests an intriguing possibility of one peptide acting as a positive cofactor and another acting as a negative cofactor to tightly modulate transcription of this locus.

Both peptides of the *sal* locus, SalA and SalZ, have a role in virulence of *S. pyogenes*. SalA and SalKR act cooperatively to promote bacterial survival in human whole blood, but not in promotion of survival in a human PMN infection model. Further work to elucidate the factor(s) present in human whole blood not present in a PMN fraction with 50% human serum that allow this effect could lead to a better understanding of *S. pyogenes* virulence in septic disease. The role of monocytes would be especially interesting to investigate, as the role of macrophages, the monocytes of tissues, is well understood as a host cell for *S. pyogenes* during infection (121-123). SalZ contributes to virulence in an *ex vivo* human whole blood model and in models of zebrafish lethal infection and dissemination to the zebrafish spleen. Further work should be done to understand the mechanism for SalZ contribution to virulence.

CHAPTER 6: GENERAL CONCLUSIONS

Roles of CpsA and LytR in modification of the *Streptococcus agalactiae* cell envelope:

The body of work described here showed that modification of either the extracellular domain of the CpsA protein or the leucine zipper motif of the CpsA protein had significant effects on the *S. agalactiae* capsule and cell wall. Moreover, strains expressing the truncated CpsA protein are attenuated in virulence, suggesting a role for the extracellular domain in pathogenesis. Similarly, complete deletion of the *cpsA* gene or insertional mutagenesis of the *lytR* gene alone or in combination had effects on the capsule and cell wall and in virulence of *S. agalactiae*. This leads to a model (Figure 37) of CpsA having multiple binding partners interacting with each of the domains, leucine zipper motif, accessory domain and LCP domain, and potential direct or indirect interactions with LytR.

The CpsA and LytR proteins are both members of the LCP family of proteins. These proteins are implicated in cell envelope maintenance in many Gram-positive species (34, 35, 37, 38, 82, 85, 86, 88-93, 112, 115, 116, 124-126). Targeting and inhibition of LCP proteins could lead to a better understanding of Gram-positive cell envelope biogenesis and virulence mechanisms. Multiple mutations of LCP family members in the same strain could provide information on how these proteins can act alone or in combination, and if they can compensate for each other.

The accessory domain is unique to the CpsA protein members of the LCP family (34). Our data indicates that the accessory domain, when expressed in the absence of the LCP domain, common to all members of the LCP family, (34) leads to a dominant

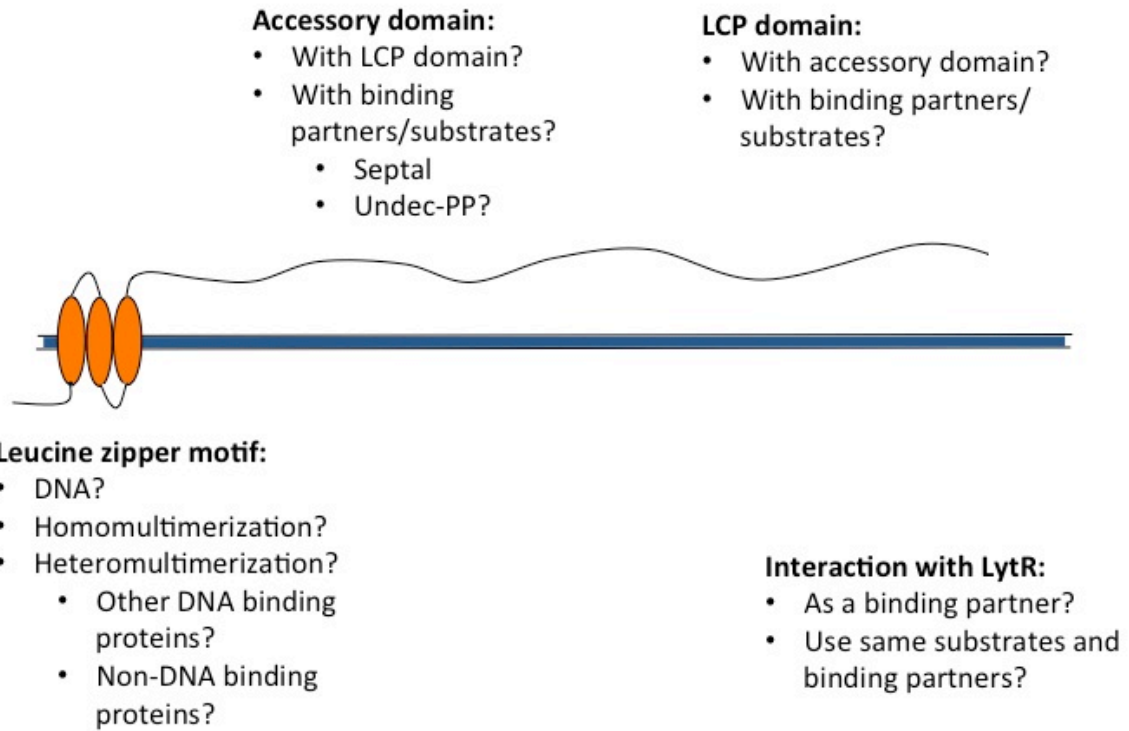


Figure 37: Model of CpsA function and hypothesized interactions.

negative effect when expressed in the wild type strain and an inability to complement a *cpsA* deletion strain. This suggests that the two extracellular domains of CpsA act independently, and suggests a model for differential regulation of capsule expression in response to environmental signaling. While the LCP domain can interact with a certain set of binding partners, during conditions requiring the expression of less capsule a different set of binding partners is engaged with the accessory domain, leading to a decrease in capsule expression. Further work is needed to determine the binding partners of the extracellular domains of CpsA.

The ligase to link the polysaccharide subunits of the capsule to the peptidoglycan cell wall is as of yet unknown. The lack of an uncharacterized gene in the locus could indicate that this function is accomplished by one of the previously characterized proteins of the *cps* locus acting in a bi-functional manner. CpsA is potentially this ligase. Further work to determine if the CpsA protein could carry out this function would be to examine if strains expressing mutant CpsA are unable, or less able, to attach capsule intermediates to the cell wall. Immuno-detection of capsule in whole cell and culture supernatants could determine if the capsule is being released from the lipid carrier but unable to be attached to the cell wall, as seen in *Staphylococcus aureus* mutants that release teichoic acids into the culture supernatant (115). Additionally, the capsule intermediates could be unable to be released from the lipid carrier. Immuno-detection of capsule in whole cell, cell wall and cell membrane fractions of strains expressing a mutant form of the CpsA protein should be performed to determine if CpsA plays a role in this process.

The *S. pneumoniae* Cps2A crystal structure contained undecaprenyl-pyrophosphate (undec-PP) (35, 82), indicating that Cps2A is capable of interacting with and potentially catalyzing a reaction with undec-PP. Inhibition of an LCP protein could be accomplished by blocking the interaction of the protein with the lipid carrier to prevent linkage of the polysaccharide to the cell wall. Our data, showing localization of FITC-CpsA peptide at the midcell/septal region, and the FITC-CpsA peptide binding only to growing bacteria, could mean the peptide is binding undec-PP or a related compound since free undec-PP is only available during active building of the cell wall. Since undec-PP is a low concentration intermediate, and important to both capsule and peptidoglycan synthesis (33) an inhibition of recycling of undec-PP by an aberrant CpsA protein could be preventing proper peptidoglycan assembly, and therefore leading to the cell wall cell division abnormalities of our truncations and leucine zipper mutants.

Many leucine zipper motif proteins function at the divisosome (105-107) and in regulation of cell size and shape (108, 109), also indicating a function for the leucine zipper motif in cell division. Our data show that mutation of the leucine zipper motif in the CpsA protein makes it unable to complement a *cpsA* deletion strain for capsule expression or cell wall morphology. Additionally, the leucine zipper CpsA mutant, when expressed episomally in the wild type strain, causes a dominant negative effect on capsule expression and cell wall morphology. Our data show that the wild type CpsA protein is capable of multimerization. Further work needs to be done to determine if the leucine zipper mutant CpsA interferes with multimerization of the wild type CpsA protein, or if the leucine zipper mutant CpsA interferes with interaction of wild type CpsA protein with its normal binding partners. In addition, we do not know the identity of the

CpsA protein binding partners. Bioinformatic searches for membrane spanning leucine zipper motifs in other GBS proteins could suggest these binding partners, followed by confirmation by cross-linking and co-immunoprecipitation and/or by purifying the proteins and doing isothermal titration calorimetry.

Our data indicating that the dominant negative function of the truncated CpsA protein can occur through (1) expression of truncated protein, (2) addition of purified protein or (3) addition of synthetic peptide to wild type cultures provides new information for understanding of cell envelope biogenesis and for therapeutics against GBS disease. Extracellular inhibition of an LCP protein through interference with extracellular domains could help identify the extracellular binding partners of these proteins and lead to a better understanding of the Gram-positive cell envelope, including how it is synthesized and regulated. Additionally, functional inhibition of LCP domain containing proteins could have a therapeutic role in treating disease.

Current strategies for prevention of GBS disease rely on antibiotic treatment of women during labor and delivery. These strategies have multiple weaknesses (4, 6, 17, 21, 127) in that they do not prevent chorioamniosis or late onset neonatal disease and have consequences to the maternal and neonatal microflora. Ability to use a dominant negative factor that acts extracellularly as a topical treatment just prior to delivery to block capsule production by GBS could render the bacteria aspirated by the neonate avirulent. Additionally, the dominant negative factor caused cell wall changes that could render the bacteria more susceptible to antibiotics or components of the immune system, which would make an infection more treatable. If a dominant negative factor is

capable of acting against multiple bacterial species, then this treatment could be a widespread therapeutic agent.

Virulence signaling of the *Streptococcus pyogenes salivaricin* locus:

Here we demonstrated that the peptides of the *sal* locus, SalA and SalZ have important roles in virulence of *S. pyogenes*. One possible role for these peptides is in transcriptional control of the locus. Transcription from the *salKR* promoter is increased in both a *salA* deletion strain and a *salKR* deletion strain, and even further increased in a *salA salKR* double deletion strain (52). This work showed that transcription of the *salKR* promoter in a *salZ*-insert strain was reduced. The differential transcription seen, combined with the inability to find specific *in vitro* binding conditions for SalR in EMSAs, suggests an intriguing scenario where each peptide is serving as a transcriptional cofactor, but in opposite roles to allow differential transcription of the *sal* locus. We would need to purify these peptides and add them to the EMSAs to determine if SalA and/or SalZ are acting as peptide cofactors in SalR binding to DNA. Peptides can act as transcriptional co-repressors or anti-repressors (66).

Alternatively, the peptides could serve a role as pheromones and act indirectly at the transcriptional level through interaction with SalK, which would change the phosphorylation state of SalR and differentially regulate transcription. Here we demonstrate that phosphomimic SalR_{D53E} had increased apparent binding of the *salKR* promoter probe *in vitro*. To determine if signaling through SalK to change the SalR phosphorylation state is the mechanism of SalA and SalZ action we would have to determine if either peptide interacts directly with SalK, doing *in vitro* and *in vivo* crosslinking, or isothermal titration calorimetry with purified SalK and purified peptide to

determine if they interact. The roles of peptides in signaling by Gram-positive bacteria is well characterized (49, 58, 60, 62-65, 67, 128) and roles in virulence specifically (49, 60, 63, 128).

Additionally, we could examine the role of the phosphorylation state of the SalR response regulator protein through allelic replacement of *salR* with a point mutant that is translated into a protein that is incapable of being phosphorylated by SalK, SalR_{D53N}, or one that acts constitutively phosphorylated, SalR_{D53E}. Chromosomal *salR* phosphomutant and phosphomimic strains could be examined for transcriptional regulation of the *sal* locus genes, and also to determine other potential targets of SalKR regulation. The ability for two component signaling systems to cross-talk *in vivo* and regulate multiple targets is increasingly appreciated in multiple bacterial species (129-135).

The synergistic effects of the SalA peptide and the SalKR proteins in promoting bacterial survival in human whole blood is another avenue for future work. Determination of the factor that is present in blood that contributes to this effect could give key insights into septic infection by *S. pyogenes*. Additionally, strains with mutations in *salA* and *salZ*, or in *salKR* and *salZ*, or in all of *salA*, *salKR*, and *salZ* should be generated to determine further potential combinational roles of these peptides and two-component system in virulence by *S. pyogenes*.

The ability to interrupt virulence signaling could lead to an important understanding of virulence mechanisms of *S. pyogenes*, and could lead to clinical interventions to treat invasive *S. pyogenes* disease. Two component systems are an

active target of intervention (51) as are interruption of quorum sensing peptide signaling (136, 137).

CHAPTER 7: FUTURE DIRECTIONS

Effects of extracellular domain of CpsA on GBS cell envelope:

Follow up work on this chapter's topic should be focused on the CpsA peptide. Firstly, narrow down what portion of this peptide is necessary and sufficient for capsule inhibition by examining wild type cultures grown with shorter versions of this peptide for capsule expression and chain length phenotypes. Additionally, determination of the binding partner(s) of the peptide would be helpful to determine the binding partners of CpsA in proper regulation of the GBS cell envelope.

Additionally, a peptide corresponding to amino acids 153-187 should be constructed and wild type cultures grown with this peptide examined for capsule expression and chain length phenotypes. Episomal expression in the wild type strain of a CpsA with truncation of these amino acids showed they too had a role in the dominant negative capsule expression phenotype. Examination of wild type bacteria grown with this peptide alone, or with this and the CpsA peptide examined in this chapter, should be undertaken to determine the effects on capsule expression and cell wall integrity. If an effect is seen, the binding partner(s) of this peptide should be determined to further understand the binding partners of CpsA in cell envelope regulation.

Other work should be undertaken to determine the effects on the cell wall. Transmission electron microscopy (TEM) to observe the cell wall in mutant strains expressing truncated CpsA, and of wild type strains treated with the peptide, should be performed. This could verify the fluorescent vancomycin data indicating cell wall thickness abnormalities and aberrant division septum formation. Additionally, the cell wall integrity can be examined by treating strains expressing truncated CpsA, or

cultured with CpsA peptide, with cell wall targeting antimicrobials, and cell membrane disrupting chemicals such as detergents and osmotic agents.

Effects of the leucine zipper motif of CpsA on GBS cell envelope:

Further work needs to be done to see if the leucine zipper motif is acting to cause specific binding of DNA at the *cpsA* promoter. This will require purification of a stable leucine zipper mutant CpsA protein to perform electrophoretic mobility shift assays (EMSAs). If the FLAG-tagged leucine zipper mutant CpsA is also found to be unstable *in vitro*, purified membrane fraction containing CpsA or leucine zipper mutant CpsA should be used in EMSAs.

Also, further work on the multimerization of CpsA needs to be done. Better cross-linking *in vivo*, either in *E. coli* or ideally in GBS lysates, needs to be performed to see clear multimers of CpsA and determine the role of the leucine zipper motif. Better *in vitro* crosslinking and isothermal titration calorimetry (ITC) should be performed to determine multimerization status of purified protein.

Additionally the binding partners of CpsA need to be determined. Co-immunoprecipitation of both full length and CpsA-117 with and without the leucine zipper motif will identify the binding partners of CpsA that bind the extracellular domain (interact with CpsA full, and CpsA-leu zip-full, but not the CpsA-117 truncations) and that bind the leucine zipper motif (those that interact with CpsA full and CpsA-117, but not the leucine zipper mutants). Additionally, binding partners that interact at the leucine zipper motif could be predicted by bioinformatics identifying transmembrane leucine zipper motifs in other GBS proteins and further analyzed.

Lastly, analysis of the role of the leucine zipper motif on cell wall stability should be performed. TEM of cultures episomally expressing leucine zipper mutant CpsA should be performed to look for cell wall thickness and cell division abnormalities. Also, mutants episomally expressing leucine zipper mutant CpsA should be examined for autolysis phenotypes and for sensitivities to cell wall and membrane targeting antimicrobials.

Role of LytR in cell envelope maintenance of GBS:

This work is still preliminary, but there are several interesting avenues to pursue. Both the CpsA and LytR proteins contain the conserved LCP domain. If this domain is the functional enzymatic part of the protein it would be interesting to see how the CpsA protein truncations perform in the *lytR::pUC* and $\Delta cpsA$ *lytR::pUC* strain backgrounds. An unexplained finding from our work with the strain expressing the CpsA-117 truncation is that episomal expression of this protein, which has the entire extracellular domain of CpsA removed, is able to complement the *cpsA* deletion strain for capsule expression, but it is unclear by what mechanism. Perhaps the LCP domain of the LytR protein is capable of functionally compensating for the missing LCP domain of the CpsA-117 protein truncation. Additionally, we were unable to explain the mechanism of the dominant negative effect of episomal expression of CpsA truncations containing all or part of the accessory domain in the absence of the LCP domain. Expression of these truncations in the *lytR::pUC* and $\Delta cpsA$ *lytR::pUC* strains could further elucidate this mechanism since there would not be the confounding LCP domain of the LytR protein present.

The synthetic CpsA peptide should also be examined with the *lytR::pUC* and Δ *cpsA lytR::pUC* strains. This could determine if the synthetic peptide is interacting with a common substrate/binding partner of both of the LytR and CpsA proteins or if it is interacting with a unique substrate/binding partner of CpsA. If found to have promiscuous activity against multiple LCP family members of the same species, this peptide could even have therapeutic implications against multiple Gram-positive bacterial pathogens.

Lastly, a strain with a triple mutation in the genes encoding all three LCP family members of *S. agalactiae* should be produced. This could further delineate the contributions of each member of the LCP family in GBS and give insight into its cell envelope biosynthesis, and cell division regulation.

Virulence signaling of the *Streptococcus pyogenes salivaricin* locus:

Specific binding conditions for SalR to the *salkR* promoter need to be determined. Optimization of the binding buffer is required. The current binding buffer contains too much glycerol, and both magnesium and EDTA, which chelates magnesium which negates the potential for magnesium to be acting as a cofactor. Magnesium was added to the binding buffer after preliminary data (P. Namprachan-Frantz, unpublished data) indicated enhanced transcription of the *salkR* promoter in alkaline phosphatase assays of *S. pyogenes* promoter-reporter strains grown in the presence of magnesium. However, magnesium is a cofactor for many nucleases, so inclusion of magnesium in the binding buffer should be done with caution, to avoid possible activity of contaminating nucleases. Additionally, all binding buffers should also contain non-specific DNA, salmon sperm or poly dAT, and protein, BSA. Also, a

range of ionic concentrations should be examined to optimize binding. Additionally, the potential for both SalA and/or SalZ to serve as transcriptional cofactors needs to be examined. Purification of these peptides and inclusion in binding reactions should be performed.

Further work could be done in examination of transcription of this locus and virulence by replacing *salR* on the chromosome with the phosphomutant and phosphomimic point mutants. These constitutively unresponsive, or responsive, proteins would allow us to see the role of SalR phosphorylation in virulence, using *ex vivo* human blood and also zebrafish models of disease, and to examine transcription of the *sal* locus, using both our promoter reporter fusions and qRT-PCR.

APPENDIX: ZEBRAFISH AS A MODEL FOR ZOO NOTIC AQUATIC PATHOGENS

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Abstract

Aquatic habitats harbor a multitude of bacterial species. Many of these bacteria can act as pathogens to aquatic species and/or non-aquatic organisms, including humans, that come into contact with contaminated water sources or colonized aquatic organisms. In many instances, the bacteria are not pathogenic to the aquatic species they colonize and are only considered pathogens when they come into contact with humans. There is a general lack of knowledge about how the environmental lifestyle of these pathogens allows them to persist, replicate and produce the necessary pathogenic mechanisms to successfully transmit to the human host and cause disease. Recently, the zebrafish infectious disease model has emerged, as an ideal host for examining aquatic pathogens, not only while in the aquatic environment, but also during infection of the human host. This review will focus on how the zebrafish has been used successfully to analyze the pathogenesis of aquatic pathogens.

1. Introduction

Aquatic pathogens are found in all water environments including marine, freshwater, brackish water, sewage, ground and even drinking water. Seawater alone harbors about 100 million bacteria per liter (138). Humans can be exposed to all of these aquatic sources through various activities, ranging from consumption of contaminated seafood, ingestion of contaminated drinking water, recreational activities

such as swimming, surfing, boating or fishing, exposure of open wounds to contaminated water sources or through handling of contaminated fish.

An important source of human pathogens from aquatic habitats is the many fish species that are used for food consumption. Fish are an important part of the human diet worldwide and can therefore serve as vectors of human disease. Extensive growth in the aquaculture industry in the last 30 years has come from an increased desire for lean protein and healthy fats, as well as depletion of fish species in natural waters from overfishing. In addition, restrictive quotas have been imposed for many wild caught fish in certain geographical areas leading to a further decline in fish availability in the food supply. To further increase overall production to meet the rising demand, fish farms often have overcrowded ponds that can serve as hot transmission sites for fish disease and serve as bacterial reservoirs that can be passed on to humans during food preparation. In many parts of the world, fish that can harbor potential bacterial pathogens live in water supplies creating a reservoir that can be passed on to humans in drinking water. Incidences have been reported in which the source of a bacterial disease outbreak in wild fish came from a nearby contaminated aquaculture facility (139). Therefore, one of our major sources of protein can also be the origin for potential human pathogens as well as serve as a major contributor to contamination of essential water supplies.

For many years, zebrafish (*Danio rerio*) have served as an important tool of biomedical research, providing great advances in our understanding of vertebrate development and disease modeling. Importantly, in recent years, zebrafish have been used to effectively model human infectious disease. Zebrafish symptoms closely reflect

those of diseased wild and farmed fish or clinical symptoms observed during human infections. Zebrafish can also become colonized by some aquatic pathogens without succumbing to disease providing an excellent model for the role of fish as an “environmental reservoir” for aquatic pathogens. “Environmental reservoirs” describes locations that allow bacterial persistence and replication in the environment while favoring pathogen transmission to susceptible hosts (140). This review will focus on recent research on zebrafish as experimental hosts of aquatic bacterial pathogens that can act as environmental vectors for human disease by colonization of both farmed and wild fish populations. Furthermore, aquatic colonization models of human pathogens can provide information on environmental reservoirs of pathogens and how they contribute to transmission of human disease. Of all the infectious diseases modeled using the zebrafish host, those diseases caused by aquatic organisms would appear to have the greatest potential to add to our knowledge of pathogenesis because of environmental niche they share.

2. Utilizing zebrafish as an infectious disease model.

Natural pathogens of fish, such as *Mycobacterium marinum* and *Streptococcus iniae*, have been utilized with the zebrafish infectious disease model to provide excellent models for understanding the pathogenesis of human *M. tuberculosis* pathogenesis (see (141-147) for review and citations within) and human streptococcal systemic infections (45-47, 120, 148-150). Using a model host allows comparison in side by-side infections of bacterial mutants with wild type bacteria to examine the role of virulence genes in aquatic and human disease. Information gained from these analyses allows development of methods to better target infection either in the vector (fish) itself or in the

human or animal host. One advantage of using the zebrafish as an infectious disease model is the ability to knockdown zebrafish genes using morpholino technology to suppress expression of a particular immune gene to determine its role in fighting disease. Furthermore, vaccine and treatment strategies studied in the zebrafish can be translated to the fish farms and to the human clinics. Consequently, the use of the zebrafish for analysis of aquatic pathogens makes it an ideal infectious disease model.

Zebrafish disease models have utilized multiple life stages of the host, from embryos to adults. Because the innate immune system is active within 24 hours post fertilization (hpf) in zebrafish, but the adaptive is not completely developed until 3-4 weeks later, important differences in required immune responses for fighting specific infections can be analyzed using the different stages of development.

Multiple methods of inoculation have also been used and depend on the ability of the pathogen to breach natural defense barriers of the zebrafish. The outer mucus layer of the zebrafish provides a natural barrier to infection, much as the mucous membranes in human tissues. The epidermis and scales of the zebrafish also act as a protective layer just as the epidermis and dead skin cell layer act as a barrier to pathogens in humans. Natural routes of infection in the model system include simply adding bacteria to the tank water; bath immersion, in which the zebrafish are placed for a limited period of time in a high concentration of bacteria and then placed into fresh tank water; and dermal abrasion prior to bath immersion. Fish can also be infected or colonized by the oro-gastric route, by being exposed to inoculated tank water or allowed to feed on inoculated food sources. All of these test the ability of the bacteria to breach natural barriers, or mimic inoculation conditions that would be encountered in the natural

aquatic environment. However, they do not allow for an accurate measurement of the amount of bacteria delivered to the host.

Other methods of inoculation include oral gavage, in which bacteria are inserted into the esophagus through a small tube attached to a syringe (151, 152). This allows inoculation into the gastrointestinal tract and is particularly important for analysis of pathogens that have a GI tropism. Although this method does provide a bit more precision in determining the amount of bacteria inoculated, some of the inoculum can escape through the gills or be lost through the mouth. Therefore, subsequent bacterial counts taken shortly after inoculation can be inconsistent.

The most common route of inoculation is injection of bacteria directly into the host, which gives reliable reproducible infection results, but is not a natural route of infection. In adult zebrafish this includes intramuscular injection into the dorsal muscle or intraperitoneal injection into the peritoneum (153). Microinjection is also used for larval infections where bacteria are inoculated into the otic vesicle, the yolk sac, tail muscle or the tail vein (154). All of these can be used to mimic a certain type of infection and to ask a specific question about the ability of the pathogen to survive in a particular tissue or to disseminate to other tissue environments.

3. Aquatic pathogens modeled in the Zebrafish

While mammalian models of human disease have proven invaluable in providing information on pathogenesis, they do not always accurately mimic disease symptoms that occur during human infections. Furthermore, some human pathogens do not readily infect mice, rats or rabbits, as they are not natural pathogens or these organisms. In the case of zoonotic infections, information on transmission from the

vector to the human host is completely lacking. Many questions remain about the ability of the pathogen to persist during the environmental stage or in an alternate host, often in a non-pathogenic state, before being transferred to humans. Using the zebrafish to address these types of questions with aquatic pathogens provides an opportunity to acquire new knowledge that could be used to develop strategies to eliminate the pathogen before human exposure.

3.1 *Edwardsiella*:

Edwardsiella tarda and *Edwardsiella ictaluri* can cause septicemia in fish populations from both freshwater and marine environments. While *E. ictaluri* only infects fish, *E. tarda* is a zoonotic pathogen that can infect both fish and humans (155). Transmission to humans is through exposure to contaminated aquatic environments and infected fish or through ingestion of contaminated fish (155). Gastroenteritis is the most common clinical syndrome observed in humans and can range from an asymptomatic carrier state to intestinal colitis (156). *E. tarda* can also cause extraintestinal infections with soft tissue infections, meningitis, peritonitis, septicemia, bacteremia, endocarditis and urosepsis (157). Although infections in humans are more rare than in fish, *E. tarda* septicemia infections occurring in individuals with impaired immune systems can be severe and result in high mortality rates up to 50% (155, 157, 158).

Along with the Aeromonads, the *Edwardsiella* bacterial species cause the most severe morbidity and mortality in aquaculture (159). Multiple species of fish are susceptible to edwardsiellosis including carp, tilapia, eel, catfish, mullet, salmon, trout and flounder. Conditions most favorable for *E. tarda* infection include high temperature,

poor water quality and high organic content (159), conditions that can often occur in aquaculture farms without proper diligence to hygienic practices. The bacteria can survive in pond water and mud for long periods of time and can increase in concentration with increasing temperature and organic load (159). The clinical pathology of edwardsiellosis includes small cutaneous lesions that can develop into large necrotic abscesses, petechial hemorrhaging of the fin and skin, rectal hernia and swelling of the abdomen due to fluid accumulation (160, 161). The ability to survive outside the host provides an environmental reservoir that can lead to outbreaks of infection in the fish population, and subsequently humans, when the conditions are favorable.

Experimental infection analyses have shown zebrafish to be an effective experimental host for *E. tarda* infection, showing similar clinical pathology to that observed in naturally infected wild and farmed fish populations (161). While larvae were susceptible to infection by bath immersion, adult zebrafish were only susceptible to infection if the dermis was abraded prior to bath immersion, or if injected with bacteria. Mortality was observed from all infection methods in both larvae and adult fish. Gross symptoms in adult fish included discoloration and petechial hemorrhages at the site of wounding, if infected via the abrasion-bath immersion method, and distended abdomen and characteristic peri-anal edema, if infected by IP injection (161). The blood of infected fish contained inflammatory cells with intracellular bacteria in macrophages, similar to that shown in kidneys of experimentally infected rohu fish (159). Infected larvae showed systemic inflammation, leading to epithelial and muscle tissue

destruction, and numerous inflammatory cells in the bloodstream, with intracellular bacteria in macrophages (161).

Although bath immersion mimics a more natural route of infection, modeling the environment of a fish farm, or site of high bacterial load in the natural environment, van Soest et al. (162) found that infection levels and especially immune responses following immersion in *E. tarda* culture are highly variable from fish to fish. However, injection into the tail vein of the larva caused a more reproducible infectious dose and a more consistent induction of immune related genes. While immersion resulted in a range of 25-75% mortality, tail vein injection resulted in 100% mortality. Therefore, while the zebrafish can be used to mimic natural infection routes of *E. tarda* that may occur in the environment, using methods that produce 100% inoculation (such as injection) allows experimental analysis of virulence pathologies and host response by bypassing the first protective barriers to infection. Pro-inflammatory responses were measured on the single embryo level and showed a strong induction of the *il-1b* and *mmp9* genes (162). Pressley et al. (2005) also showed a strong upregulation of IL-1B in both *E. tarda* infected larvae and adult zebrafish, as well as an increase in TNFa (161). The results of a microarray performed on RNA isolated from infected zebrafish larvae revealed a increased expression of genes involved in defense and regulation of the immune response (162). Results from these types of analyses with zebrafish provide new information about host response to *E. tarda* infection allowing development of more effective treatments.

Using a responsive experimental host also allows for analysis and identification of major virulence genes in the pathogen. Very little was known about the pathogenic

mechanisms of *E. tarda* until recently. Using the zebrafish infectious disease model, several groups have been able to identify key factors in *E. tarda* pathogenesis. Xiao et al. (2012) showed *E. tarda* encodes two catalases, enzymes that allow the bacteria to breakdown hydrogen peroxide. Hydrogen peroxide is often the first line of defense used by the innate immune system against invading pathogens. Comparison of zebrafish infections with an *E. tarda* wild type strain and a strain that had a knockout of one of the catalase genes showed partial attenuation, with an approximate 10-fold increase in LD₅₀. However, a strain with a deletion of both catalase genes showed a 100-fold increase in LD₅₀ (163). Similarly, Wang et al. (2012) showed that deletion of the *rpoN* gene, which encodes an alternative sigma factor implicated in multiple species as a transcriptional regulator of virulence genes, had an 8-fold increase in LD₅₀ compared to infection with the wild type strain. The $\Delta rpoN$ strain also had reduced ability to survive peroxide, acid, osmotic, and starvation stresses and produces less biofilm and chondroitinase, all of which could be implicated in virulence (106). The invasin protein of *E. tarda* was examined for its role in virulence during zebrafish infection. Both deletion and overexpression of the invasion gene was analyzed. While deletion showed decreased virulence, most likely through reduced hemolytic activity and biofilm formation, overexpression of the invasin protein demonstrated higher adherence and internalization levels in cultured zebrafish epithelial cells (164).

Yu et al. (2012) took a different approach by analyzing the role in virulence of an antibiotic resistance plasmid (165). Virulence genes are often encoded on these plasmids, leading to transfer of virulence genes between bacterial populations and increased survival of bacteria in environments with high antimicrobial concentrations

such as fish farms. An *E. tarda* strain cured of an antibiotic resistance plasmid showed attenuation in mortality in zebrafish as well as goldfish and Japanese flounder. Subsequent sequencing of the plasmid identified 84 open reading frames, revealing that multiple virulence genes other than the genes for antibiotic resistance are encoded on the plasmid harbored by *E. tarda* (165). All of these analyses provide new information on genes required for pathogenesis, identifying new targets for antimicrobial therapies.

A major reason for the success of the zebrafish infectious disease model is because their immune system is very similar to humans (150). This means that they can also be a good model for vaccine development and analysis of immunization against infections. In an effort to find a vaccine for *E. tarda* infections, Yang et al. (2013) examined the zebrafish immune response using a live attenuated *E. tarda* vaccine strain for immunization and a fully virulent *E. tarda* strain for the subsequent challenge. The vaccination led to 78% of fish surviving a challenge 4 weeks post-immunization whereas only 12% of naïve fish survived challenge. Immunity was mediated through TLR5 signaling and activated a CD8+ T-cell response through cross-presentation of extracellular antigen. CD8+ responses were more induced during the challenge phase than the immunization phase. Vaccination also induced specific antibody in the fish serum, indicating that this vaccination activated both cell and humoral mediated immunity (166).

3.2 *Aeromonas*

Aeromonas species have had a devastating effect on the aquaculture industry with reports of major losses in fish populations worldwide resulting in great financial loss (167). Disease states in fish from *Aeromonas hydrophila* infections range from

hemorrhagic septicemia, red sore disease and ulcerative infections in multiple species of fish (168). Although the *Aeromonads* are mostly considered associated with aquatic environments i.e., found in fresh water, seawater, drinking water and groundwater, they are also ubiquitous in the environment, and are associated with a number of recreational habitats, animals and consumable products that can lead to human exposure and potential infection (168). There are a plethora of clinical syndromes, both intestinal and extraintestinal, that *Aeromonas* can cause in humans ranging from relatively mild gastroenteritis to infections with high mortality rates such as septicemia, necrotizing fasciitis, and myonecrosis ((168) and the references within). Serious wound infections can occur from simple abrasions if the affected area is exposed to contaminated water sources, such as streams, lakes and recreational waters. Therefore, aquatic habitats can serve as an environmental reservoir for human *Aeromonas* infection, which is now considered an emerging pathogens for humans (169).

A. hydrophila is also a natural pathogen of zebrafish, making it an excellent model for the study of pathogenesis. A recent outbreak of hemorrhagic disease in laboratory zebrafish was isolated and identified to be *A. hydrophila* (170). Naïve fish challenged with this strain by either IP injection or a wound-immersion route quickly developed the same symptoms as fish in the original outbreak. Importantly, injection with just the extracellular secreted products gave similar mortality, indicating that the major virulence factor(s) is secreted. Live bacteria and their extracellular secreted products were hemolytic against fish blood and cytotoxic to cultured zebrafish kidney cells. Pro-inflammatory cytokines (TNF α , IL-1 β and IFN γ) and iNOS were strongly

induced by infection with live bacteria as well as with extracellular secreted products alone. These results strongly indicate that *A. hydrophila* causes disease by triggering massive inflammation, mediated by the extracellular secreted products (170). In support of these results, when Li et al. (2011) focused on three known virulence factors of *A. hydrophila*: the aerolysin, the cytotoxic enterotoxin and a serine protease, it was found that these three factors act synergistically in causing disease (171). Examining isolates encoding none of or only 1 or 2 of these virulence genes had similar LD₅₀ when tested in a zebrafish model, whereas isolates with all three virulence genes had a reduced LD₅₀. Isolates positive for all three virulence genes were also more hemolytic, more cytotoxic towards Vero cells, and had more proteolytic activity in their supernatants, suggesting that disease is caused by tissue destruction by these virulence factors (171). However, there were strain specific differences, indicating that other virulence factors, beyond the three tested, also play a role in disease.

Much work has been done on finding ways to combat *A. hydrophila* infection in aquaculture to preserve fish populations as well as circumvent transmission to humans. Antibiotic treatment is one of the major options for treating an infection. However, use of inappropriate antibiotic treatment in the food supply has been highly controversial. One reason is that inappropriate antibiotic use can lead to development of antibiotic resistance in pathogens through selective mutations or transfer of antibiotic resistance genes between bacteria. Cantas et al. recently utilized the zebrafish infectious disease model to examine the role of antibiotic use in treating *A. hydrophila* infection (172). Oral gavage was used to inoculate adult zebrafish with *A. hydrophila* leading to systemic disease. Infected zebrafish were then treated with antibiotics that were either

effective for the infection or at sub-inhibitory levels leading to ineffective treatment. Expression of innate immunity genes in the gut of the zebrafish was then measured under the different conditions. In addition, expression of bacterial antibiotic resistance plasmid transfer genes was examined to determine how the antibiotic treatments affected potential transfer of antibiotic resistance. Infection alone increased expression of IL-1 β and IL-8, but not TNF α or complement factor C3. Infection plus effective antibiotic treatments increased all four examined immune genes, as did ineffective treatment with trimethoprim, sulphonamide or flumequine, but ineffective doses of tetracycline reduced expression of TNF α and C3 below levels measured in untreated infected fish. In addition, ineffective antibiotic treatment can also activate transfer genes of a bacterial antibiotic resistance plasmid, while transfer genes were repressed in bacteria during effective antibiotic treatment. Taken together, using zebrafish and *A. hydrophila* as a model, this study demonstrated the danger in ineffective antibiotic treatment leading to activation of resistance plasmid transfer genes, and in the case of tetracycline, repression of immune genes, whereas effective antibiotic concentrations not only kill the pathogen, but block transfer of resistance plasmids and can activate innate immunity genes to help the immune system clear the infection. This supports observations that single high dose antibiotic therapy can clear infection (172).

The zebrafish model has also been used to explore alternatives to antibiotic treatments to *A. hydrophila* infection. Many bacteria use “quorum sensing” a signaling system based on population density to communicate with each other. *A. hydrophila* use quorum sensing to regulate virulence gene expression using the signaling molecule acyl homoserine lactone (AHL) (81, 173, 174). One alternative to antibiotic use would be to

block the quorum sensing signaling between bacteria using a treatment with an AHL degrading enzyme (lactonase), acting as a broad spectrum inhibitor of virulence. Zebrafish exposed to *A. hydrophila* infection by bath immersion that were subsequently fed with a fish food supplemented with an AHL lactonase showed significantly reduced mortality compared to fish not treated with the enzyme providing a new potential therapy for infection (175).

Vaccination methods in fish populations are also being pursued as a mechanism for combating *A. hydrophila* infection. This reduces the dangers from broad scale antibiotic use in ponds, but is more labor-intensive as each fish needs to be treated. The zebrafish model has provided an excellent model for testing vaccination methods for many types of aquatic pathogens, including *Aeromonas*. One way in which to provide immunity to a population without having to vaccinate every fish is to use a vaccine that can be provide immunity that is transferred from mother to offspring. Wang et al. (2009) demonstrated that vaccination of breeding females with formalin-killed *A. hydrophila* provided significant protection to the embryos (176). This was not mediated by maternal transfer of antibody, but rather by maternal transfer of complement components C3 and Bf. Vaccination of breeding females caused a rise in C3 and Bf levels in both the whole body homogenate of the adult fish and in the extract of eggs produced by that fish. Treatment of the fish with an anti-C3 or anti-Bf antibody reversed these effects. Embryos from vaccinated and unvaccinated fish were challenged with live, fully virulent *A. hydrophila*. Embryos from vaccinated fish survived significantly more than those from unvaccinated fish. Embryos from vaccinated fish were able to kill

the bacteria, providing evidence that the maternal derived complement components are able to lyse the pathogen (176).

In a follow-up study, vaccination of female zebrafish with the hapten-carrier trinitrophenylated bovine serum albumin TNP-BSA raised a specific antibody response in the fish (106). These antibodies were transferred to the offspring of vaccinated females. Embryos from females vaccinated with TNP-BSA were less susceptible to *A. hydrophila* than embryos from unvaccinated females. This protection was IgM specific, as an anti-IgM treatment blocked protection. Embryos of vaccinated mothers were able to kill *A. hydrophila* suggesting that the protection is maternal antibody mediated bacterial lysis. These were not *A. hydrophila* specific antibodies, indicating that these maternal antibodies were able to act non-specifically in protecting the embryo from water-borne infection. This mechanism of specific maternal antibodies acting non-specifically against bacterial pathogens, in addition to maternally derived natural antibodies, may protect embryos from water-borne infection until development of the adaptive immune system (106).

3.3 Streptococcus

Streptococcosis in fish populations was first confirmed in Japan in the late 1950s (177). Since that time, multiple species of streptococci have been found to infect fish populations, but the major cause of economic losses from streptococcosis in aquaculture and wild fish population are *Streptococcus iniae* and *Streptococcus agalactiae*. Both of these pathogens can also cause disease in humans and the aquatic habitat can act as a reservoir for human infection. Here we will discuss reports of the

zebrafish infectious disease model to experimentally determine pathogenic mechanisms in both human and aquatic isolates of streptococci.

3.3.1 *Streptococcus iniae*

The first report of *Streptococcus iniae* infection was in 1976 in a captive Amazon freshwater river dolphin in which subcutaneous lesions called “golf ball disease” were described (178). However, in the last 35 years, *S. iniae* has emerged as a major finfish pathogen with a host range of over 27 species of fish from both freshwater and marine environments (179). While wild fish populations are just as susceptible to *S. iniae* infections, the most devastating effects of this pathogen have been observed in aquaculture with an estimated economic loss of over 100 million worldwide (180). Although the type of disease presentation varies with fish species, most often a systemic infection occurs with accompanying meningitis and panophthalmitis (181). In almost all cases, infection results in high morbidity and mortality.

S. iniae is also a zoonotic pathogen with the first reports of human infection occurring in 1995 after the handling of fresh fish from aquaculture (182). Infection in humans is usually a result of handling of contaminated fish and sustaining an injury allowing access of the pathogen to the underlying dermis layer, usually of the hand. Resulting infection manifests as bacteremic cellulitis with the rare complications of endocarditis, meningitis, arthritis, sepsis and toxic shock in immunocompromised individuals (182-186). Although there have only been about 30 reports of *S. iniae* infections in humans, it has been suggested that infections are most likely underreported because clinical identification relies on biochemical testing and *S. iniae* is currently not listed in commercial or clinical databases (185, 187).

The first publication using zebrafish as an infectious disease model for streptococcosis demonstrated that the histopathology as well as the clinical symptoms of *S. iniae* disease mimicked that which was observed in farmed fish populations (149). Zebrafish succumbed to a systemic infection within 4 days post intramuscular injection with an LD50 of 5×10^3 cfu. *S. iniae* was able to be isolated from the skin, heart, gall bladder and brain (149). Subsequently, a large-scale screen investigating virulence factors in *S. iniae* highlighted the advantage of using the zebrafish infectious disease model (45). A signature-tagged mutagenesis screen was performed to determine mutants that could not survive in the heart or the brain of the zebrafish at 24 hours. The screen identified 41 mutants that were attenuated in the zebrafish host, 50% of which had homology to virulence genes from other streptococcal pathogens. These results confirmed the effectiveness of the zebrafish disease model for identifying virulence genes required for infection in the host. Importantly, 10 of the transposon insertions were found to be in the capsule operon of *S. iniae*, confirming the importance of capsule for systemic infections (45). By comparing the sequence of the capsule operon of the 9117 virulent *S. iniae* strain to a commensal strain of *S. iniae* isolated from healthy fish, Lowe et al. (2007) was able to show that the commensal strain had a large deletion in the capsule operon encompassing several important capsule synthesis genes (81). In addition, the ability of various capsule mutants to survive in the presence of the wild type strain (competitive indices) or the ability to disseminate and survive in different tissue environments was examined using the adult zebrafish, providing new information on regions of the capsule operon required for virulence (81).

To better determine the role of the innate immune system in protection from *S. iniae* infection, a zebrafish larvae model was employed to visualize host-pathogen interactions in real time. An *S. iniae* wild type strain and a $\Delta cpsA$ capsule mutant strain were stained with the CellTracker red CMPTX dye prior to injection. Three zebrafish larvae strains were used as hosts: the Tg(*mpeg1:dendra2*) (47) zebrafish line in which the macrophages are expressing EGFP; the Tg(*mpx-dendra2*) (188) zebrafish line in which the neutrophils are expressing EGFP; and the Tg(*mpx:mCherry-2A-rac2-d57n*) zebrafish line in which neutrophils are expressing the inhibitory Rac2(D57N) mutation making them unable to respond to inflammatory stimuli, including otic infection (141). Injections were into the otic vesicle so as to observe recruitment of macrophages and neutrophils to the site of infection, as the otic cavity is usually free of leukocytes (189). Injections of the wild type *S. iniae* into larvae were lethal within 24 hours, while over 90% of larvae injected with the $\Delta cpsA$ mutant survived. Both neutrophils and macrophages were visibly recruited to the site of infection and were observed to phagocytose bacteria. While depletion of both macrophages and neutrophils using Pu.1 morpholino injection led to an increased survival of the larvae, using a strain with impaired neutrophil function, but normal macrophage function, revealed that neutrophils are essential for control of *S. iniae* wild type infection (47).

The *cpsY* gene, which is directly upstream of the capsule operon of *S. iniae* was originally thought to be involved with capsule expression because of its homology to the LysR transcriptional regulator family (190). A *cpsY* deletion strain was also highly attenuated in the zebrafish infectious disease model and showed an inability to disseminate to the brain, a key factor in *S. iniae* pathogenesis in fish (81). However, it

was determined that CpsY was not regulating capsule in *S. iniae* (81) or *S. agalactiae* (191) and was instead involved in methionine biosynthesis and uptake (192). To elucidate the role of CpsY in pathogenesis, Allen and Neely (2011) combined in vitro whole blood and human neutrophil assays along with in vivo zebrafish infections to determine that CpsY is required for survival within neutrophils (120). Subsequent research confirmed that in addition to methionine biosynthesis and uptake, CpsY stimulates cell wall stabilization through peptidoglycan O-acetylation and repression of autolysins (193). Therefore, as was shown by the larval infection model detailed above (47), neutrophils play a key role in controlling infection of *S. iniae*; however, the pathogen has evolved specific mechanisms that allow survival in that environment.

In an effort to learn more about key virulence genes required for pathogenesis of *S. iniae*, Locke et al.(2008) performed pyrosequencing of an *S. iniae* strain isolated from the brain of a diseased hybrid striped bass (K288) (148). Their analysis revealed an M-like protein (called SiM), which had high homology to the well-studied surface-anchored M protein from *Streptococcus pyogenes* that is required for virulence (194, 195). In addition, they identified a gene that encodes a protein homologous to the C5a peptidases found in both *S. pyogenes* and *S. agalactiae* that cleaves the chemoattractant C5a of the complement system (196). In vivo analyses in zebrafish and hybrid striped bass of *S. iniae* mutants in these two identified genes showed that the SiM protein plays a role in virulence, while the C5a peptidase did not. They further tested the SiM deletion strain as a vaccine candidate in the hybrid striped bass model and found it to be highly protective from a lethal dose of the wild type strain 90 days after vaccination (148).

Lopez-Munoz utilized the zebrafish-*S. iniae* disease model to determine the role of zebrafish IFNs during infections (197). Their results demonstrated that while both the group I and group II zIFNs could protect against viral infection, only the group I zIFN was able to protect zebrafish from *S. iniae* infection through the induction of proinflammatory genes. Interestingly, they also found that unlike with mammalian IFN- γ , zIFN-g was unable to produce the proinflammatory response to infections (197).

3.3.2 *Streptococcus agalactiae*

Streptococcus agalactiae, better known as Group B *Streptococcus*, (GBS) is a Gram-positive pathogen responsible for human and agricultural disease. Of all the streptococcal species, *S. agalactiae* appears to have the broadest host range for pathogenesis, having been isolated from both cold-blooded aquatic organisms as well as warm-blooded terrestrial animals. In humans, it is the leading cause of early onset neonatal sepsis and meningitis in the developed world (198). GBS can also cause infections in non-pregnant adults and is considered as an emerging pathogen in immunocompromised and older individuals (199). Outside of human infections, GBS is a major contributor to disease in agriculture, particularly bovine mastitis and fish sepsis.

Massive outbreaks of streptococci caused by GBS have been observed in both farmed and wild fish populations worldwide (200). There are also many reports examining the serotypes of strains isolated from different hosts to determine if certain serotypes are species specific (201, 202). There are only 9 serotypes of GBS and those that are found to cause the most invasive disease in humans have also been isolated from aquatic mammals and fish (203). One study identified eating colonized fish as a risk factor for human colonization of GBS (204).

Patterson et al. (2012) used the streptococcal-zebrafish model (149) to investigate disease characteristics of GBS (205). Zebrafish infected with high doses of GBS by either the intramuscular or intraperitoneal route succumb to infection, whereas fish infected with a low dose can clear the infection. Bacteria are found within the blood immediately after IP injection and dissemination to the brain was observed within 2 hours post injection. Inflammation within the brain was observed grossly as cerebral edema with higher expression of inflammatory cytokines IL-1 β and IL-6 within the brain compared to non-infected zebrafish (205). Their study accurately models the symptoms and response to meningitis as observed in human GBS patients. Mutations in virulence genes of GBS lead to attenuation in the zebrafish model, validating that factors important for human disease are also important for fish disease. Deletion of the transcriptional activator of capsule production *cpsA* leads to increased survival of fish infected by the IM route (38) and *cpsA* mutants are attenuated in the ability to disseminate to the spleen, heart and brain (Fig A1, unpublished data from our lab). Mutations in the hemolysin *cyIE* or a capsule transglycosylase *cpsD* or a two-component system responsible for virulence regulation *covRS* were attenuated in their ability to cause meningitis in the zebrafish, whereas the hypervirulent in humans COH1 strain caused increased concentration of GBS in the blood and brain, resulting in increased lethality in zebrafish (205).

3.4 *Mycobacteria*

Mycobacterium marinum has been analyzed extensively in zebrafish as a model for human tuberculosis infections caused by *Mycobacterium tuberculosis* (see (141-147) for review and citations within). However, *M. marinum* is also a human and fish

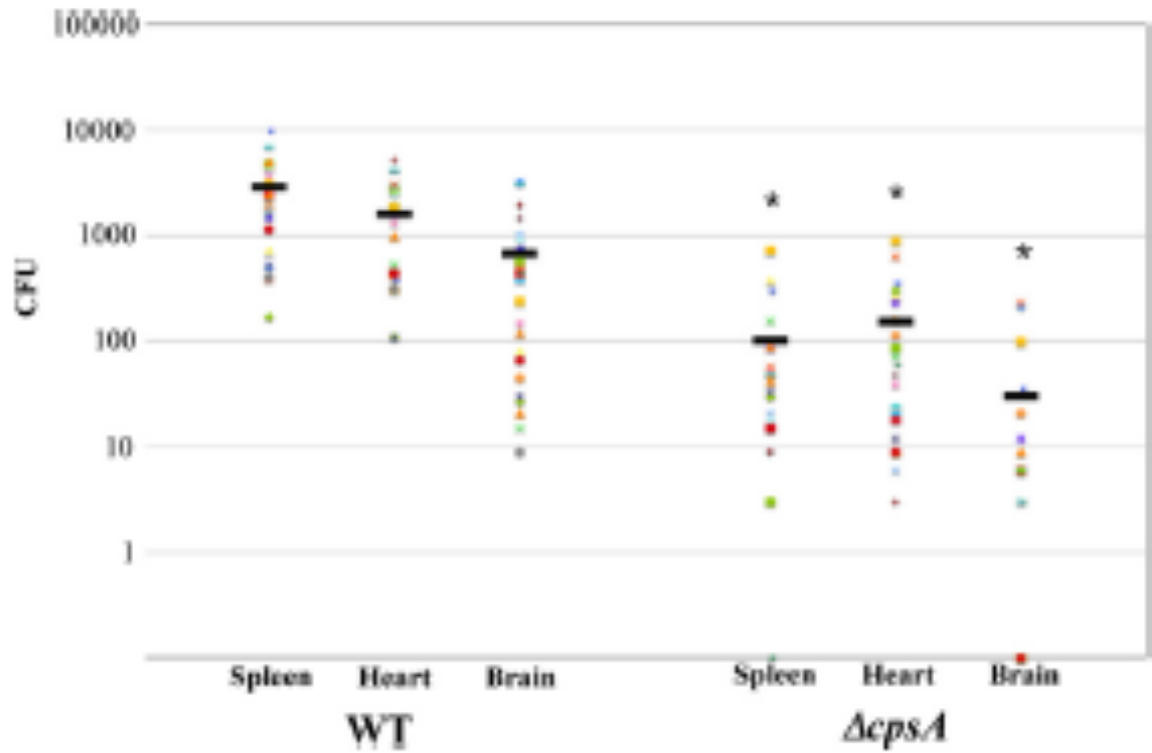


Figure A1: Adult zebrafish were injected intramuscularly with 10^6 CFU of *S. agalactiae* strain 515 or its isogenic $\Delta cpsA$ derivative. Four hours post injection, fish were euthanized and spleens, hearts and brains were isolated. Organs were homogenized and CFUs enumerated by viable plate count. Each dot represents 1 fish. (* $p < 0.01$).

pathogen in its own right. As a human pathogen, *M. marinum* most often causes a granulomatous skin and soft tissue infection in fish hobbyists named “fish tank granuloma.” This normally results from superficial wounding during routine fish tank maintenance. Additionally, infections have been linked to swimming in contaminated aquatic environments (206, 207). These infections can become severe requiring prolonged multi-antibiotic therapy and/or surgical interventions (206, 207). In patients being treated with immunosuppressant drugs, these infections can be more prevalent and severe (208). Superficial infection can rarely disseminate to bones and joints or cause a pulmonary infection (206, 207). Mycobacteria are also natural pathogens of marine and freshwater fish (see (209, 210) for review and citations within).

Fish kept in aquaculture farms and zebrafish research colonies are susceptible to natural infection by several species of mycobacteria, including *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. haemophilum* and *M. peregrinum*. Most species are opportunistic pathogens but *M. marinum* and *M. haemophilum* are highly pathogenic. Bacteria are acquired from other infected fish or infected biofilm. (for more extensive discussion see (211) and citations within).

To further examine the virulence of mycobacterial species isolated from research facilities Watral and Kent experimentally infected, by intraperitoneal injection, zebrafish with several strains of *M. marinum*, *M. abscessus*, *M. peregrinum*, and *M. chelonae* (212). Only fish infected with *M. marinum* exhibited symptoms of disease and succumbed to infection. However, histopathologic examination of infected fish found granulomatous lesions in the peritoneum, kidneys and ovaries of fish infected with *M. marinum* and non-*marinum* species, indicating that these fish can be sub-clinically

infected with mycobacteria. Live bacteria were only consistently recovered from the fish infected with *M. marinum*, but some individual fish were asymptomatic carriers of other the other mycobacterial species. Symptoms of *M. marinum* disease in experimentally infected fish were skin erythema, raised scales, swollen abdomens and rarely, skin lesions (212).

To study possible natural transmission routes, Peterson et al. (2013) examined inoculation of *M. marinum* and *M. chelonae* by the oro-gastric route by using the single cell eukaryotic ciliate *Paramecium caudatum* as a vector (213). Larval, juvenile and adult fish exhibited more extragastric (mainly kidney and spleen) granulomas containing *Mycobacteria* when fed paramecia infected with *M. marinum* or *M. chelonae* than when the bacteria were incorporated into a food gel matrix or free bacteria were added to the water. Gastrointestinal bacteria were observed for all infection routes, suggesting that bacteria are more virulent after passing through a ciliate host, and are better able to cause disseminated infection (213).

In many mycobacterial species the Type VII secretion system has been implicated in virulence. To examine the effect of the *M. marinum* type VII protein secretion system ESX-5 in establishment of granulomas in zebrafish, Weerdenburg et al. (2012) used the zebrafish model. Their analysis demonstrated that this protein secretion system serves a complex role in virulence (214). When the ESX-5 mutant was microinjected into zebrafish larvae 28 hours post fertilization with 100 cfu into the caudal vein, establishment of initial infection was mildly attenuated compared to injection with the wild type strain. Fewer bacteria were isolated from the infected larvae and granuloma formation was delayed in fish infected with the ESX-5 mutant than with

wild type bacteria. However, adult fish infected with the ESX-5 mutant succumb more rapidly to infection and have higher bacterial loads in the spleen and lung than fish infected with wild type bacteria. Since larvae do not yet have a functional adaptive immune system, the role of the adaptive immune system was examined to see if it was responsible for the discrepancy between attenuation in larvae and hypervirulence observed in adult fish. However, infection with the ESX-5 mutant into Rag1 deficient adult fish, which are deficient in an adaptive immune response, showed that the hypervirulent phenotype was not depended on the adaptive immune system. Histopathologically, fish infected with the ESX-5 mutant bacteria developed granulomas sooner and in higher numbers than fish infected with wild type bacteria. Additionally, pro-inflammatory cytokines (IL-1 β , TNF- α and IFN- γ) were more highly expressed in ESX-5 granulomas. Co-infection of wild type and ESX-5 bacteria, to determine if the secreted factors from the wild type could complement the hypervirulence of the ESX-5 mutant, showed that the ESX-5 mutant outcompeted the wild type strain. These results suggest that the ESX-5 protein secretion system functions to modulate *M. marinum* virulence and potentially contribute to persistence (214).

In addition to a type VII secretion system, the Sec2A alternative protein translocation system is another putative virulence factor of *M. marinum*. Sec2A mutants were attenuated in a larval zebrafish model of *M. marinum* infection (215). Fish were infected by microinjection into the caudal vein 24 hours post fertilization. Five days post-infection, bacterial loads were five times higher in fish infected with wild type than sec2A mutant bacteria. Mutant bacteria showed less aggregation in larvae and leukocytes formed smaller and less compact aggregates in fish infected with mutant

bacteria. Complementation of the *sec2A* mutation restored wild type levels of bacterial load and aggregation with leukocytes (215).

In an effort to develop a vaccine to control *M. marinum* infection, Cui et al. (2012) examined the effectiveness of an attenuated strain of *M. marinum* with reduced replication in macrophages. In addition, heat killed *M. marinum* and extracellular secreted products were tested. Results showed that while all three vaccination methods lead to the production of antibodies, only the live attenuated strain protected zebrafish from challenge with virulent *M. marinum* (216).

3.5 Vibrios

Of the human pathogens that can be found in seawater environments, *Vibrios* are the predominant species (217). Infection is most commonly acquired by wound contamination from exposure to contaminated water or aquatic organisms, the ingestion of contaminated water or consumption of raw or undercooked seafood (138). The clinical syndromes most commonly associated with *Vibrio* infection from aquatic environments are gastroenteritis, soft tissue infection and primary bacteremia (218). Soft tissue infections from wounds most commonly occur from recreational water activities such as swimming, surfing or boating, followed by fishing injuries and handling of seafood (219). Although food borne infection is the most commonly recognized clinical syndrome from *Vibrio* aquatic exposure, soft tissue infections can result in high morbidity and mortality, leading the Centers of Disease Control and Prevention to recently add non-cholera vibriosis as a national notifiable disease (219).

For non-food borne *Vibrio* infections, *Vibrio vulnificus* is the most common aquatic-associated infections in humans, followed by *Vibrio alginolyticus* and *Vibrio*

parahemolyticus (219). All three of these organisms have been modeled in the zebrafish (220-223).

3.5.1. *Vibrio alginolyticus*

Vibrio alginolyticus causes septicemia in fish, as well as gastrointestinal, wound and mucosal infections in humans. To determine the role in pathogenesis in vivo of the twin-arginine translocation system (Tat) used by *V. alginolyticus*, He et al. (2011) utilized the zebrafish infectious disease model (171). The Tat system is used by the bacterium for translocating folded proteins and secretion of specific virulence factors along with roles in growth and motility. Construction and analysis of a strain deleted for the genes in the twin-arginine translocation system impacted biofilm formation and swarming motility and decreased secretion of extracellular proteases. Loss of these functions resulted in a reduction in virulence in the zebrafish compared to infection with the wild type strain (171). Similarly, Wang et al. used the zebrafish model to analyze mutants in iron uptake systems by demonstrating that the TonB1 and TonB2 systems are also required for systemic virulence of *V. alginolyticus*. Deletion of one locus resulted in an approximately one-log increase in LD₅₀ and a double deletion resulted in over a 25-fold increase in LD₅₀ (31).

3.5.2 *Vibrio vulnificus*

Vibrio vulnificus causes the greatest majority of soft tissue infections from marine microorganisms (138). However, the most lethal infection caused by *V. vulnificus* is primary septicemia, with a greater than 50% mortality rate (218).

V. vulnificus infection of zebrafish is lethal and shows a specific increase of innate immunity genes following *V. vulnificus* infection (66). Treatment of zebrafish with

the antimicrobial peptide epinecidin-1 from Grouper (*Epinephelus coioides*) is able to protect zebrafish from *V. vulnificus* infection. Co-injection of epinecidin-1 conferred the best protection, but pre-treatment (one hour prior to injection of bacteria) or post-treatment (one hour after bacterial injection) also conferred protection. Fish surviving infection with epinecidin co-treatment were challenged again with bacteria 30 days post infection and had reduced mortality compared to naïve fish, indicating that epinecidin co-treatment acts similar to a live-attenuated vaccine. Microarray analysis for inflammatory genes indicated that infection, epinecidin and co-treatment lead to distinct transcriptional changes. Epinecidin is acting as both an antibacterial and immunomodulatory molecule to confer protection to *V. vulnificus* infection (66).

3.5.3 *Vibrio cholerae*

Another *Vibrio* species that is responsible for a great deal of morbidity and mortality worldwide from consumption of contaminated seafood or water is *Vibrio cholerae*. Disease from *V. cholerae* manifests mainly as a gastrointestinal pathogen, causing the disease of cholera, which leads to massive watery diarrhea resulting in dehydration and loss of electrolytes. Cholera afflicts an estimated 5 million people annually worldwide, causing over 100,000 deaths. Without rehydration therapy, cholera is lethal in 50-60% of patients; however quick treatment results in less than 1% mortality (224).

Like other *Vibrio* species, *V. cholerae* can be found associated with aquatic organisms (shellfish, insect egg masses, and plankton) as well as free swimming in water (225-228). Much research has been done on the human pathogenesis of *V. cholerae*, and recently there has been increased interest on the environmental lifestyle

and aquatic reservoirs of the organism, with hope of determining key factors required for transmission to humans. Current animal models for *V. cholerae* include the infant mouse model (229), the rabbit intestinal ileal loop model (230-232). Unfortunately, neither the mouse nor the rabbit are natural hosts for *V. cholerae* and therefore, do not mimic human disease. Senderovich recently identified non-O1 *V. cholerae* in the intestinal tracts of multiple species of wild-caught fish (228), suggesting that *V. cholerae* may use vertebrate fish as an environmental reservoir, allowing for bacterial replication and transmission to water sources and other aquatic organisms.

Not much is known about the environmental lifestyle of *V. cholerae* or the key factors required for the pathogen to survive for long periods in an aquatic environment. Since previous mammalian models were not successful in reproducing human cholera symptoms, recent work investigated the efficacy of using the zebrafish host model as a model for cholera disease, and/or model as an environmental reservoir for *V. cholerae* (38). Runft et al. found that zebrafish gut could be highly colonized over long periods of time by pandemic O1 serogroup *V. cholerae* strains through simple addition of the bacteria to the water. Furthermore, no prior manipulation of the natural intestinal flora is needed as is required with the other *V. cholerae* animal models, which suggests that colonization of the intestine in natural fish populations could easily occur with *V. cholerae*. Histology of infected zebrafish intestines illustrated that *V. cholerae* was closely attached to the intestinal epithelium and formed many micro-colonies in this environment (38). In an effort to mimic uptake of bacteria from both the water and the natural environment, providing a delivery vehicle of a contaminated food source was explored. Zebrafish rapidly eat freshly hatched brine shrimp as a natural food source.

V. cholerae express chitin binding proteins and were found to bind readily to brine shrimp after a 15 minute incubation (Fig A2A and A2B). Addition of contaminated brine shrimp to an experimental tank of zebrafish resulted in an initial increase in colonization by 2 logs at 2 hours post inoculation compared to water inoculated fish (Fig A2C). However, by 24 hours post infection, levels of *V. cholerae* in the intestine were actually lower than the water-inoculated fish. One possible explanation for this observation is that *V. cholerae* adhere directly to the feeding appendages of brine shrimp and, therefore, many of the *V. cholerae* are excreted by the fish without ever establishing colonization of the epithelial surface.

One of the most interesting findings from this study was the ability of infected fish to pass the infection to naïve fish. Zebrafish were placed in water contaminated with *V. cholerae* for 3 hours, followed by multiple washing steps. An exposed fish was then placed into an experimental tank with fresh water and multiple naïve fish. Importantly, previous work showed that *V. cholerae* cannot be isolated from the outer surface of the fish. After 24 hours, the naïve fish population, exposed to the contaminated fish, was colonized with *V. cholerae*. This type of disease transmission cannot be analyzed using the mammalian host models. Moreover, this occurrence is very likely to happen in the natural environment and may be one way in which *V. cholerae* maintains its presence in an aquatic habitat, by transfer from host to host (38).

This new model for *V. cholerae* provides a more natural route of infection of any previous animal model. Furthermore, it goes beyond most animal models as it provides a method for exploring the environmental lifestyle of the organism and transmission characteristics of the pathogen from infected to non-infected host. Further study needs

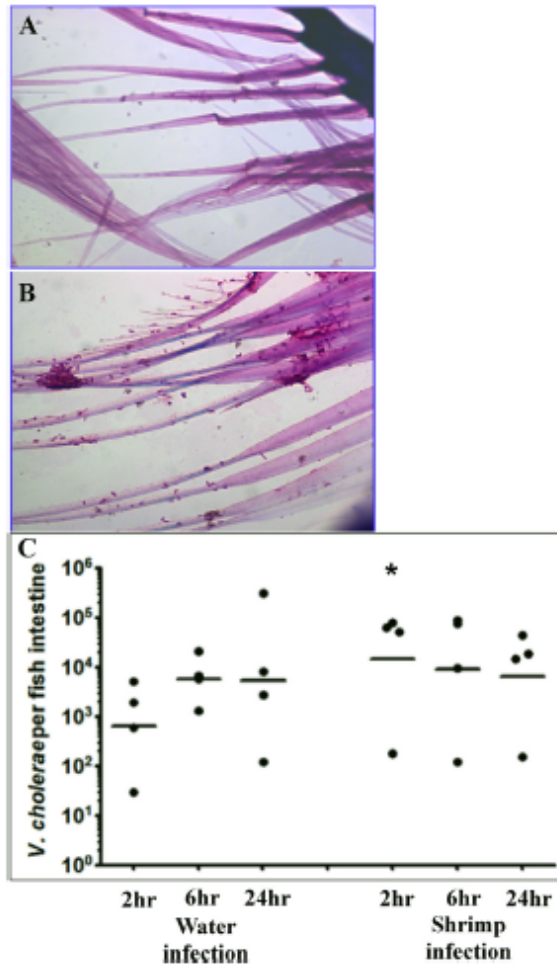


Figure A2: Brine shrimp cultures are produced using brine shrimp eggs (Artemia cysts) and a brine shrimp hatchery (Aquatic Eco-systems). Freshly hatched brine shrimp (< 24 hours post hatch) were removed from the hatchery and washed 3X in tap water to remove excess salt. One hundred microliters of washed brine shrimp were added to 1 ml of 10^8 cfu of *V. cholerae* and mixed gently on a rotator for 15 minutes at room temperature. A) uninfected shrimp B)infected shrimp C) Time course of colonization for fish infected after water exposure or using brine shrimp as a vehicle. Each dot represents the data from one fish and the horizontal bar indicates the mean bacterial load per fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenate 24 hr post infection.

to be done to determine how colonization of the fish gut may prime the organism for infection of humans or allows the organism to maintain physiologically competent for transmission and subsequent infection of humans.

4. Summary

The zebrafish infectious disease model has emerged as a highly useful tool for analyzing pathogenesis caused by a number of organisms. More recently, analyses of aquatic pathogens in the zebrafish model have provided new information on how fish can act as environmental reservoirs for both human and aquatic infections. The zebrafish presents a perfect model in which to study these interactions because of its aquatic lifestyle as well as its ability to respond to infection that accurately mimics disease characteristics observed during human infections. Aquatic pathogens can cause life-threatening soft tissue infections, food borne disease and bacteremia in humans as well as other aquatic species that are consumed or handled by humans. Here we focused on those pathogens that are found in the aquatic environment and serve as environmental reservoirs for infection of fish species and, importantly, as a mode of transmission to humans.

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ABSTRACT**CELL ENVELOPE MODIFICATIONS BY LCP FAMILY PROTEINS OF GROUP B
STREPTOCOCCUS AND VIRULENCE SIGNALING BY PEPTIDES OF THE
SALIVARICIN LOCUS OF *STREPTOCOCCUS PYOGENES***

by

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Streptococci are important human and animal pathogens. Group B *Streptococcus* (GBS) is a zoonotic pathogen that is the leading cause of human neonatal sepsis and meningitis. In order to cause systemic disease GBS must produce a polysaccharide capsule. Capsule expression is controlled in part by the protein CpsA. We demonstrate that deletion of the *cpsA* gene, or expression of proteins with modifications to the extracellular or leucine zipper domains leads to reduced capsule expression and abnormalities in the bacterial cell wall and in cell division. Capsule and cell wall/cell division phenotypes are also seen when modified proteins are expressed in a wild type bacterial strain, indicating the modified protein exerts a dominant negative effect. Additionally, we demonstrate that the dominant negative effect can act through addition of purified modified CpsA protein or synthetic CpsA-derived peptide to wild type cultures. Further investigation of mutation of the *lytR* gene, which encodes for a protein related to CpsA, showed that LytR also has roles in bacterial capsule expression and cell wall/cell division phenotypes. *Streptococcus pyogenes* is a human pathogen that

causes a range of diseases from superficial to life threatening. Peptide signaling serves important roles in many pathogenic bacterial species. We demonstrate that the peptides encoded in the *salivaricin* locus by *S. pyogenes*, SalA and SalZ, have key roles in controlling virulence of *S. pyogenes*. Data suggests that the SalA peptide acts synergistically with the two component signaling system also encoded in the *sal* locus, SalKR, in regulation of transcription and in survival in a model of septic infection. The SalZ peptide was also shown to have roles in transcription of the *sal* locus and virulence of *S. pyogenes*. Taken together, the work described here demonstrates key mechanisms for pathogenesis by *Streptococci*.

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H.M. Rowe, B.R. Hanson, D.L. Runft, Q. Lin, S.M. Firesteine, and M.N. Neely.
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