Exploration of novel factors associated with Group B Streptococcus virulence

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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Abstract:

Group B Streptococci (GBS) is the leading cause of invasive diseases in neonates, pregnant women and non-pregnant adult. In order to cause invasive disease, GBS encodes several virulence factors that are involved in the disease process. One such factor is the moonlighting glycolytic enzyme, phosphoglycerate kinase (PGK), a surface-expressed protein whose mechanism of becoming expressed on the GBS surface has not been known. The surface localization of PGK appears to contribute to bacterial virulence through its ability to bind strongly to host plasminogen. Determining the role of surface-expressed GBS-PGK through deletion of the gene for PGK is hampered by PGK's central role in glycolysis. Consequently, it was necessary to first identify the binding ligand of PGK on the GBS surface, then prevent the expression of PGK on the GBS surface. My results identified two genes in GBS, *eveA* and *eveB,* external virulence effector A and B*,* that are involved in GBS virulence, as well as in GBS-PGK surface expression. Mutagenesis of the *eveA* and *eveB* genes in GBS resulted in a significant decrease in the surface expression of GBS-PGK, as well as the mutants displayed different GBS phenotypes as compared to the wild type strain, such as decrease in ß-hemolysis, CAMP factor and the production of orange pigmentation. The mutants also showed reduced ability to invade epithelial cells and to resist the phagocytic activity of phagocytic cells within fresh human blood. In order to determine whether the EveA and EveB proteins on GBS surface acting as a binding ligand for GBS-PGK on the bacterial surface, a Far Western Blot was conducted on the purified proteins, EveA and EveB, using the GBS-PGK protein as a probe. Since the EveB protein is a large protein (99.4 kDa) with eight transmembrane domains, the protein was designed to be expressed as two fragments,

EveB (up) and EveB (down). The results demonstrate that purified proteins EveA and EveB(up), but not EveB(down) were found to bind the PGK protein on the blot. The immunoreactivity of the purified EveA and EveB proteins with human sera from patients with GBS infection allowed us to determine that EveA and EveB are immunogenic proteins. Using polyclonal rabbit antibodies raised against EveA or EveB proteins in *in vitro* studies of invasion or opsonophagocytic killing assays, it was investigated that EveA and EveB are not protective. Treating epithelial cells with EveA or EveB(down) prior to infection with GBS inhibited GBS invasion, suggesting EveA and EveB are important in the invasion process. Interestingly, the addition of purified EveA and EveB(down) proteins exogenously to GBS cultures inhibited bacterial growth indicates that EveA and EveB proteins have some antimicrobial activities on GBS. However, their activities in bacterial killing are very low and their minimum inhibitory concentrations (MICs) was measured as 32 and 16 μg/ml, respectively. In this thesis, the effect of polyols in altering GBS phenotypes was studied and the results identified that 1% of erythritol, but not sorbitol or mannitol, was able to stimulate the surface expression of GBS-PGK, increase GBS resistance to phagocytic cells and increase bacterial invasion into HeLa cells suggest that erythritol plays a role in altering GBS phenotypes. These findings show that the presence of GBS in environments enriched with erythritol can affect the nature of the bacteria and transform it from a non-harmful commensal bacterium into a pathogenic one. The data presented in this thesis advances our understanding of the factors in GBS, as well as in the host that are involved in GBS pathogenesis.

Acknowledgments:

"If you are not thankful to people, you are not thankful to God"
- Prophet Mohammed

First and foremost, praises and thanks to the God Almighty, for giving me strength and for his showers of blessings throughout my research work to complete the project successfully.

It is a pleasure for me to thank those people who have contributed to and made this work possible. First of all, I would like to express the deepest appreciation to my research supervisor, Dr. Gregory Tyrrell for giving me the opportunity to work on the research project in his laboratory, as well as for his patient guidance, encouragement and advice that he provided throughout my time as his student. Without his guidance, this dissertation would not have been possible.

I would like to take this opportunity to thank my committee members, Dr. Tracy Ravio and Dr. Carmen Charlton, for their encouragement and for providing me with valuable advice.

I would also like to thank members of the Dr. Xiao-Li Pang's laboratory, especially Dr. Ran Zhuo, Dr. Min Cao, Dr. Maria Eloisa Hasing, Dr. Yuanyuan Qiu, Jiaao Yu and Chen Liu for their time and assistance in the laboratory, as well as for their encouragement and moral support, which made my stay and studies in Edmonton more enjoyable. To Dr. Min Cao, thank you for spending countless hours in the laboratory teaching me and answering my questions regarding tissue culture. I would like to express special thanks to Ruthanna Okorosobo for being such a wonderful friend and for taking time to proof-read my writing. To LeeAnn Turnbull, thank you for your technical

expertise and assistance in the laboratory. Thanks to previous members of Dr. Tyrrel's laboratory, Faisal and Areej. Thank you all and I wish you the best of luck in your future endeavors.

To Dr. Monika Keelan, thank you for being caring and providing continual guidance to us graduate students in the Department of Laboratory Medicine and Pathology at the University of Alberta.

I am extremely grateful to my parents for their love, prayers, care and the sacrifices they made in educating and preparing me for my future. I express my thanks to my sisters and brothers for their support and valuable prayers. As my family, your presence beside me always means a lot.

I am deeply thankful to my wonderful husband, Waled, for his love, understanding and for motivating me to complete my studies. Thank you Waled for standing by me, for making me laugh when I needed it most, for listening to my research drama or me just complaining. To my sons, Jameel and Qasim, thank you for allowing me time away from you to work on my research and write my thesis. My heartfelt thanks to you, my family.

Finally, this work would not have been possible without the financial support provided by Taif University, supported by the Kingdom of Saudi Arabia, Ministry of Higher Education, which offered me a scholarship for my graduate studies.

Table of Contents

List of Tables:

List of Figures

List of Abbreviations:

α	Alpha
β	Beta
µg	microgram
µl	microliter
µm	micrometer
°C	degrees Celsius
+ve	Positive control
6XHis	Six histidine residues
A	Absorbance
AMP	Antimicrobial peptide
ATP	Adenosine 5'-triphosphate
BAP	Blood Agar Plate
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BLAST	Basic local alignment search tool
Bp	Base pair
BSA	Bovine serum albumin
BMEC	Brain microvascular epithelial cells
C	Control
C-	Carboxy-
CDC	Center for Disease Control, Atlanta, Georgia
CFU	Colony forming units
cps	Capsule biosynthesis operon
cyl	β-hemolysin/cytolysin
CovSR	Control of virulence Sensor and Regulator
dH_2O	deionized water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ELISA	Enzyme linked immunosorbent assay
EOD	Early onset disease
ECM	Extracellular matrix
EveA	External virulence effector A
EveB(up)	External virulence effector B (up)
EveB(down)	External virulence effector B (down)
erm	Erythromycin resistant gene
FBS	Fetal bovine serum
Fig.	Figure

x g	Acceleration due to gravity (9.8 m/s^2)
g	Gram(s)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Group A streptococcus
GBS	Group B streptococcus
h	Hours
HBMEC	Human bran microvascular endothelial cells
HK	Histidine Kinase
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IAP	Intrapartum antimicrobial prophylaxis
kDa	Kilodalton
kb	Kilobase
KCl	Potassium Chloride
LB	Luria-Bertani
LOD	Late onset disease
LTA	Lipoteichoic acid
M	Molar
mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
ml	Milliliter
mM	Millimolar
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MS	Mass spectroscopy
N	Normal
N-	Amino
NaCl	Sodium chloride
NaH2PO4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NBT	Nitro-blue tetrazolium chloride
Ni-NTA	Nickel-nitrilotriacetic acid
NT	Non-typeable
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PBST	0.1% tween 20 in Phosphate buffered saline
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
rGBS-PGK	Recombinant group B streptococcal phosphoglycerate kinase expressed as an N-terminal histidine tagged protein in *Escherichia coli*
rEveA	Recombinant group B streptococcal external virulence effector A expressed as an C-terminal histidine tagged protein in *Escherichia coli*
rEveB(up)	Recombinant group B streptococcal external virulence effector B (up) expressed as an C-terminal histidine tagged protein in *Escherichia coli*
rEveB(down)	Recombinant group B streptococcal external virulence effector B (down) expressed as an C-terminal histidine tagged protein in *Escherichia coli*
RR	Response regulator
rpm	Rounds per minute
SDS	Sodium dodecyl sulfate
Sag	*Streptococcus agalactiae* gene
ST	Serotype
TBS	Tris buffered saline
TBST	0.1% tween 20 in Tris buffered saline
TH	Todd Hewitt
tPA	Tissue-type plasminogen activator
TCS	Two-component regulatory system
Tn*917*	Transposon 917
uPA	Urokinase plasminogen activator
WT	Wild Type

Chapter 1

Introduction

1.1. Group B streptococcus:

Lancefield Group B Streptococcus (GBS), also known as *Streptococcus agalactiae,* is an encapsulated Gram positive, β-hemolytic, catalase negative, non-motile and facultative anaerobic bacterium that grows in chains. GBS is part of the normal microbiota that colonizes the gastrointestinal and urogenital tract of 20-30% of healthy human adults (1,2). A study by Kwatra *et al.* demonstrated that 50% of pregnant women were colonized with GBS over a longitudinal study (3). The bacteria can cause severe neonatal and adult infections. GBS was mainly recognized as a cause of bovine mastitis in the early 1930s (4). However, in the 1960s, GBS emerged as a leading cause of neonatal infections (5,6). GBS has also been isolated from various mammals and fish as a commensal or pathogen (7,8).

GBS strains share a common antigen on their surface called sialic acid-rich capsular polysaccharide antigen (CPS). There are 10 antigenically and structurally unique GBS-CPS serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX) that have been characterized (9,10). Although it is known that all GBS serotypes are capable of causing invasive infections, five serotypes (Ia, Ib, II, III and V) account for the majority of disease both in neonates and adults (11,12). Each CPS consists of variously arranged monosaccharides and a sialic acid residue on the branching terminus of the repeating unit (13,14). This antigen is one of the most virulent factors in GBS that is involved in GBS survival in the host.

1.1.1. Laboratory identifications:

In most clinical laboratories, GBS identification starts with phenotypic methods. GBS bacteria grow readily on blood agar plates after overnight incubation and which results in a narrow zone of β-hemolysis surrounding the colonies. The zone size of hemolysis maybe different from strain to strain, and in some strains, it is difficult to detect and can only be observed when the colony is removed from the blood agar. The β-hemolytic GBS can be distinguished from other β-hemolytic streptococci by its production of an orange carotenoid pigment called granadaene. The β-hemolysin and granadaene in GBS are encoded by a single genetic locus, *cyl* operon, and their expression is always linked to each other (15–17). As GBS is the only streptococci that produces granadaene (16), the orange pigmentation can be used as a method for detecting GBS. Detection of pigmented β-hemolytic GBS colonies can be carried out Granada agar plates. Granada medium contains molecules such as folic acid pathway inhibitors and starch to trigger the production of pigment in the media (16). Although most GBS strains are hemolytic and produce granadaene, some of the human GBS isolates are non-hemolytic and do not produce pigment (18–21). For such GBS strains, which are non-hemolytic and do not produce pigment, the methods for identification by detection of hemolysis and pigment production is not reliable and their identification has to rely on other assays.

GBS is also known to produce a cytolytic toxin called CAMP (Christie, Atkins, Munch-Petersen) factor. The *cfg* gene that encodes the CAMP factor, a diffusible extracellular protein, is also present in non-hemolytic, non-pigmented GBS strains and

can be used as a method for GBS identification (22). This cytolysin is different from the GBS β-hemolysin and *in vitro*, only lyses red blood cells that are pre-treated with staphylococcal β-lysin (sphingomyelinase). The CAMP factor acts synergistically with the β-lysin produced by *Staphylococcus aureus* to produce a zone of enhanced lysis (arrowhead-shaped) of erythrocytes on blood agar plates at the junction between GBS and *S. aureus* when they are placed perpendicular to each other (23). However, GBS is not the only β-hemolytic streptococci that can produce a positive CAMP test; Group A Streptococci (GAS) (24) and *Streptococcus Porcinus* (25) strains can also produce false positive CAMP test reactions. Therefore, this test is not 100% specific. Molecular identification of GBS using the *cfb* gene can be more specific for GBS identification than the phenotypical CAMP test method (22).

GBS possesses a group B cell wall-associated carbohydrate antigen, as was classified by Rebecca Lancefield (26). This antigen has been a useful tool in identifying GBS in the clinical laboratories. Streptococci grouping test based on testing latex agglutination can provide rapid identification of important streptococcal pathogens, including GBS. Commercial kits are available for this testing. Latex particles are coated with specific antibodies directed towards Lancefield antigens A, B, C, D, F, and G. The kits contain substrates that allow for rapid extraction of the antigens mixed with the coated latex particles. Positive reactions are visualized by agglutination in the presence of homologous antigen. This serological grouping test is widely used for routine diagnostic purposes due to its simplicity, rapidity and specificity.

1.1.2. Group B streptococcus diseases:

The gastrointestinal tract is the main reservoir for GBS and represents the source of vaginal colonization (27). GBS must overcome several levels of vaginal immunity before vaginal colonization, including the epithelial layers, mucus, acidic pH, antimicrobial peptides, antibodies, microbicidal immune cells, and a vaginal microbiome dominated by lactobacilli (28). GBS colonizes the urogenital tract of more than 30% of healthy women asymptomatically. It was estimated that 50% of infants born from colonized pregnant women become colonized, and approximately 1% of these infants develop disease (29–31). During pregnancy, GBS present in the vagina may access the neonate *in utero* through an ascending infection becoming an invasive pathogen resulting in severe neonatal diseases.

1.1.2.1. Neonates:

GBS diseases in neonates are usually described as being early- or late-onset, depending on the age of the infant at the time of disease manifestation. Early-onset GBS disease (EOD) results from ascending infection from colonized mothers to their infants *in utero* or during the birthing process when the neonate aspirates contaminated vaginal fluids (18). EOD develops within the first week of life and manifests as respiratory distress and pneumonia that rapidly progresses into sepsis (18). Late-onset disease (LOD) develops in infants up to several months of age (7 - 90 days) and is characterized by bacteremia with a high risk of progression to meningitis (32). While maternal colonization is a prerequisite for EOD, the acquisition of GBS in LOD is less clear. It may be acquired from maternal transmission, nosocomial transmission, prematurity,

contaminated breast milk or community sources (33–39). Although the mortality rate of LOD is lower than EOD, the morbidity of LOD is higher. If infants survived the meningitis caused by LOD, almost 50% will develop chronic neurological disability, including seizures, mental retardation, hearing loss and blindness (31,40).

1.1.2.2. Adults:

Although GBS infection is serious in neonates, GBS has also emerged as an important cause of invasive infection in adults. GBS infection in adults usually becomes serious in individuals with low immunity, such as the elderly and pregnant women. In the United States, the incidence of invasive GBS disease in adults doubled from 3.6 cases/100,000 persons in 1990 to 7.3 cases/100,000 persons in 2007, with a significantly higher case fatality rate at 15% (41).

1.1.2.2.1. Pregnant women:

In general, asymptomatic GBS is usually harmless in healthy women. However, it can cause serious infection in pregnant women. During pregnancy, the immune system is slightly decreased, rendering pregnant women more susceptible to infection (42). In some cases, the asymptomatic GBS carrier can be switched to invasive GBS, thereby causing urinary tract infection, bacteremia, endometritis, chorioamnionitis, preterm births and stillbirth (43–47). Approximately 10% of preterm births are due to a GBS infection (48,49). GBS infection in women of childbearing age are rarely fatal.

1.1.2.2.2. Non-pregnant individuals:

Invasive GBS diseases have been increasingly described in the elderly and immunocompromised patients. Diabetes, obesity, cancer, and HIV increase the risk for invasive GBS disease (50–54). In non-pregnant adults, the common clinical syndromes include bacteremia, skin and soft tissue infection and pneumonia (41). Severe clinical syndromes, such as meningitis, streptococcal shock syndrome and endocarditis are rare and often associated with severe morbidity and mortality (55). In patients older than 65 years of age, the incidence of adult GBS disease is much higher, at approximately 30 cases/100,000 persons (12,41). The mortality rate for invasive GBS disease is higher in the elderly than in neonates and is approximately 10% (11). The incidence of invasive GBS disease is 3 times greater in older adults living in nursing homes than in older adults in community settings (56).

1.2. Virulence factors contribute to GBS disease pathogenesis:

GBS encodes several virulence factors that enable it to cause disease (Fig.1.1). It is unclear how this pathogen regulates the expression of these virulence factors during infection. Although GBS is a commensal organism that resides in the lower gastrointestinal and maternal urogenital tracts, it can become an invasive pathogen that disseminates into a number of host niches, including the intrauterine compartment, neonatal lung and neonatal brain (Fig.1.1). GBS is able to overcome the changes in the host's environment. In response to the host's environment during infection, GBS expresses different gene products for successful infection, and the expression is accomplished via signal transduction systems (STS). In response to external signals,

these systems regulate the function of DNA-binding transcription factors. The two-component regulatory system (TCS) is the most common STS in bacteria (57). TCS consists of membrane-bound histidine protein kinase (HK) and cytosolic response regulator (RR). The HK is the sensor of an environmental signal that induces auto-phosphorylation, and subsequently, it phosphorylates its cognate RR. Phosphorylation of the RR induces a conformational change that alters its function, thereby modifying its DNA binding affinity and changing the expression of various genes which allows GBS to then adapt to the environmental signal (57,58). In GBS, the most well characterized TCS is CovSR (Control of virulence Sensor and Regulator), which regulates coordinately up to 27 % of the entire genome (59–61). The CovSR tightly controls GBS gene/virulence factor expression during infection.

1.2.1. Biofilm formation:

The first step in the bacteria's pathogenesis, GBS colonization and persistence, are critical for disease development in humans. The adherence capacity of GBS to host niches facilitates bacterial cell aggregation and biofilm formation. A biofilm is an aggregation of cells in a sessile community surrounded by a self-produced matrix, referred to as extracellular polymeric substances, which is composed of polysaccharide, protein and DNA (62). Biofilm matrix composition of some pathogens such as uropathogenic *E. coli* may not include all the three polymeric substances and only composed of carbohydrates and proteins with very less extracellular DNA (63). Biofilm formation is an important virulence factor in bacterial persistence and development of

chronic infections. Bacteria tend to build biofilms in the host environment as protection from nutrient deprivation and clearance by the host's immune system.

The GBS bacterial capsule and pili have been investigated as being involved in biofilm formation (64,65). GBS are classified into ten serotypes, Ia, Ib, and II to IX, based on unique GBS capsular polysaccharides (9,10). Although there are differences in oligosaccharide composition and linkage pattern of GBS capsular polysaccharides, GBS capsules share common structural features in that they all display a terminal sialic acid residue linked to *O*-3 of D-galactopyranosyl (9). Since sialic acid is a major component of host glycoproteins, the host fails to recognize GBS as non-self. Therefore, capsular sialic acid permits GBS to evade host immune responses through molecular mimicry (66). Studies have investigated the involvement of GBS capsular polysaccharides in biofilm formation (64). Isogenic mutation in the GBS capsular polysaccharide impaired the ability of the bacteria to form biofilms (64). In addition to the capsule, GBS encodes small cell surface appendages known as pili (67–69). The genes encoding the GBS pilus machinery are clustered in two genomic pilus islands and are known as pilus islands 1 and 2 (PI-1 and PI-2, respectively). The PI-2 locus exists in two variant forms: PI-2a and PI-2b. Each GBS strain carries one or two islands (70,71). Dramsi *et al*. investigated that PI-2a mediates adherence of GBS to the pulmonary epithelial cell line (68). Other studies indicated that most of the GBS strains that form biofilms carry PI-2a (72). In addition, studies using GBS isogenic mutants lacking PI-2a, but not PI-1 and PI-2b, showed that PI-2a plays a role in pili facilitating host cell contact and forming biofilms (65,72). Remarkably, antibodies raised against pilus island 2a components inhibited the formation of biofilm in a dose-dependent manner (72).

The host environmental conditions have been demonstrated to play a crucial role in GBS biofilm formation (73,74). Within the host, exposure of GBS to the acidic environment of the vagina seems to be the signal sensed by the bacteria to develop a biofilm. Several studies demonstrated enhanced GBS adherence to vaginal epithelial cells and significantly higher biofilm formation under low pH in comparison to neutral pH (75–78). Additionally, Xia *et al.* investigated that GBS biofilm formation is enhanced by the presence of human plasma (64).

In GBS, acidic pH and the presence of plasma act as important environmental factors that regulate the expression of bacterial surface-associated structures, such as pili and the bacterial capsule, which are both involved in promoting bacterial biofilm formation (64,65,72).

1.2.2. Adherence and invasion of host cells:

GBS encodes several virulence factors that allow its persistence in the acidic vaginal environment and avoidance of clearance by host immune defenses. Some of these factors are involved in adherence and invasion into host epithelial cells, thereby enabling persistent colonization and eventual infection. Adherence and invasion into host cells are mediated by GBS interacting with the host extracellular matrix (ECM) components, such as fibrinogen, fibronectin and laminin, on the host cell surface and promotes entry into the host (79,80). Examples of functionally characterized adhesins that mediate GBS interaction with host ECMs and adherence within the host are the fibrinogen-binding proteins (Fbs), the streptococcal fibronectin-binding protein A (SfbA), the laminin-

binding protein (Lmb), the GBS immunogenic bacterial adhesin (BibA), and the group B streptococcal C5a peptidase (ScpB).

1.2.2.1 Fibrinogen-binding proteins:

In GBS, five fibrinogen-binding proteins have been characterized to date. FbsA, FbsB, the serine-rich repeat glycoproteins, Srr1 and Srr2, and FbsC or BsaB (81–86). Studies have shown that invasive GBS isolates demonstrate greater fibrinogen-binding capabilities in contrast to colonizing ones (87). FbsA has been shown to mediate GBS adherence, while FbsB was shown to promote entry of GBS into host cells (81,82). Srr proteins of GBS are comprised of highly glycosylated, surface-localized (contain a signal peptide and LPXTG cell-wall anchoring motif), and serine-rich proteins that have adhesive functions (88,89). The differences between Srr1 and Srr2 in GBS is the alternation in the amino acid sequences with serine in the repeat region (89).
It has been reported that Srr1 and Srr2 interacts with host fibrinogen and binds to epithelial cells (84,90). Deletion of Srr1 in GBS resulted in decreased adherence *in vitro* and decreased vaginal colonization in mouse models, suggesting that Srr1 mediates vaginal colonization and persistence (91,92). Srr1 and Srr2 were also reported to mediate invasion of microvascular endothelial cells (83,84). FbsC or BsaB (bacterial surface adhesin of GBS, also known as FbsC) were recently characterized as interacting with host fibrinogen leading to increased adherence to cervicovaginal epithelial cells and biofilm formation (85,86). Furthermore, the virulence abilities of GBS mutants containing the FbsC deletion were reduced in mouse infection models (85).

1.2.2.2 Fibronectin binding proteins:

In addition to the Fbs family, the fibronectin-binding ability presented by the group B ScpB (Streptococcal C5a peptidase) protein seems to be involved in GBS binding to human epithelial cells (93–95). ScpB is a surface-associated serine protease that can both cleave and inactivate the human complement component C5a and mediates bacterial binding to fibronectin (93–95). In addition, it has been reported that ScpB is also involved in cellular adhesion, since isogenic mutation of the *scpB* gene significantly reduced the invasion into human epithelial cells *in vitro* (93). Recently, a novel GBS fibronectin-binding protein, SfbA (Streptococcal fibronectin-binding protein A) has been identified and appears to be involved in binding to the ECM fibronectin and invading human brain microvascular endothelial cells (96). Deletion mutation of the *sfbA* gene in a mouse model reduced the ability of the mutant to invade the blood brain barrier to cause meningitis (96). *In vitro* evidence by Stoner *et al*. supported the contribution of SfbA in the GBS invasion into human fetal astrocytes, which are physically associated with brain endothelial cells (97). SfbA has also been shown to contribute to GBS invasion of vaginal and cervical epithelial cells, which may be involved in GBS colonization of the vagina (96). Besides the involvement of BsaB in binding to the host ECM fibrinogen, BsaB has also been investigated as binding to the host fibronectin and laminin, as well as participating in GBS binding to epithelial cells and in biofilm formation (86). BsaB and FbsC revealed to be identical proteins encoded by the same gene (85,86).

1.2.2.3 Laminin-binding proteins:

GBS adhesion to host ECM laminin seems to have a distinct function in bacterial colonization of the central nervous system. Laminin-binding protein (Lmb) has been found to play an essential role in GBS colonization of damaged epithelial cells and subsequent translocation into the blood stream (98). Mutation of the *lmb* gene results in significantly reduced GBS adherence and invasion into human brain microvascular endothelial cells (98,99). Studies have shown that GBS strains associated with meningitis display higher levels of expression of Lmb (100).

1.2.2.4 GBS immunogenic bacterial adhesion:

GBS adherence to host cells plays an important role in its pathogenesis. GBS immunogenic bacterial adhesion (BibA) has been investigated to be involved in GBS adherence to human epithelial cells (101,102). Isogenic mutation of the *bibA* gene showed significant decrease in GBS adherence capacity to lung and cervical epithelial cells (101). Additionally, the BibA protein was reported to bind to a regulator of the classic complement pathway known as C4-binding protein and enhance bacterial survival in the human blood by resisting opsonophagocytic killing by human neutrophils (101,102). Therefore, BibA seems to be a multifactorial virulence factor involved in both adhesion to host cells and resistance to phagocytic killing.

1.2.2.5 Pili:

GBS was recently revealed to express pili, which facilitate attachment, colonization, biofilm formation, translocation and invasion into host cells. The GBS cell-

wall anchored pili contain motifs that are composed of three pilin proteins: the backbone protein (PilB subunit), the two ancillary proteins (PilA subunit), and the pilus base (PilC subunit) (68,69,103,104). Studies have shown that the PilB subunit is involved in bacterial invasion and paracellular translocation, whereas the PilA subunit, the pilus-associated adhesion, was shown to be involved in cellular adherence and colonization (68,91,103,105). Dramsi *et al*. showed that GBS mutants lacking PilA and PilC, but not PilB, had a significant reduction in epithelial cell adherence (68). Among the various sequenced GBS genomes, two genetic loci encoding pili (PI-1 and PI-2) were identified. PI-2a and PI-2b are two variants of the pilus island 2 (PI-2). Jiang *et al*. reported that PI-1 is not involved in bacterial adhesion to host cells; however, it is involved in GBS evasion of innate immunity mechanisms (106). While PI-2a has been reported as being involved in GBS adherence and biofilm formation, PI-2b was recently investigated as having an important role in bacterial adherence and invasion into epithelial and endothelial cells (65,72,107). The PI-2b pilus was found to have a crucial role in GBS infection and penetration of the blood brain barrier (107).

1.2.2.6 Alpha C Protein:

Another surface-anchored protein known as alpha C protein (αCP) has been shown to mediate GBS invasion of human cervical epithelial cells (108,109). In a neonatal mouse model of infection, inactivation of the αCP gene renders the mutant less virulent as compared to wild type (109). The αCP protein contains a glycosaminoglycan-binding domain that interacts with the host cell glycosaminoglycan and promotes bacterial internalization into the host cell (110). GBS invasion into host cells can also be

promoted by binding the αCP protein to $\alpha_1\beta_1$-integrins on the epithelial cell surface (111).

1.2.2.7 Invasion-associated gene:

Using a GBS transposon mutant library to screen for GBS mutants, the invasion-associated gene, *iagA*, was identified as an important virulence factor for GBS meningitis (112). The *iagA* gene encodes a membrane glycolipid known as diglucosyldiacylglycerol, which functions as a membrane anchor for lipoteichoic acid (LTA) (112,113). In an *in vitro* study of an *iagA* mutant, GBS shed LTA into the medium and inhibited bacterial invasion into brain microvascular epithelial cells (BMEC) (112). Additionally, in a murine model of meningitis, the *iagA* mutant was attenuated, which demonstrated decreased BMEC penetration and meningitis infection as compared to the wild type (112). However, for the GBS challenge in a mouse model, the *iagA* mutant strain could survive in the bloodstream and develop bacteremia at similar efficiencies as the wild-type GBS strain (112). Thereby suggesting that the *iagA* gene product is essential for epithelial cells and BMEC invasion and is not a factor for meningitis.

1.2.2.8 Pore-forming toxins:

GBS produces β-hemolysin/cytolysin which is encoded by the genes of the *cyl* operon (114). The *cylE* gene in the *cyl* operon encodes an N-acyltransferase necessary for β-hemolysin/cytolysin production (114). The other genes in the *cyl* operon, *cylA, cylB, cylJ* and *cylK*, were shown to be involved in the post-translational modification and secretion of the β-hemolysin/cytolysin (115,116). The GBS β-hemolysin/cytolysin

protein is a pore-forming toxin that results in the cellular damage of epithelial and endothelial cells in the lung and blood brain barrier (BBB), thereby promoting GBS invasion (117–121). In a rabbit model of GBS-induced pneumonia, the efficiency of a *cylE* mutant to invade pulmonary barriers and produce systemic infection was less than in the wild type strain (122). In addition, the role of β-hemolysin/cytolysin was recently confirmed by Randis *et al*. in a mouse model of GBS infection; they demonstrated that mutation of the *cylE* gene resulted in a significant reduction in GBS dissemination, fetal injury, and preterm birth (123). Similarly, in a non-human primate model, Boldenow *et al*. showed that, compared to non-hemolytic GBS, the hemolytic GBS induces GBS invasion of the amniotic cavity, fetal injury and preterm labor (124). Moreover, β-hemolysin/cytolysin has been shown to impair cardiac function, promote liver failure and induce host inflammatory responses that contribute to neurological sequelae (125–127). β-hemolysin was shown to have antioxidant activity, since hyper-hemolytic GBS strains are resistant to the antimicrobial activity of neutrophil extracellular traps (128). It was reported that β-hemolysin also plays a role in GBS vaginal colonization due to the fact that in non-hemolytic GBS, GBS vaginal colonization was reduced (123,129). These findings suggest that β-hemolysin/cytolysin is important in promoting GBS dissemination in uterine, placental, and fetal tissues during pregnancy. However, recent study by Gendrin *et al*. showed that a nonhemolytic GBS strain (GB37) isolated from a septic neonate exhibits hypervirulence (130). This study demonstrated that the nonhemolytic GBS strain can exhibit hypervirulence due to a substitution of tryptophan to leucine (W297L) in the CovS results in constitutive kinase signaling, leading to decreased hemolysis and increased activity of the GBS hyaluronidase, HylB (130).

CAMP factor is another pore-forming toxin, secreted by GBS, which has been suggested as important for GBS pathogenesis. In 1944, Christie, Atkins and Munch-Peterson first described the CAMP factor as a co-hemolytic protein involved in the lysis of sheep red blood cells via the combined action of sphingomyelinase from the *Staphylococcus aureus* β-toxin (131). CAMP factor was shown to bind to glycosylphosphotidylinositol-anchored proteins on host cell membranes and induce cell lysis by oligomerization and pore-forming action (132,133). The role of CAMP factor was studied in GBS animal models, and it was demonstrated that purified CAMP factor is lethal to rabbit and induces septicemia and death in mice (134,135). However, in a series of *in vitro* and *in vivo* assays, Hensler *et al*. evaluated the indirect evidence implicating CAMP factor in GBS pathogenesis by studying the virulence properties of the CAMP factor mutant strain (136). In a mouse model of infection, Hensler *et al*. demonstrated that the CAMP factor-deficient GBS strain retained full virulence and has equivalent phagocytic resistance and endothelial cell invasiveness as the wildtype (136). These studies indicate that CAMP factor in its nature may not be essential for GBS pathogenesis. It was hypothesized by Rajagopal *et al*. that β-hemolysin/cytolysin and CAMP factor may be compensate each other during systemic infection and CAMP factor may only be essential for GBS pathogenesis in host niches when β-hemolysin/cytolysin activity is reduced (33). Consistent with this hypothesis, GBS oppositely regulates β-hemolysin/cytolysin and CAMP factor using CovR/S system (60,61,137). Thus, the GBS pore-forming toxins β-hemolysin/cytolysin and CAMP factor are essential component of GBS pathogenesis as they promote bacterial entry into host cells and facilitate their intracellular survival and systemic dissemination.

1.2.2.9 Hyaluronidase:

GBS secretes hyaluronidase or hyaluronate lyase (HlyB) encoded by the *hlyB* gene that is important for vaginal colonization (138). The HlyB protein degrades hyaluronic acid, the major component of human tissue and promotes bacterial dissemination during the GBS infection process (139,140). Hyaluronic acid has been found to be present in higher concentrations in the placenta, amniotic fluids and the lung; these may represent preferential targets during the early stages in the pathogenesis of vertical transmission and early-onset infection (140–142). In a GBS mice model of infection, the isogenic GBS hyaluronidase mutant led to increased clearance of GBS from the mouse vagina compared to the wild type (138). In another study by Vornhagen *et al*., a GBS *hlyB* mutant showed reduced ability to ascend from the vagina to the uterus and reduced ability to invade fetal tissues and cause preterm birth and fetal demise (143). Similarly, compared with neonates that were asymptomatically colonized with GBS, a high concentration of hyaluronidase was found in neonates with bloodstream infections (144). Vornhagen *et al*. also showed that clinical GBS strains associated with neonatal infections have increased hyaluronidase activity compared to commensal strains obtained from rectovaginal swabs of healthy women (143). Thus, HlyB plays an important role in GBS infection associated fetal injury.

1.2.2.10 Surface expressed glycolytic enzymes:

A number of bacterial glycolytic enzymes have been reported as being associated with the outer aspect of the bacterial cell wall. Gram positive organisms such as Streptococci seem to have on their cell surface most of the enzymes contained within the

glycolytic pathway (145–148). Typically, these glycolytic enzymes are cytoplasmic proteins lacking signal sequences or hydrophobic anchors and have unknown mechanisms for being exported onto the bacterial surface. These surface-expressed glycolytic enzymes have been found to act as moonlighting proteins with glycolytic functions in the cytoplasm and as virulence factors when expressed on the cell surface. For example, GBS expresses the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the cell surface that binds and converts host plasminogen to plasmin, which then promotes host invasion and systemic spread of GBS through degradation of the host extracellular matrix fibronectin (149,150). It has also been reported that surface localization of GAPDH on GBS facilitates host colonization (151). Phosphoglycerate kinase (PGK) is another glycolytic enzyme that has been reported as being expressed on the GBS surface (152,153). The surface-expressed PGK binds the host actin and plasminogen and may be involved in bacterial adhesion, internalization and dissemination within the host (152,153). Furthermore, during the *in vitro* study, in addition to being anchored to the bacterial surface, PGK was also found to be secreted in the culture media, indicating that PGK may have the opportunity to interact with the host cell actin cytoskeleton through pore-forming hemolysins resulting in disruption of the host actin cytoskeleton (153). Thus, these studies suggest that surface localization of glycolytic cytoplasmic proteins may play a role in bacterial virulence. Not only with the metabolic enzymes of the glycolytic pathway, there are now a significant number of bacterial moonlighting proteins that appear to play a role in the virulence properties of bacteria such as enzymes of other metabolic pathways (154,155) and molecular chaperones and protein folding catalysts (156,157).

1.2.3. Resistance to host immune responses:

Once GBS binds and invades the host cellular barriers to reach the deep tissues, it is essential for GBS to disrupt the host's innate immune defenses in order to facilitate GBS survival in the host. Host phagocytic cells, including neutrophils and macrophages, play a critical role in innate immune responses to clear the infection; however, these cells are often deficient in newborns (158–160). To this end, GBS encodes a number of virulence factors that prevent its recognition by the host, provide resistance to opsonophagocytosis or neutralize the bactericidal activities of neutrophils and macrophages (128,161–165). The following are some of the factors that facilitate GBS immune evasion were studied in GBS.

1.2.3.1. Capsular polysaccharide:

The GBS capsular polysaccharide (CPS) represents one of the defense factors in the bacteria. GBS bacteria are classified into 10 specific serotypes known as CPSs (Ia, Ib, II-IX) that are created by different arrangements of four monosaccharides (glucose, galactose, N-acetylglucosamine and sialic acid) into unique repeating units with a terminal sialic acid molecule attached to galactose (166). Since sialic acids are typically abundant on vertebrate glycoproteins, the presence of sialic acid on the GBS CPS provides bacterial evasion from the host immune responses via molecular mimicry (161). The sialic acid-rich CPS also protects GBS from host phagocytosis by preventing the deposition of complement factor, C3 onto the bacterial surface (167,168). In animal models of infection, the isogenic GBS mutants lacking CPS or sialic acid are more

susceptible to neutrophilic killing and are less virulent than the wild type GBS (167,168). Thus, the sialic acid-rich capsular polysaccharide inhibits the alternative complement pathway by preventing C3 deposition onto GBS and protects the organisms from phagocytic killing (167). In addition, Takahashi *et al*. investigated the role of sialic acid-rich CPS in the inactivation of the complement-derived chemoattractant, C5a (169). The study reported that in highly sialylated GBS strains, the production of C5a is significantly reduced compared to less sialylated or less virulent GBS strains (169). The reduction of C5a production resulted from the rapid inactivation of C5a that occurred due to the sialic acid-rich CPS working synergistically with the ScpB protein, which acts as a C5a peptidase (169). The higher sialic acid content of GBS strains inhibits both opsonophagocytic killing and C5a production in the absence of type-specific antibody (169).

1.2.3.2. Superoxide dismutase:

During infection, the bacteria will try to resist the host immunity by releasing factors that facilitate bacterial survival. In GBS, once engulfed and contained in the phagosome of phagocytic cells, the bacteria encounter the rapid release of toxic reactive oxygen species (ROS) produced during the oxidative burst. Unlike *S. aureus*, GBS is unable to produce catalase; however GBS is able to resist killing by ROS and survive inside phagolysosomes (170–173). GBS encodes the enzyme superoxide dismutase (SodA) that converts the superoxide anions (O^{-2}) into oxygen (O_2) and hydrogen peroxidase (H_2O_2), which are subsequently metabolized by peroxidases (162). The SodA enzyme in GBS enables the bacteria to resist oxidative stress during infection. Mutation of the *sodA* gene in GBS significantly impaired the bacterial survival in the bloodstream

and brain of mice due to the resulting high bacterial sensitivity to macrophages (162). However, the survival of the *sodA* mutant in the mouse liver or spleen was not significantly impaired (162). These results indicate that SodA provides (i) GBS resistance to host ROS and (ii) GBS survival in specific host niches.

1.2.3.3. NADH Peroxidase:

Recently, a study by Korir *et al*. investigated that NADH peroxidase, encoded by *npx*, is important for GBS survival within the macrophages (174). The study demonstrated that expression of *npx* is highly upregulated during survival inside macrophages and that Npx plays a role in defending against oxidative stress and is important for reducing ROS production inside macrophages.

1.2.3.4. Hemolytic pigment:

The biosynthesis of GBS β-hemolysin/cytolysin has been associated with the production of an orange, carotenoid pigment that is genetically linked to the *cyl* operon (15,114,175). It was reported that deletion of the *cylE* gene renders GBS non-hemolytic and non-pigmented, whereas in hyper-hemolytic strains, where the *cylE* is over expressed, GBS demonstrated increased pigmentation (17,114). The orange, carotenoid pigment, also known as granadaene, has been shown to promote pathogen resistance to ROS (128). It was reported that *cylE* deficient GBS was more readily cleared from a mouse's bloodstream, human whole blood, and isolated macrophage and neutrophil cultures (128). The granadaene neutralises hydrogen peroxide, superoxide, hypochlorite and singlet oxygen, thereby shielding the bacteria from several elements of phagocytic

ROS killing (128). Liu *et al*. concluded that these observations suggest a mechanism by which the linked *cylE* gene-encoded phenotypes, β-hemolysin/cytolysin (sword) and carotenoid (shield), act in partnership to impede the immune phagocytic defenses (128).

1.2.3.5. Bacterial mechanisms to resist antimicrobial peptides:

During infection, the first defense mechanism by the innate immune system is the production of cationic antimicrobial peptides (AMPs) that are critical in combating bacterial infections (176). The AMPs, such as cathelicidins and defensins, are expressed by many host immune cells and epithelial cells (177). These AMPs are released into phagolysosomes, wherein they contribute to the killing of engulfed microorganisms. The initial interaction between AMPs and the bacterial cell surface is electrostatic. The positively charged AMPs are attracted to the negatively charged microbial cell surface, and the AMPs then assemble to create membrane pores or disrupt the membrane integrity (178). GBS has several mechanisms for resisting the activity of AMPs. One of these mechanisms is modifying the surface charge of the bacteria. To resist the effect of AMPs, GBS incorporates positively charged D-alanine residues into the cell-wall lipoteichoic acids (LTA), thereby reducing the surface electronegativity and enabling GBS to repel the positive charge on AMPs (163). Poyart *et al*. investigated the role of D-alanyl LTA in the virulence of GBS (179). In a mouse and neonatal rat model, a knockout mutant of the *dltA* gene that encodes the D-alanine-D-alanyl carrier ligase was severely impaired and resulted in an increased susceptibility to host defensins and macrophages, in comparison to the wild type strain (179).

Penicillin binding proteins (PBPs) on the bacterial surface have also been reported to assist GBS in resisting host AMPs. Typically, PBPs are involved in cell wall peptidoglycan biosynthesis, which are targets for the β-lactam family of antibiotics, such as penicillin (180). In a neonatal sepsis model of infection, compared to the wild type strain, a GBS mutant of the *ponA* gene, which encodes the PBP1a protein, had attenuated virulence, was susceptible to cathelicidins and defensins, and more susceptible to killing by alveolar macrophages and neutrophils (164,181,182). Surprisingly, the *ponA* mutant did not demonstrate a significant sensitivity to penicillin, change in its surface charge or change in its cell wall peptidoglycan, as compared to wild type strain (164,182,183). Despite the action of PBPs in bacterial virulence, their mechanism(s) related to the virulence of GBS has not been determined.

In addition to the contribution of GBS pili in biofilm formation, adherence and invasion of the pathogen into host cells, pili have been found to mediate GBS resistance to AMPs (68,91,103,105,184). The PilB protein, the major GBS pilus backbone subunit, has been shown to play an integral role in protecting GBS from the host phagocytic killing (184). In a mouse model of infection, an isogenic GBS pilB knockout strain was susceptible to killing by isolated macrophages and neutrophils, as well as by cathelicidin AMPs. The mutant was more readily cleared from the mouse bloodstream and was less virulent compared to the wild type strain (184). Interestingly, in a mouse challenge model, overexpression of the *pilB* gene in a non-pathogenic strain of bacteria known as *Lactococcus lactis* enhanced bacterial resistance to phagocytic killing, increased bloodstream survival, and conferred virulence (184). However, as with the PBPs, the mechanism by which pili enable GBS to resist AMPs is not clear.

1.2.3.6. Virulence factors with multiple functions:

GBS possesses a number of virulence factors that prevent the key components of effective host opsonophagocytic killing. Some of these virulence factors have been found to have multiple functions. For example, the fibronectin-binding protein, ScpB has been found to mediate GBS binding to host fibronectin, to assist in cellular adherence and invasion, and to interrupt the host complement activation by splitting the neutrophil chemoattractant, C5a (93,185–187). Similarly, the GBS immunogenic bacterial adhesin, BibA, is involved in the bacterial binding to human epithelial cells; however, it was also found to aid GBS survival in human blood through binding the C4 binding protein and preventing opsonophagocytic killing by human neutrophils, thereby conferring anti-phagocytic activity (101,102). In addition, the cell-surface associated protease, CspA cleaves human fibrinogen and coats the bacteria with fibrin, which interferes with complement deposition and provides protection for the bacteria from opsonophagocytic killing (170). The CspA protein has also been demonstrated to cleave a variety of CXC chemokines such as GRO-alpha, GRO-beta, GRO-gamma, neutrophil-activating peptide 2 (NAP-2), and granulocyte chemotactic protein 2 (GCP-2) in order to prevent the attraction to and activation of neutrophils at the site of infection (188). Moreover, the surface beta protein was reported to protect GBS from opsonophagocytosis by binding to the middle region of complement inhibitor factor H so as to inhibit C3b deposition onto the bacterial surface (189). The β-hemolysin/cytolysin protein has also been reported to provide GBS the ability to survive in human blood through induction of cytolysis and apoptosis of the phagocytes (128).

1.3. GBS adaptation to environmental changes:

1.3.1 GBS adaptation to high pH

Although GBS is a commensal bacterium residing in the vagina and the gastrointestinal tract of healthy adults, GBS can convert from commensal to pathogenic and cause invasive infections. In the transition from commensal organisms into pathogens, GBS will face various host environments and require coordinated control of the transcriptional responses to these changes. For neonatal infections, GBS moves from the acidic pH environment of the vagina to the neutral pH environment of human blood, wherein resistance from the host immune system can occur. By using a comparative global gene expression analysis, Santi *et al*. examined GBS responses to acid stress in comparison to neutral pH conditions (190). Santi and colleagues demonstrated that the GBS global response to acid stress included the differential expression of genes involved in the transport, metabolism, stress response, and virulence of the bacteria, and that many of the genes involved in the GBS response to pH are known to be controlled by the CovRS two-component regulatory system (190). Some of the virulence factors that were previously determined to be under the control of CovRS are β-hemolysin/cytolysin, ScpB, BibA, Pili, Fbs proteins and Lmb (60,61,106,190,191). Santi *et al*. determined that, of the genes regulated by the CovRS system, the genes encoding the BibA and pili proteins were overexpressed at high pH levels (190). These results suggested that the translocation of GBS from the acidic condition of the vagina to the neutral pH of the neonatal lung switches on the GBS virulence genes and facilitates GBS conversion from a colonizing to an invasive phenotype. Since the CovRS system in GBS is a major

virulence regulator, the contribution of this system in coordinating GBS gene expression has been extensively investigated (59,137,192). Patras *et al*. reported that maintaining GBS vaginal colonization and avoiding host immune responses is achieved by the CovRS system that controls GBS virulence gene expression (193). The GBS genomic adaptation was also reported to take place during GBS progression to disease. Almeida *et al*. compared the genomic profile of GBS samples causing infections in newborns with that of the GBS colonizing their mothers; multiple mutations were detected in the samples from the newborn, including a CovRS mutation that affected the pathogenesis of GBS (194).

1.3.2 GBS adaptation to human blood:

GBS adaptation to human blood was also investigated using a whole genome transcriptome analysis after incubation with whole human blood (195). During GBS growth in whole blood, Mereghetti *et al*. found a significant increase in the expression of genes encoding proteins related to oxidative stress, such as SodA and proteases, which facilitate bacterial resistance to phagocytic cells (195). In addition, the transcription of GBS virulence factors involved in cellular adherence and evasion of host immune responses, such as FbsA and BibA, were significantly upregulated in human blood (195). Interestingly, the transcription of genes encoding the glycolytic enzymes aldolase, enolase and GAPDH, which have been determined to have role in the binding or activation of host plasminogen, were also upregulated in human blood than in the laboratory growth media (195). In addition, increased expression of transcriptional

regulators, including CovS was also observed in GBS during growth in human blood. All illustrate the complex responses used by GBS to adapt to human blood (195).

1.3.3 GBS adaptation to human amniotic fluid:

Sitkiewicz *et al*. studied the GBS adaptations for growth in the human amniotic fluid (AF) environment and how GBS causes severe intrauterine infections (196). Global gene expression analysis of GBS grown in human AF illustrated a number of mechanisms that GBS uses to adapt to the host (196). In this study, GBS grew rapidly to a high cell density within the human AF. In addition, the expression of some virulence genes were up-regulated during GBS growth in human AF; this included the genes encoding the oligopeptide-binding protein, *oppA*, which encodes a protein involved in cellular adhesion (197), and the genes encoding the capsule, β-hemolysin/cytolysin, CAMP factor and CspA. Furthermore, growing GBS in AF affected the transcription of GBS glycolytic enzymes (195), in particular, there was increased transcription of GAPDH (196).

1.3.4 GBS adaptation to glucose:

GBS infections have also been reported to occur in adults with serious underlying conditions, including diabetes (50,198,199). In diabetic patients, GBS has been reported to take advantage of this condition by crossing the endothelial barrier and promoting invasion (52). Di Palo *et al*. examined the GBS adaptation to glucose, which mimics hyperglycemic conditions in the host (59). At prolonged incubation times (more than 30 mins.), the GBS growth rate was higher at 55mM glucose (59). Comparative global gene

expression analysis of GBS grown in the presence of 55mM glucose versus in a glucose-free environment demonstrated that, there was increased expression of the genes involved in bacterial colonization, such as *sag2021*, which encodes a GP-340 binding protein (200), and *cydC*, which was previously reported to play a role in virulence and GBS growth *in vivo* (201); these genes were found to be up-regulated in the presence of glucose (59). The up-regulation of transcriptional regulators was observed in response to the availability of glucose sources (59); these regulators include the putative Mer family of regulators, which have been identified in *Streptococcus pneumoniae* as required for defense against nitric oxide stress and required for survival in blood (202,203). A wide range of glucose-dependent genes have been found to be under the CovRS regulation, which indicates that effectors of this system are involved in the GBS response and adaptation to high glucose conditions (59). Similar to the investigation reported by Santi and colleagues (190), pH-dependent genes were also found to be regulated by glucose stress conditions, suggesting that GBS may activate common pathways when exposed to stressful conditions and may have similar adaptations to stressful external stimuli, such as low pH and high glucose conditions (59).

Therefore, the data from several independent studies reinforce the role of CovRS as a central GBS regulator controlling the switch from mucosal colonization to invasive infection. Furthermore, the increased expression of several virulence genes induced in the presence of neutral pH, human blood, human AF and glucose, suggest that GBS rapidly adjusts to the various conditions present in the host environment.

1.4. Preventative strategies for GBS infection:

In the 1970s, GBS was reported as the predominant pathogen causing invasive neonatal disease. By the 1980s, GBS-causing neonatal infections had an estimated incidence of 0.5-2 per 1000 live births, with a mortality rate of 20-25% (204). Since then, numerous efforts have been made to prevent and treat GBS disease in infants and pregnant women. In 1996, the national consensus guidelines for GBS disease prevention that was issued by the Centers for Disease Control and Prevention (CDC) recommended the use of intrapartum antimicrobial prophylaxis (IAP) for GBS-positive pregnant women (205). The guidelines recommended the universal screening of pregnant women for rectovaginal GBS colonization at 35–37 weeks gestation and administering IAP to GBS carriers during labor and delivery (205). IAP is recommended to pregnant women who were GBS positive at the time of screening time or who present with at least one of the risk factors, including prolonged rupture of membranes, intra-amniotic infection, maternal fever, preterm delivery, women with GBS bacteriuria, previous infants with invasive GBS disease or GBS isolation at any time during the pregnancy. For women with no risk factors or with negative GBS results, the IAP is not recommended. Currently, the IAP is the most applied and effective strategy used to prevent early-onset GBS disease (EOD), which is caused by vertical transmission of GBS from mother to the infant, and the IAP reduces the risk of perinatal sepsis. After the introduction of IAP, the incidence rate of infants with EOD declined up to 0.21 per 1000 live births in the USA in 2015 (206). Despite the implementation of prophylactic measures, the incidence of GBS late-onset disease (LOD) has remained stable, with an average rate of 0.3-0.4 per 1000 live births (2,11,207). In addition, the identification of pregnant women at risk for GBS

perinatal infection and the administration of IAP is very difficult in many low- and middle-income countries; IAP administration is also not possible for women who have home deliveries. Moreover, the widespread use of IAP raises concerns about the emergence of antibiotic resistance in GBS isolates or in other microorganisms affecting pregnant women and newborns. Likewise, the use of antibiotics during pregnancy has been shown to have significant effects on neonatal health (208,209). However, the prevention or treatment of GBS infection in human adults was not addressed by these CDC guidelines.

The probiotic approach has become the current focus in medical research due to the effect of probiotics in reducing vaginal GBS colonization. Recent studies reported that pre-treatment of vaginal epithelial cells with probiotic *Lactobacillus species* significantly reduced GBS adherence to and colonization of human epithelial cells (210–212). It was also reported that repeated doses of probiotics, three to seven doses, were needed to attain a protective effect *in vivo* (211,212). In addition, Patras *et al*. studied the ability of *Streptococcus salivarius*, a predominant member of the native human oral microbiota, to control GBS colonization (213). The study showed that administration of *Streptococcus salivarius* to pre-colonized GBS-infected mice significantly reduced GBS vaginal colonization through a yet unidentified antimicrobial mechanism (213). In another study, Hanson *et al*. determined that yogurt ingestion was inversely related to GBS colonization (214). In this study, the probiotic group of pregnant women who received oral probiotic once daily had lower quantitative GBS colony counts compared to the control group (214). All these results highlight the demand to continue to exploring this field in order to identify an efficient and feasible probiotic therapy to prevent GBS colonization.

Additional GBS prevention and therapeutic measures are needed, especially since GBS infections in human neonates and in high risk adult populations are still persistent. Vaccination seems to be the most effective intervention compared to perinatal antibiotic prophylaxis, and the vaccines could prevent neonatal GBS infections through transplacental antibody transfer and could potentially reduce new vaginal colonization. However, no licensed GBS vaccines are currently available (215). Maternal antibodies are effective in reducing the incidence of neonatal invasive infections caused by GBS. In the 1930s, Rebecca Lancefield demonstrated that using CPS-specific polyclonal rabbit antibodies can provide protection for mice against GBS infections (216). This was later supported by findings from various researchers who showed that increased levels of maternal serotype specific capsular antibodies decrease the susceptibility to invasive GBS disease in newborns (217–220). In most GBS vaccine clinical trials, the CPS has been the main target for the vaccines; however, vaccines targeting the GBS capsule alone are not effective due to their poor immunogenicity. The immunogenicity of GBS type-specific CPS antigens was successfully increased through conjugation. Thus, conjugate capsule vaccines are now being tested in clinical trials (14,215). Although there are 10 known GBS capsular polysaccharide serotypes (Ia, Ib, II-IX), most invasive GBS disease is caused by five serotypes: Ia, Ib, II, III and V (221). Despite the safety and immunogenicity of monovalent CPS conjugate vaccines that have been evaluated in healthy women, monovalent vaccines with serotype-specific immune responses are not adequate to provide protection against the dissimilar GBS serotypes found in invasive infections (222). Therefore, multivalent CPS conjugate vaccines would provide a wider range of vaccine coverage. Phase I and II clinical trials of trivalent GBS conjugate

vaccines based on serotypes Ia, Ib and III were conducted, and the safety and immunogenicity among healthy non-pregnant and pregnant women has been confirmed, with a consideration for a phase III trial (223–225). A pentavalent vaccine based on serotypes Ia, Ib, II, III, V is also now being investigated for preclinical development (226).

Due to geographic diversity in GBS serotypes, lack of coverage against non-typeable GBS strains, and potential problems of serotype switching, efforts are now being directed towards the development of broad coverage protein-based GBS vaccines that target various highly protective antigens (227,228). For a vaccine to be considered promising, the vaccine proteins should be surface-exposed, conserved and expressed in a wide range of GBS strains, strongly immunogenic, and able to confer protection from GBS disease (55). However, phase variation considers as a major obstacle for generating effective vaccines against targets. Phase or antigenic variation refers to the mechanism by which a pathogen, such as a bacterium, alters the proteins on its surface to avoid a host immune response. It also refers to a reversible switch between an on/off expressing phase, resulting in variation in the level of expression of one or more proteins between individual cells of a clonal population. This mechanism results in the expression of the heterogenic phenotype of a clonal bacterial population. Thus, for protein-based vaccines design, proteins should be preferentially targeted that are expressed in all isolates and in each individual cell and that show no antigenic variation. Thus, vaccine candidates based on GBS surface proteins have been investigated; these proteins include ScpB, Lmb, surface immunogenic protein (Sip), leucine rich repeat protein (LrrG), Rib and the tandem repeat containing the alpha and beta components of the C protein complex

(98,229–236). The immunogenicity of the Rib and alpha C proteins against GBS disease has been determined by phase I clinical trials, and these proteins have been shown to confer protection against invasive GBS disease in murine models and human studies (215,233,237,238).

Reverse vaccinology based on identifying a conserved sequence encoding components of GBS proteins could assist in the discovery of potential vaccine targets (239). Investigators used reverse vaccinology to identify conserved GBS sequences belonging to the pili proteins expressed on the bacterial surface (67). Margarit *et al*. previously showed that all GBS isolates carried at least one or a combination of two pilus islands, PI-I and/or PI-II, and PI-II has 2 variants, PI-IIa and PI-IIb. Subsequently, Margarit and colleagues suggested that pilus-based vaccines composed of the three pilus components, one from each pilus type (PI-I, PI-IIa, and PI-IIb), could be effective in preventing GBS infections and providing a broad range of protection (70). The backbone subunit of the PI-IIa pilus subunit (BP-2a), which is present in six immunogenically different but structurally similar variants, showed the ability to elicit high opsonophagocytic titer (70). However, due to the antigenic variation of the BP-2a pili subunits in GBS, coverage against all GBS strains would have been impossible; however, structural vaccinology was successfully applied to overcome this problem (55,70,240). The crystallographic structure of the BP-2a pili revealed four IgG-like domains: D1, D2, D3, and D4 (240). The individual domains were tested as subunit vaccines, but only the D3 domain elicited a similar protection as the whole BP-2a component (240). Since the other BP-2a variants showed a similar structural organization, the protective D3 domains from six variant BP-2a pilus subunits were combined together to construct a 6xD3

vaccine candidate (55,240). In challenged mice, this pili vaccine construct conferred strong protection against all six strains expressing a BP-2a variant (240).

Other promising vaccine candidates that have been identified by reverse vaccinology are the BibA and Sip proteins (101,102,241). It was recently reported that IgG titres specific to BibA and Sip were higher in pregnant women colonized with GBS compared to women with negative GBS cultures at ≥37 weeks of gestation (242). Thus, there is an association between BibA and Sip induced-antibodies and the reduced probability of maternal recto-vaginal GBS acquisition during pregnancy (242).

Although several vaccine candidates are under clinical development, phase variation confounds several of attempts to make effective GBS vaccines. A key issue of phase III clinical trial that is required for vaccine licensure is the low baseline incidence of the primary clinical endpoints of GBS infections in both neonates and elderly.

In order to protect mothers and their infants from GBS infections, the World Health Organization (WHO) Initiative for Vaccine Research has prioritized the development of Group B streptococcus (GBS) vaccines for maternal immunization (243). GBS vaccines would be a promising strategy for the prevention of GBS infections in newborns, as well as in adults with underlying diseases. Thus, the successful development and introduction of a GBS vaccine may result in converting GBS into predominantly harmless commensal bacteria of the mucosa.

1.5 Thesis overview:

Human fatal puerperal sepsis was the first case of GBS disease identified in 1938 (244). By the 1970s, GBS had emerged as the predominant pathogen causing invasive

neonatal disease in many regions (245–247). Since then, many efforts had been made towards preventing and treating GBS disease in infants and pregnant women. One of these efforts was the administration of intrapartum antimicrobial prophylaxis (IAP) to GBS-colonized women during labor and delivery. The IAP was effective in reducing early-onset GBS disease (EOD) by 90% in the United States (248). However, it was estimated that the cases of GBS late-onset disease (LOD) remained the same, even after the implementation of IAP (207,249). Likewise, GBS has emerged as an important cause of invasive infections in non-pregnant adults, particularly among the elderly and adults with underlying medical conditions, such as diabetes. The incidence rates of invasive GBS disease in non-pregnant adults is on the rise (41).

Understanding the mechanisms of GBS infections, by which they interact with host cells, would facilitate the development of novel therapies and vaccines. In the last two decades, efforts have shifted towards the investigation of GBS virulence factors that contribute to its ability to cause infection. Many virulence factors have been identified as being involved in epithelial cell colonization, biofilm formation, translocation and invasion in the host. Interestingly, one group of bacterial virulence factors that have been identified as having a role in GBS virulence is the glycolytic enzymes. These enzymes were thought to only have one important function, which is bacterial glycolysis; however, the enzymes were found to occupy a different cellular compartment than they normally occupy. The term that was introduced to describe this ability of a protein to have more than one biological action was moonlighting. A number of examples of bacterial moonlighting proteins have been identified, and many of them appear to play a role in bacterial virulence. An example of this is the GBS phosphoglycerate kinase (PGK)

protein. This enzyme was previously identified in our laboratory as being expressed on the GBS surface (152). In addition to the PGK role in glycolysis, this enzyme was also believed to contribute to bacterial virulence through its ability to bind to host plasminogen (153). The fact that PGK binds to plasminogen suggests a potential role for PGK in aiding dissemination of the bacteria within the host through the breakdown of host extracellular matrix proteins and endothelium. Modulation of the host plasminogen system by GBS could play a role in the penetration of the blood-brain barrier and the subsequent development of meningitis.

Due to the essential role of PGK in glycolysis, determining the function of surface-expressed GBS-PGK by traditional knockout mutagenesis seems impossible. It is unknown how the glycolytic enzymes are transported to the bacterial surface, since these enzymes are anchorless and have no signal peptides within their sequences that could explain their extracellular location on the bacterial surface. The GBS gene involved in surface expression of PGK was identified in our laboratory using Tn*917* transposon mutagenesis (250). Transposon mutation in the GBS gene, *sag1003*, resulted in a significant decrease in GBS-PGK expression on the bacterial surface, with increased levels of PGK in the culture supernatant as compared to the parent strain. This result suggested that the decreased expression of PGK on the surface of the mutant was a result of decreased attachment to the bacterial surface. Since the *sag1003* gene codes for an ATP permease that has 8 transmembrane regions, we hypothesized that a portion of this protein is likely exposed to the outer surface of the bacteria and could act as a binding ligand for GBS-PGK. It was important to determine whether other proteins besides Sag1003 play a role in binding to the surface GBS-PGK. Thus, one goal of this work

(discussed in chapter 2) was to determine the binding ligands for the surface-expressed GBS-PGK, in which by inactivation of the binding ligand genes, we would be able to understand the potential virulence functions of surface-expressed GBS-PGK.

Interestingly, two proteins, Sag1003 and Sag0912, revealed to be located on the GBS cell surface have been identified as being involved in the surface expression of GBS-PGK. Another goal of this study was to assess the virulence of the constructed mutant of Sag1003 and Sag0912 and compare the result to the parent strain of GBS. Mutation of *sag0912* and *sag1003* genes in GBS significantly reduced (i) the expression of PGK on the bacterial surface, (ii) the expression of β-hemolysin and CAMP-factor, (iii) the antiphagocytic activities and (iv) the invasion into human epithelial cells compared to the wild type strain. Based on these results we have designated the proteins Sag0912 and Sag1003 as EveA and EveB (External Virulence Effector A and B), respectively. Based on these exciting results, we have shifted our focus to studying the function of these two protein in GBS virulence (discussed in chapter 2, 3 and 4).

The signals dictating the switch of GBS from commensalism to virulence are mainly unknown. One factor that could be associated with GBS virulence during pregnancy is the presence of polyols. Polyols (of which erythritol, sorbitol and mannitol are a member) are sugar alcohols derived naturally from some fruits and have been found in the placentas of ruminants and in the human coelomic and amniotic fluids (251–254). The presence of erythritol in the placentas of ruminants has been proposed as an explanation for the accumulation of bacteria found at this site, eventually leading to abortion (255,256). Given the wide range of host niches encountered by GBS during its disease cycle, we have attempted to address changes in GBS virulence in response to the

presence of polyols (discussed in chapter 5), which could thereby affect the fetus by penetrating the placentas and the amniotic membranes, causing serious neonatal infections.

Determining the role of surface-expressed GBS-PGK through deletion of the gene for PGK is hampered by PGK's central role in glycolysis. Creation of such a GBS-PGK mutant strain would provide a valuable tool with which to explore the biological significance of PGK expressed on the surface of GBS, particularly, as it relates to plasminogen binding. Mutation of the *pgk* gene was attempted using an alternative approach involving auxotrophic complementation (AC), a system developed to facilitate the traditional knock-out system (257,258). The AC system is based on a strain auxotrophic for an essential metabolite, obtained by mutating the corresponding chromosomal gene. However, this system requires extra nutrients in the *in vitro* minimal media. Using this approach, a *pgk* knockout mutant of GBS was constructed and the mutant required the use of media supplemented with 10% fetal bovine serum in order to maintain bacterial growth. A preliminary study was first conducted (discussed in chapter 6) in order to facilitate identifying the function of PGK in GBS virulence.

Thus, the broad objective of my thesis was to determine the factors involved in GBS pathogenesis, including the surface expression of GBS-PGK. The identification process was achieved either at the molecular level, such as identifying the genes involved in GBS-PGK binding to the bacterial surface, or by determining the effect of external environments, such as the polyols, on GBS growth. Characterizing the proteins, EveA and EveB, as well as studying the effect of polyols on GBS virulence will contribute to the understanding of how GBS causes invasive disease. In addition, the GBS *pgk* mutant

strain that was constructed in this study will further elucidate the biological significance of PGK in GBS virulence. Overall, the work presented here has the potential to lead to the development of potential therapeutic or vaccine components for preventing invasive GBS disease.

1.6 Hypothesis and objectives:

General hypothesis of the thesis: Group B streptococcus bacteria expresses on the surface the glycolytic enzyme phosphoglycerate kinase (PGK) and two transmembrane proteins, Sag912 and Sag1003, that play important roles in pathogenesis of these bacteria.

Hypothesis chapter 2: GBS-PGK binds to GBS surface through interaction with the transmembrane proteins, Sag1003 and Sag0912 on the GBS surface. Thus, GBS mutant strains of Sag1003 and Sag0912 will have reduced amounts of GBS-PGK on the mutants' surfaces.

Objectives chapter 2:

1. To determine if GBS-PGK binds to the GBS surface via Sag0912 and/or Sag1003.
2. To identify the role of GBS surface proteins, Sag0912 and Sag1003 in GBS virulence by constructing GBS mutant strains of *sag0912* and *sag1003*.

Hypothesis chapter 3: Sag0912 and Sag1003 (EveA and EveB, respectively) are immunogenic in humans.

Objective chapter 3:

1. To identify the immunoreactivity of recombinant EveA and EveB proteins using sera from GBS-infected patients.

Hypothesis chapter 4: The EveA and EveB proteins play roles in GBS invasion into human epithelial cells and the antibodies produced against EveA and EveB are protective.

Objectives chapter 4:

1. To demonstrate the neutralization effect of polyclonal rabbit antibodies produced against EveA and EveB.
2. To investigate the role of purified EveA and EveB in GBS invasion into human epithelial cells.

Hypothesis chapter 5: The presence of polyols (erythritol, sorbitol and mannitol) enhances expression of GBS virulence factors, including the surface expression of GBS-PGK.

Objective chapter 5:

1. To determine if different concentrations of polyols alters the GBS phenotypes in various assays.

Hypothesis chapter 6: The presence of PGK on the surface of GBS binds to plasminogen. Due to the essential role of PGK in glycolysis, determining the function of surface expressed GBS-PGK by traditional knockout mutagenesis is not possible. Thus, auxotrophic complementation of *pgk* GBS mutant with 10% fetal bovine serum will maintain the growth of mutant.

Objective chapter 6:

1. To determine if GBS-PGK that is surface expressed binds to plasminogen.

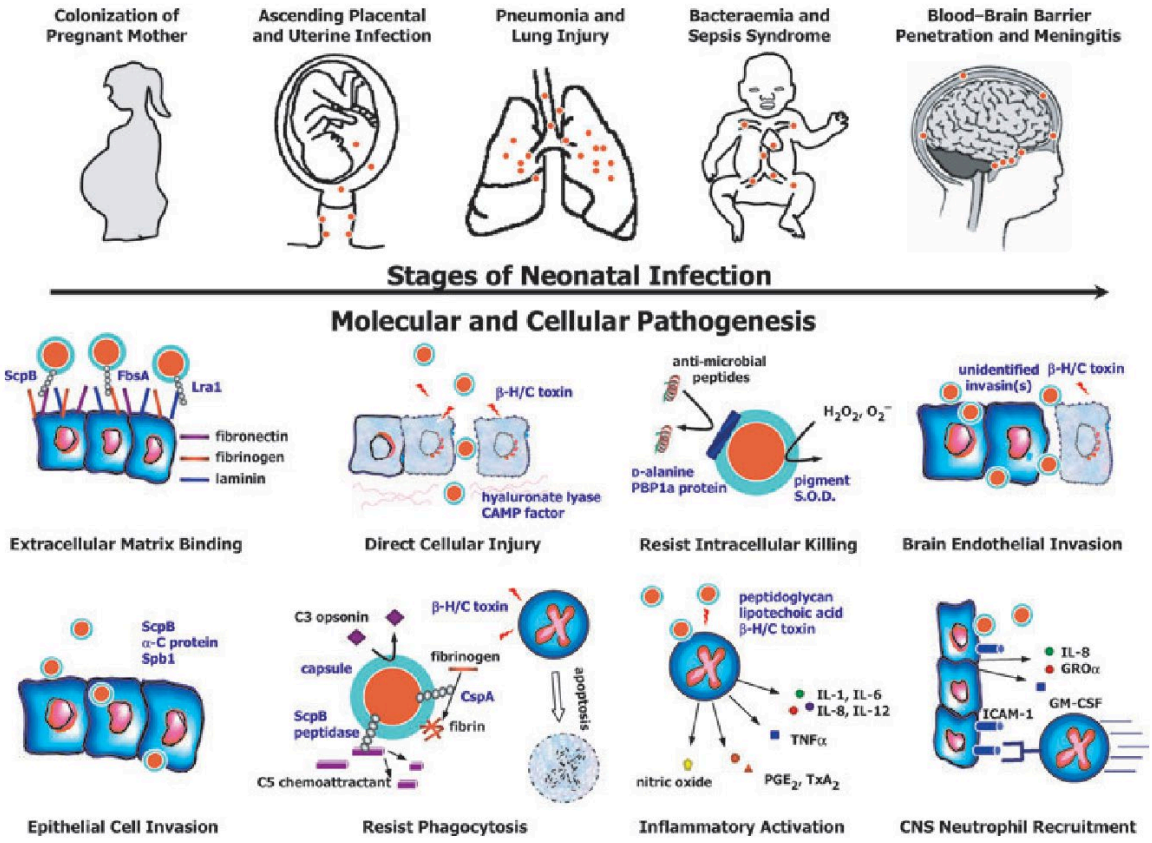

Figure 1.1. Stages in the molecular and cellular pathogenesis of neonatal Group B Streptococcal (GBS) infection. (Adapted from Doran *et al.* 2004;(1)).

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Chapter 2:

Identification of two Group B Streptococcal genes which bind GBS-PGK and are important for GBS virulence

2.1. Introduction:

Lancefield group B streptococci (GBS), also known as *S. agalactiae*, is the leading cause of neonatal sepsis, meningitis and in some cases, death (1–3). GBS has also been associated with invasive diseases in the elderly and immune-compromised patients (4–6). GBS displays an array of multifunctional proteins on its surface to mediate GBS adherence to and invasion into the host. These proteins include fibrinogen-binding proteins (Fbs), the laminin-binding protein (Lmb), the group B streptococcal C5a peptidase (ScpB), the streptococcal fibronectin-binding protein A (SfbA), the GBS immunogenic bacterial adhesin (BibA), and the pili (7–14).

In addition, glycolytic enzymes that are generally located in the cytoplasm, such as glyceraldehyde 3 phosphate dehydrogenase (GAPDH), α-enolase and phosphoglycerate kinase (PGK) have been identified on the surface of GBS (15–18) and other streptococcal species (19–22). Surface-expressed glycolytic enzymes have been shown to bind to a number of host proteins and act as potential virulence proteins (19,23–25). In GBS, surface-expressed PGK has been shown to bind to actin and plasminogen (16), thereby suggesting that GBS-PGK contributes to GBS virulence.

Surface-expressed GBS-PGK and other glycolytic enzymes are anchorless surface proteins (26). It is unknown how these proteins are expressed on the bacterial surface as the C-terminal hydrophobic tails and N-terminal signal sequences which are required for protein export to the cell surface are absent from these glycolytic enzymes. It is not possible to study the function of surface-expressed GBS-PGK through the traditional knockout mutagenesis of GBS-PGK as the gene coding for PGK is essential for microbial survival. However, by identifying GBS gene(s) involved in the surface expression of

GBS-PGK, then one can be able to prevent the surface expression of GBS-PGK and evaluate the role of GBS-PGK.

Previous work in our lab using Tn*917* transposon mutagenesis identified a gene in GBS, *sag1003*, that is involved in GBS-PGK surface expression (27). Surface-expressed GBS-PGK was significantly reduced in the mutant NCS13 *sag1003*::Tn*917* compared to the parent NCS13 strain. It was confirmed that a reduction in GBS-PGK surface expression was due to decreased attachment to the bacterial surface, and not in secretion. Homology analysis predicts the *sag1003* gene to encoded a permease protein with 8 transmembrane domains (Fig. 2.1) involved in ATP-dependent efflux of antimicrobial peptides.

Initially, the objective of this study was to use Far Western Blot with GBS cell fractions to determine if GBS-PGK binds to a portion of the transmembrane domains within the Sag1003 protein that is exposed on the outer surface of the bacteria. These outer proteins could act as a binding ligand for the surface-expressed GBS-PGK. Interestingly, I found that recombinant GBS-PGK also bonds to another protein within the cell wall fraction. The protein was identified by mass spectroscopy as Sag0912, a hypothetical GBS protein with unknown function. As both GBS proteins Sag 912 and Sag1003 bound GBS-PGK, these two genes, *sag0912* and *sag1003* were identified as potentially being involved in in GBS-PGK surface expression and in changing the expression of some virulence factors in GBS (to be presented in this chapter). Therefore, *sag0912* and *sag1003* have been designated as external virulence effector A (*eveA*) and external virulence effector B (*eveB*), respectively. Further experiments were performed to explore the roles of EveA and EveB proteins in GBS virulence.

2.2. Material and Methods:

2.2.1. Bacterial strains, cell lines and growth conditions:

All GBS strains, *Streptococcus pyogenes* and *Streptococcus pneumoniae* were grown in Todd- Hewitt broth (TH) (Difco Laboratories, Detroit, MI) and the *E. coli* strain was grown in Luria-Bertani broth (LB) (Gibco-BRL, Gaithersburg, MD). The bacteria were grown at 35°C without antibiotics, except for *NCS13 eveA::erm*, *NCS13 eveB::erm* and *NCS13 eveB::Tn917* mutants, which were grown in the presence of 10 μg/ml of erythromycin. A collection of 14 strains of GBS representing the ten capsular serotypes (ST), ST-Ia (97SR400), ST-Ib (97SR570), ST-II (97SR138), ST-III (COH-1), ST-IV (97SR331), 2 strains of ST-V (97SR384) and (NCS13), ST-VI (9842), ST-VII (7271), ST-VIII (JM9), ST-IX (ATCC 27412), non-capsulated serotype (COH-13) and 2 of non-typable strains (18SR431) and (17SR409) were used in this study. *S. pyogenes* (ATCC 19615), *S. pneumoniae* (ATCC 49619) and *E. coli* DH5α were used as a control. Stock cultures, kept at -80°C, were rapidly thawed and grown for 24 h on blood agar plates (BAP) (Dalyn Biologicals, Calgary, Canada) at 37°C in a humid incubator under microaerobic conditions. The *eveA* and *eveB* genes were initially identified from GBS serotype V strain NCS13. NCS13 is an invasive isolate derived from a soft tissue wound of an 82-year-old male; this isolate was obtained from the National Centre for Streptococcus, Provincial Laboratory of Public Health for Northern Alberta (Edmonton, Canada) and has been used by the laboratory as a highly invasive GBS strain (28).

The human epithelial cell line, HeLa 229 (ATCC CCL-2.1), obtained from the American Type Culture Collection (ATCC; Manassas, VA), was grown in OPTI-MEM I reduced serum medium supplemented with 4% fetal bovine serum (FBS; Gibco BRL,

Burlington, ON).

2.2.2. Subcellular fractionation of GBS:

A method for subcellular fractionation of GBS was used as previously described (16). A 100 ml culture of GBS NCS13 was grown overnight on TH broth at 37ºC. A one ml aliquot of the overnight culture was transferred to a micro-centrifuge tube and centrifuged at 14000 x g for 5 minutes at 4ºC. The supernatant was removed and the cell pellet was resuspended in 100 µl of lysozyme (50 mg/ml in distilled water) and incubated for 30 minutes at 37ºC to generate the whole cell lysate. The remaining culture was centrifuged at 5000 x g for 20 minutes at 4ºC and the supernatant was filtered through a 0.22 µm filter and collected as the extracellular fraction. The supernatant fraction and sterile TH broth (negative control) were precipitated using 70% ammonium sulfate and resuspended in 1/10 volume TBS. The pellet was resuspended in 10 ml of ice-cold spheroplast-forming buffer (20% sucrose, 1 mM $Mgcl_2$, 33 mM Tris (pH 8.0), treated with 1 mg/ml lysozyme at 37ºC for 2 h and centrifuged at 14000 x g for 15 minutes. The supernatant was collected as the cell wall fraction. The cell pellet was resuspended in distilled water, sonicated in a water bath sonicator for 10 minutes and collected as the cytoplasmic fraction.

2.2.3. Production and purification of GBS-PGK:

Recombinant GBS-PGK (r-GBS-PGK) was expressed and purified from previously constructed *E. coli* M15 pQE30/*pgk* as an N-terminal hexahestidyl tagged

protein under native conditions using the QIA expressionist kit (Qiagen; Mississauga, ON, CA) as described previously (16). The *E. coli* M15 pQE30/*pgk* strain was constructed by cloning the PCR amplified *pgk* gene from the genomic DNA of the characterized GBS strain NCS13 (16,17,28) using primers PGK-pstF and PGK-hinR (Table 2.1) into the expression plasmid pQE30 and transformed into chemically competent *E. coli* M15 (16). To express GBS-PGK, 10 ml of the overnight culture was used to seed 100 ml of LB broth supplemented with 60 µg/ml carbenicillin and 25 µg/ml kanamycin. Following a 1 h incubation at 37ºC, expression of the N-terminal histidine tagged GBS-PGK (r-GBS-PGK) was induced by the addition of 2 mM of isopropyl ß-D-1-thiogalactopyranoside (IPTG). Following further incubation for 4 h at 37ºC, the bacterial culture was harvested by centrifugation at 5000 x g and the bacterial pellet was resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and treated with lysozyme (1 mg/ml) for 30 minutes on ice. The bacterial cells were then sonicated in an ice water bath for 15 minutes and centrifuged 10000 x g for 20 minutes. The supernatant was then collected and incubated with 1 ml of Ni-NTA resin (Qiagen) for 1 h at 4ºC. The resin was loaded into a 1 ml polypropylene column (Qiagen) and washed 8x with 1 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The purified protein was eluted 6x with 500 µl of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0).

2.2.4. Far Western Blot to visualize the interaction between rGBS-PGK and GBS proteins:

Subcellular localization of proteins that bind to rGBS-PGK was performed as previously described (16). Briefly, GBS cell fractions (cell wall, cytoplasm, and extracellular) and whole cell lysate were subjected to 10% SDS-PAGE and transferred onto a 0.45mM nitrocellulose membrane (Bio-Rad). TH broth and human plasminogen (0.5 µg) (Sigma–Aldrich, St. Louis, MO, USA) were also separated and transferred to be used as negative and positive controls, respectively. The membrane was blocked with 5% skim milk diluted in Tris buffered saline with 0.1% tween 20 (TBST) for 1 h at room temperature, washed 1X with TBST and incubated with rGBS-PGK (25 µg/mL) overnight at 4°C in TBST. The membrane was washed 1X with TBST and probed with anti-GBS-PGK antibodies (1:1500 in TBST), generated previously (16), for 2 h at 4 °C. The blot was washed 3X with TBST and then incubated in 1:10,000 goat anti-rabbit IgG Alkaline Phosphatase conjugate (Promega) for 1 h at room temperature. The blot was again washed 3X with TBST and developed using 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP/NBT) commercial liquid substrate system (Promega).

2.2.5. Peptide sequencing:

For peptide sequencing, GBS whole cell lysate and cell wall fractions were separated on 10% SDS-PAGE and stained with Coomassie Blue. The 15 kDa and 42 kDa bands that corresponded to the bands on the immunoblot that bound rGBS-PGK were excised from the gel, kept in dH_2O and sent to Alberta Proteomic and Mass Spectrometry

Facility, Department of Biochemistry, University of Alberta, Edmonton, Alberta. Peptide sequencing was analyzed using tandem mass spectroscopy.

2.2.6. Construction of *eveA* and *eveB* mutants:

Construction of a single deletion mutation of *eveA* or *eveB,* NCS13 *eveA::erm* or NCS13 *eveB::erm,* was accomplished via insertional inactivation with an erythromycin resistance gene using an allelic exchange strategy. To achieve this, the upstream (5') and downstream (3') DNA fragments (500 bp each) of either *eveA* or *eveB* genes were amplified by PCR from the GBS NCS13 genomic DNA, along with an erythromycin resistance cassette (*ermB*) (800 bp) from the pTV1-OK plasmid. To construct the *eveA* mutant, the forward and reverse primers PF25/PR26 (upstream fragment), PF27/PR28 (downstream fragment) and PF22/PR24 (*ermB* gene) were used in this reaction (all primer sequences used are shown in Table 2.1). The upstream and downstream fragments of the *eveA* gene were digested with restriction enzymes KpnI and BamHI for 1 h at 37˚ C, respectively. The *ermB* gene was double digested with KpnI and BamHI. The DNA fragments were then purified using a commercial DNA extraction and purification kit (Qiagen, Mississauga, ON, Canada). The purified DNAs were ligated together at room temperature for 18 h with T4 DNA ligase (New England Biolabs) and amplified by PCR with the primers PF25/PR28 to generate a 1.8 kb construct, *5'eveA-ermB-5'eveA*. As well, NCS13 *eveB::erm* was constructed in order to confirm that the loss of surface GBS-PGK expression in the NCS13 *eveB*::Tn*917* mutant was due to the loss of EveB expression and not due to an additional spontaneous mutation elsewhere in the genome. To construct the *eveB* mutant, the forward and reverse primers PF9/PR10 (upstream fragment),

PF11/PR12 (downstream fragment) and PF1/PR2 (*ermB* gene) were used in this reaction (Table 2.1). The upstream and downstream fragments of the *eveB* gene were digested with restriction enzymes PstI and KpnI for 1 h at 37˚C, respectively. The *ermB* gene was double digested with PstI and KpnI. Following the DNA purification, the purified DNAs were ligated and amplified by PCR with the primers PF9/PR12 to generate a 1.8 kb construct, 5′*eveB-ermB-5′eveB*. The constructs were then transformed into GBS electrocompetent cells by electroporation and plated onto THY agar containing 10 μg/mL of erythromycin. PCR amplification was performed to confirm the presence of the desired mutations.

2.2.7. Phenotypic assays:

The zone of β-hemolysis and CAMP expression of each of the GBS strains were detected on BAP (Oxoid, Nepean, ON, Canada). Pigment production of the GBS strains was assayed on Granada agar plates (Hardy diagnostics, CA, Santamaria, USA). BAP or Granada agar plates were incubated at 37°C for 18-24 h. One colony of each overnight GBS culture grown on BAP was streaked on BAP or Granada plates. Photographic acquisitions of the BAPs were taken with light from below. Photographic acquisitions of the Granada plates were obtained with side lighting from above.

2.2.8. Measuring GBS-PGK surface expression:

Expression of GBS-PGK on the surface of GBS mutant strains was assayed using an ELISA, as previously described with some modifications as follows (27). Overnight growth cultures of GBS strains grown in TH media were washed once with 1 mL of TBS

and re-suspended in 1 mL of TBS. One hundred microliters of washed bacterial cultures were fixed to the wells of a 96-well polystyrene plate (Maxi-sorp; NUNC, Thermo Fischer Scientific, Nepean, ON, Canada) by 2 h incubation at 37°C. The wells were washed once with TBS and blocked with 5% skim milk in TBST for 1 h. After blocking, the wells were washed 3X with TBST and incubated for 1 h with anti-rGBS-PGK antibodies (100 µl; 1:3000) diluted in blocking buffer. The wells were washed 3X with TBST and incubated 1 h with anti-rabbit IgG-Horse-Raddish Peroxidase conjugate antibodies (Sigma-Aldrich) (100 µl; 1:10,000) diluted in blocking buffer. The wells were then washed 3X with TBST and developed with 50 µL of 3,3',5,5'-Tetramethylbenzidine (TMP) (Sigma-Aldrich) for 30 minutes at room temperature before the reaction was stopped with 50 µL of 2M sulphuric acid (H_2SO_4). The absorbance at 450 nm (A_{450}) was measured using a Revelation 4.25 microplate reader (Dynex Technologies, Chantilly, VA, USA). The A_{450} values obtained were compared with the average A_{450} measurement from the GBS NCS13 wild type strain to determine the relative A_{450}.

2.2.9. Plasminogen binding to GBS:

Binding of plasminogen to the surface of GBS mutant strains was analyzed using an ELISA, as previously described. One milliliter of overnight growth cultures of GBS strains were washed once with 1X TBS and re-suspended with 1 mL of 1X TBS. One hundred microliters of bacterial suspensions were aliquoted onto a 96-well microtitre plate (Corning Incorporated, Corning, NY, USA) and incubated for 2 h at 37°C. The wells were blocked for 1 h at 37°C with 5% skim milk diluted in TBST. After blocking, the immobilized cells were washed once with TBST and incubated 1 h at 37°C with

plasminogen (0 or 0.1 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) diluted in blocking buffer. After washing 3X with TBST, a further 1 h incubation was performed in the presence of mouse anti-human plasminogen (SBF1) (Thermo Scientific, Rockford, IL, USA) (100 μl; 1:300) diluted in blocking buffer. The plates were washed again with TBST and incubated for 1 h at 37°C in the presence of anti-mouse IgG HRP-linked antibody (100 μl; 1:300) (New England Biolabs, Ltd., Whitby, ON, Canada). The plates were then developed with 50 μL of TMP (Sigma-Aldrich). After 30 min at room temperature, the reaction was stopped with 50 μL of 2M sulphuric acid (H_2SO_4) and the absorbance was measured at 450 nm with a Revelation 4.25 microplate reader.

The A_{450} measurements from the plates incubated with 0 μg/ml of plasminogen were subtracted from the A_{450} measurements obtained from the plates incubated with 0.1 μg/ml of plasminogen to control for the non-specific binding of the antibodies to the bacterial surface.

2.2.10. Measuring the anti-phagocytic activity of GBS:

To determine the ability of GBS mutant strains to resist phagocytosis and survive in heparinized human blood, experiments were performed as previously described with minor modifications (29). Overnight cultures of GBS strains were washed once with 1X TBS buffer. A 0.5 McFarland standard of GBS cultures was made and then diluted in TH broth (1:10,000) to obtain a concentration of 1.5 X 10^3 CFU/ml for the initial inoculum of the assay. One hundred microliters of the suspension containing approximately 150 CFU was added to 1 mL of freshly heparinized blood in sterile test tubes and was incubated at 37°C under gentle rotation (4 rmp). After 3 h of incubation, 570 μl aliquots of the mixture

were plated on BAP to determine the number of CFU after growth in human blood. The plates were incubated overnight at 37°C. Growth of GBS in human blood is expressed as relative percent of survival by calculating the number of CFU obtained at the end of the incubation period divided by the number of CFU obtained at time zero (T_0) and multiplied by 100. The growth of the GBS NCS13 wild type strain culture in TH was used as a control for antiphagocytic activity. Blood in this experiment was collected from healthy human volunteers in 6 mL Sodium Heparinized Vacutainer tubes (BD, Oakville, ON, Canada).

2.2.11. Invasion assay of GBS:

To determine the ability of GBS mutant strains to invade into tissue culture cells, a standard antibiotic protection assay was performed, as previously described (30). Tissue culture monolayers were grown to confluence in 24-well plates (Corning Costar TC-Treated multiple well plates, Sigma-Aldrich, USA). GBS cultures grown overnight in TH broth were washed once with 1X TBS and a 0.5 McFarland standard of the GBS cultures was made and then diluted 1:100 in TH broth. One hundred microliters (1.2×10^5 CFU) of the 1:100 dilutions (giving a multiplicity of infection of 1:1) were added to the monolayer. The plates were centrifuged at 100 x g for 5 minutes at room temperature and incubated at 37°C in 5% CO_2 for 2 h. The monolayer was washed 3 times with 1X PBS and incubated for 2 h with fresh media containing 5 μg/ml of penicillin and 100 μg/ml of gentamicin to kill the remaining extracellular organisms. The monolayers were washed again with PBS, trypsinized, lysed with 0.1% Triton X-100, and plated on TH agars for quantitation of the intracellular CFU. Relative percent invasion was calculated as:

Number of CFU (GBS mutant strain) invaded into HeLa cells divided by the number of CFU (GBS NCS13 wild type) invaded into HeLa cells, multiplied by 100.

2.2.12. GBS genomes and Sequence analysis of EveA and EveB:

The sequences of seven GBS genomes were screened for the presence of *eveA and eveB* genes. The genome sequences were retrieved from the NCBI FTP site (http://www.ncbi.nlm.nih.gov/Ftp/). The EveA and EveB protein sequences were searched by BLAST using as query the protein sequences of previously identified EveA and EveB (Sag912 and Sag1003, respectively) from GBS 2603 V/R. Multiple sequence alignments were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.2.13. Detection of *eveA* and *eveB* by PCR:

In order to identify the presence of *eveA* and *eveB* genes in the genomic DNA of all the GBS strains and the other three species, *S. pyogenes*, *S. pneumoniae* and *E. coli*, PCR amplification using the primers PF25/PR28 for *eveA* and PF72/PR73 for *eveB* was performed. The PCR reactions consisted of 100 µL of PCR reaction mixtures containing 2 mM $MgCl_2$, 0.2 mM dNTPs, 1X PCR reaction buffer, 100 pmoles each of the forward and reverse primers, 0.5 µL of Taq DNA polymerase and 2 µL of template DNA dissolved in dH_2O. The PCR program consisted of 30 cycles each of denaturing (94°C, 30 seconds), annealing (57°C, 30 seconds), and extension (72°C, 180 seconds) followed by a final extension at 72°C for 10 minutes. A total of 10 µL of each PCR product was separated by electrophoresis on a 1% agarose gel to confirm successful amplification.

2.2.14. Detection of transposon Tn*917* insertion site in *eveB* by PCR:

To detect the insertion site of the transposon Tn*917* in the *eveB* gene (*sag1003*) in NCS13 *eveB*::Tn*917* using PCR, different primer pairs, PF68/PR69, PF70/PR71 and PF70/PR73 were used. The DNA used for the detection was extracted from the NCS13 *eveB*::Tn*917* mutant strain or from the NCS13 wild type strain (as a control). PCR reactions were performed as previously explained in section 2.2.13. Obtaining the correct PCR amplicon size using specific primer pairs will confirm the presence of the intact nucleotide sequences of the gene in the chromosome, while obtaining no PCR product when amplifying the specific region using specific primer pairs will indicate the interruption of the region by the insertion of the Tn*917* transposon.

2.2.15. Statistical analysis:

Data was analyzed using the Students t test and a p-value <0.05 was considered statistically significant. Data points correspond to the average value of all replicates and error bars represent the standard deviation.

2.3. Results:

2.3.1. Far Western blot analysis and peptide sequencing:

The exact nature of the transportation and attachment of GBS-PGK to the surface of GBS is unknown, as it is not associated with any known secretion or signaling motif. Previous work in our lab using Tn*917* transposon mutagenesis identified a gene, *sag1003*, involved in binding GBS-PGK to the surface of GBS (Fig. 2.1) (27). Although the NCS13 *sag1003*::Tn*917* mutant displayed reduced binding of GBS-PGK to the bacterial cells, it had not been demonstrated that this was directly attributable to the loss of the Sag1003 protein. To determine if GBS-PGK bound to Sag1003, a Far Western blot assay for GBS NCS13 cell fractions was established. The Far Western blot was used to identify the protein(s) on the GBS surface to which the rGBS-PGK bound. Whole cell and fractionated GBS (cell wall, cytoplasm, extracellular) were subjected to 10% SDS-PAGE, and a western blot was performed using recombinant GBS-PGK as the probe, as previously described (16). Since PGK was previously shown to bind to plasminogen (16), plasminogen was loaded and used as a positive control. A 100 kDa band corresponding to rGBS-PGK binding to plasminogen was clearly visible (Fig. 2.2). Also, r-GBS-PGK was found to bind to the protein bands of 15 KDa and 43 kDa in both whole cell lysates and the cell wall fractions (Fig. 2.2). The corresponding protein bands were excised from a Coomassie blue stained gel, and following an in-gel tryptic digestion, each sample was subjected to mass spectrographic analysis. For the 15 kDa protein, the peptides identified from the tryptic digest were: SYSFMFTSQRHDLCQLMMTSKR and NLTLSYYR. A BLAST search analysis identified these peptides as being part of the protein sequence for Sag912 (43 KDa) from GBS 2603 V/R (Fig. 2.3). The Sag912 protein was identified as a

hypothetical protein with unknown function, and is predicted to be a polysaccharide-binding protein, based on an I-TASSER prediction (31). Analysis of Sag912 did not reveal the presence of a cell wall-anchoring motif (LPXTG), nor the existence of a 25-amino acid signal peptide at the NH_2-terminal. However, topology prediction of Sag912 amino acid sequences using the HMMpTM predictor (http://bioinformatics.biol.uoa.gr/HMMpTM) revealed the presence of single transmembrane domain that stretches from residue #106 to #125 (32) (Fig. 2.4). Other three predictors of protein structure, TMHMM server v. 2.0, Phobius and CW-PRED, predicted that Sag912 is non-cytoplasmic and located outside the cytoplasmic membrane (33–35). For the 43 kDa protein band, there was more than one specific hit for the protein sequences, hence making it difficult to identify. Therefore, two proteins revealed to be located on the GBS cell surface have been identified, Sag912 and Sag1003, which were later designated as EveA and EveB, respectively.

2.3.2. Mutations of *eveA* and *eveB* in GBS NCS13 strain:

To be certain of the roles of these two proteins, Sag912 (EveA) and Sag1003 (EveB), in PGK binding to the GBS surface, knockout mutagenesis of both genes using insertional inactivation with an erythromycin cassette was performed. Construction of a single deletion mutation of *eveA,* NCS13 *eveA::erm* was accomplished via insertional inactivation with an erythromycin resistance cassette. Briefly, the upstream (5') and downstream (3') DNA fragments of the *eveB* gene were amplified by PCR from the GBS NCS13 genomic DNA, in addition to an erythromycin resistance cassette (*ermB*) from a pTV1-OK plasmid. The fragments were ligated together, following restriction enzyme

digestion, and amplified by PCR with the primers P25/P28 to generate a 1.8 kb construct that was transformed into GBS NCS13. Likewise, NCS13 *eveB::erm* was also constructed. The constructs were transformed into GBS electrocompetent cells separately and transformants were selected on THY agar containing erythromycin. The mutations in *eveA* and *eveB* were successfully constructed, and the integration of erythromycin cassettes into the genes was confirmed by PCR amplifications (Fig. 2.5).

2.3.3. Phenotypic changes of *eveA* and *eveB* mutants:

Mutation of *eveA* and *eveB* genes resulted in different phenotypic changes in the mutant strains. One of these phenotypic changes was reduction in ß-hemolysis. All three GBS mutants, NCS13 *eveA::erm,* NCS13 *eveB::erm* and NCS13 *eveB::*Tn*917* exhibited visibly reduced hemolysis surrounding the colonies after 24 h of growth on BAPs at 35°C (Fig. 2.6). It was also noted that GBS orange pigmentation was reduced in all three GBS mutant strains (Fig. 2.7). Reduction of orange pigmentation was detected by growing mutant strains on Granada agar plates for 24 h at 35°C. Likewise, the production of CAMP factor by GBS mutant strains on BAP was tested. CAMP factor expression in NCS13 *eveA::erm* and NCS13 *eveB::erm* mutants was significantly reduced compared to the wild type strain and the NCS13 *eveB::*Tn*917* mutant (Fig. 2.8).

2.3.4. Reduction of GBS-PGK surface expression in *eveA* and *eveB* mutants:

Expression of GBS-PGK on the surface of the mutant strains was determined by ELISA. All three mutants of GBS NCS13 were assayed, using anti-rGBS-PGK antibodies, to quantify the level of GBS-PGK expressed on the bacterial surface.

Inactivation of *eveA* or *eveB* genes in GBS affected the expression of GBS-PGK (Fig. 2.9). Expression of GBS-PGK on the bacterial surface of NCS13 *eveA::erm,* NCS13 *eveB::erm* and NCS13 *eveB::*Tn*917* was significantly reduced (P=0.022, P=0.023 and P=0.02, respectively), resulting in an A_{450} measurement of 35%, 35% and 36%, respectively, as compared with that of the wild type NCS13 strain. The A_{450} reading from the wild type NCS13 strain was defined as 100%.

2.3.5. Reduction of plasminogen binding to the surface of *eveA* and *eveB* mutants:

The PGK protein in GBS was previously identified as a plasminogen-binding protein (16). The mutants NCS13 *eveA::erm,* NCS13 *eveB::erm* and NCS13 *eveB::*Tn*917* were found to have significantly reduced amounts of surface-expressed GBS-PGK compared with that of the wild type NCS13. To determine if the reduction of GBS-PGK surface expression would result in the reduction of plasminogen binding to the surface of the mutant strains, the plasminogen binding to the surface of the bacterial strains was assayed following incubation of the bacterial cells with 0.1 μg/ml of plasminogen. All three mutant strains showed a significant reduction in plasminogen binding, resulting in 28% (P=0.008) for NCS13 *eveA::erm,* 44% (P= 0.015) in NCS13 *eveB::erm* and 67% (P= 0.038) in NCS13 *eveB::*Tn*917* of plasminogen binding to the surface of the mutants (Fig. 2.10). The A_{450} reading for the wild type NCS13 strain was defined as 100%.

2.3.6. Reduction of anti-phagocytic activity of *eveA* and *eveB* mutants:

The anti-phagocytic properties of the wild type and mutant GBS strains were determined by measuring their ability to multiply in fresh human blood during a 3 h incubation period. The number of live bacteria present after incubation was determined by counting the CFU on BAP. The results were expressed in the form of relative percent of survival, which is the number of CFU at 3 h divided by the number of CFU at time zero, multiplied by 100. The percentage of survival from the wild type GBS NCS13 strain was defined as 100%. The results revealed that, while the wild type GBS NCS13 strain survived in the blood (100%) as expected, the mutant strains NCS13 *eveA::erm* and NCS13 *eveB::erm* were readily killed (94%, P =0.0000028, and 95% survival, P = 0.0000017, respectively), thereby indicating that the anti-phagocytic activity of the mutant strains were completely inhibited (Fig. 2.11). The mutation of the *eveB* gene in NCS13 *eveB::*Tn*917* resulted in a percent of survival of 63%, with a significant reduction of anti-phagocytic activity (P= 0.04) compared with that of the wild type strain.

2.3.7. Reduction of invasion of HeLa cells by *eveA* and *eveB* mutants:

Since surface-expressed GBS-PGK mediates GBS invasion into the cervical epithelial tumor cell line (30), it was hypothesized that the reduction of the cell surface GBS-PGK might adversely affect GBS invasion into HeLa cells. To test this hypothesis, NCS13 *eveA::erm,* NCS13 *eveB::erm* and NCS13 *eveB::*Tn*917* strains were examined for their ability to invade HeLa cells. The GBS invasion assay revealed that the mutant strains, NCS13 *eveA::erm,* NCS13 *eveB::erm* and NCS13 *eveB*::Tn*917* invaded the HeLa cells at rates (95%, 88% and 89%, respectively; P= 0.0000064, P=0.000078 and

P=0.000027, respectively) that were significantly less than that of the wild-type strain (Fig. 2.12).

2.3.8. Detection and identification of *eveA* and *eveB* genes in GBS strains:

The *eveA* gene from the GBS NCS13 strain, thirteen other GBS strains, *S. pyogenes*, *S. pneumonia* and *E. coli* DH5α were amplified from purified chromosomal DNA via PCR. Using *eveA* and *eveB* primers, on agarose gel electrophoresis, all 14 GBS strains produced PCR products with bands of approximately 1 kb and 3 kb for the *eveA* and *eveB* genes, respectively, as predicted if *eveA* or *eveB* genes were present; however, *S. pyogenes*, *S. pneumonia* and *E. coli* DH5α lacked this characteristic band (Fig. 2.13).

2.3.9. Comparison of EveA and EveB proteins:

To evaluate the level of amino acid conservation, EveA and EveB amino acid sequences from seven GBS strains, which already had sequenced genomes, were examined. The seven strains were serotype Ia strain (A909), serotype Ib strain (NCTC8187), serotype II strain (NCTC11079), serotype III strains (NEM316) or (COH-I), serotype IV strain (NCTC11930), serotype V strain (2603 V/R) and serotype VI strain (GBS-M002). The reason for choosing different GBS serotypes was to detect whether EveA and EveB proteins are serotype-specific or non-serotype specific. The amino acid sequences were found to be highly conserved in all seven of the GBS strains (Fig. 2.14 & Fig. 2.15). This suggests that the EveA and EveB proteins are non-serotype specific and conserved in different serotypes of GBS strains. The EveA and EveB predicted proteins were 100% identical in each of the seven strains surveyed.

2.3.10. Disruption of the C-terminal domain of *eveB* by Tn*917*:

The observation that NCS13 *eveB*::Tn*917* behaves differently from NCS13 *eveB::erm* in terms of the production of CAMP factor and anti-phagocytic activity led us to investigate the differences between the two *eveB* mutants by identifying the exact location of the transposon insertion into the *eveB* gene. Although it was known that the *ermB* gene was inserted exactly in the middle of the *eveB* gene in the NCS13 *eveB::erm* mutant strain, the exact insertion site of the transposon Tn*917* into the *eveB* gene in the NCS13 *eveB*::Tn*917* strain was not previously confirmed. Thus, traditional PCR amplification of the specific regions of the *eveB* gene was used to identify the insertion site of the Tn*917* transposon into NCS13 *eveB*::Tn*917*. The PCR products for the specific regions in the *eveB* gene obtained from the DNA template of NCS13 *eveB*::Tn*917* using the primer pairs PF68/PR69 and PF70/PR71 had sizes similar to that of the PCR products from the NCS13 wild type stain using the same primer pairs. This suggests that the N-terminal region and middle domains of the *eveB* gene in the NCS13 *eveB*::Tn*917* mutant strain were intact (Fig. 2.16). However, the PCR amplification of the *eveB* gene from the NCS13 *eveB*::Tn*917* mutant using the primer pair PF70/PR73 resulted in no PCR product compared to the product obtained from the NCS13 wild type strain using the same primer pair, thereby indicating that the Tn*917* was inserted into the C-terminal domain of the *eveB* gene (Fig. 2.16).

2.4. Discussion:

Studying the roles of GBS virulence factors is important for understanding the host-pathogen interactions that contribute to GBS pathogenesis. Since the role of surface-expressed GBS-PGK in bacterial virulence cannot be studied by traditional knockout mutagenesis, it was important to identify the gene(s) involved in the surface expression of GBS-PGK, then a knockout of those genes could be created. In this study, two genes in GBS, *eveA* and *eveB*, were identified as having roles in the surface expression of GBS-PGK. Mutation of the *eveA* and *eveB* genes in GBS resulted in a significant decrease in surface-expressed PGK on the GBS mutant surfaces. The binding of plasminogen to the surface of all the mutants was also significantly reduced compared to the wild type strain. Reduction in the plasminogen binding to the surface of the mutants confirmed the role of surface-expressed GBS-PGK as a plasminogen-binding protein, as was previously investigated (36). Whether the EveA and EveB proteins on the surface of GBS play a role in the surface expression of PGK directly (as a binding ligand for surface expressed GBS-PGK) or indirectly (as a regulator of the expression of GBS-PGK) will be discussed in chapter 3. This question can be answered through further experiments using a Far western immunoblotting to determine the binding interaction between GBS-PGK and the EveA or EveB proteins. The *eveB* gene (*sag1003*) has previously been suggested as having a role in antimicrobial peptide resistance, and it was suggested that a mechanism used by GBS to resist antimicrobial peptides might be related to the expression of GBS-PGK on the bacterial surface (27). In addition, it was suggested that a portion of the EveB transmembrane protein that is exposed to the outer surface of the bacterial membrane could theoretically act as a binding ligand for GBS-PGK, since the NCS13 *eveB*::Tn*917*

mutant strain was found to have less surface expressed GBS-PGK and more GBS-PGK secreted into the supernatant. However, the result of the Far Western blot did not identify any protein band belonging to EveB. could be that because EveB is a transmembrane protein not entering the SDS-PAGE gel during electrophoresis or the protein is unable to be transferred to the nitrocellulose membrane.

In this study, two *eveB* mutants, NCS13 *eveB*::Tn*917* and NCS13 *eveB*::*erm,* were used to run different experiments. The two mutants showed some similarities in terms of reduction of GBS-PGK surface expression, plasminogen binding to the surface of the mutants, ß- hemolysis, orange pigmentation and invasion. However, the CAMP factor production and anti-phagocytic activities of the two *eveB* mutants were not the same. The NCS13 *eveB*::*erm* mutant showed no production of CAMP factor and had almost lost its anti-phagocytic activity compared to the NCS13 *eveB*::Tn*917* mutant that showed normal production of CAMP factor and significant decrease of anti-phagocytic activity, but not completely lost as was seen with NCS13 *eveB*::*erm* mutant. One explanation for the opposite phenotype in CAMP factor production and anti-phagocytic activity between the NCS13 *eveB::erm* and NCS13 *eveB*::Tn*917* mutants is that in the NCS13 *eveB*::Tn*917* mutant, the transposon Tn*917* was inserted near the end of the gene and altered the C-terminal region of the *eveB* gene, which is important for proper function of the protein for the invasion, while the N-terminal region is still expressed in the transposon mutant and that N- terminus is enough for the expression of CAMP factor and anti-phagocytic activity.

The observation that *eveA* and *eveB* mutant strains showed reduction in ß-hemolysin and orange pigmentation suggest that these genes may be involved in

regulating the expression of the CovSR two-component system (Control of virulence Sensor and Regulator, also known as CsrSR) in GBS. The expression of ß-hemolysin and orange pigmentation in GBS are encoded for by the *cyl* operon, which is regulated directly by the CovSR system (37–44). In addition to the reduction of hemolysis and pigmentation by the three mutant strains, resistance to phagocytic killing and the ability to invade epithelial cells were significantly reduced in all the mutants. These observations suggest dual functions for the EveA and EveB proteins, particularly in bacterial resistance to phagocytic killing by human phagocytic cells and in bacterial invasion into human epithelial cells. These observations could also confirm the roles of the *eveA* and *eveB* genes in directly or indirectly controlling the regulation of the CovSR system. The CovSR system is a master regulator of GBS virulence gene expression; it regulates the expression of various virulence factors, such as proteins involved in bacterial resistance to phagocytic cells, as well as in the adhesion and invasion into human epithelial cells (43,45,46). The mechanism by which the EveA or EveB proteins could control the regulation of the CovSR system in GBS is unknown. It is possible that these proteins on the surface of GBS may interact with the membrane histidine kinase, CovS in order to inhibit its phosphorylation, and consequently, inhibit the phosphorylation of its cognate cytoplasmic response regulator, CovR. The cellular regulator of the CovSR system is becoming increasingly recognized. A study by Firon *et al*. identified a GBS transmembrane protein of unknown function, called Abx1, as an additional partner of the CovSR system that is necessary to regulate the activity of CovS via a protein-protein interaction (47). The presence of the *eveA* and *eveB* genes in all the surveyed GBS strains

confirm the conservation and likely importance of these genes in relation to expression of various GBS virulence traits.

The results of my work also suggest a relationship between the expression of PGK on the GBS surface and the expression of other GBS virulence factors. When the expression of GBS-PGK was decreased on the bacterial surface, the expression of the virulence-associated GBS protein, ß-hemolysin/cytolysin is also decreased. Whether this relationship is controlled directly by the presence of PGK on GBS surface that acts as a signal to activate the expression of the bacterial virulence genes, or indirectly by the newly identified EveA or EveB proteins regulating the expression of GBS virulence genes, needs to be investigated. Further research to study the role of the EveA and EveB proteins in GBS virulence is essential to understanding the mechanisms used to influence virulence.

Table 2.1. Primers used in this study. The underlined sequences indicate the restriction sites.

Primer	Sequence (5'—3')	Restriction site	Description
P1	CTC CTG CAG TTA CAA ACA AAT CGT TTA ACT TC	PstI	Forward primer for erythromycin gene (*ermB)*
P2	TAC GGT ACC GAA TTA TTT CCT CCC GTT AAA	KpnI	Reverse primer for *ermB* gene
P9	AAA ACA AAT CAA TCG GTT AGA TA		Forward primer for 5' fragment of *eveB* gene
P10	CTC CTG CAG GAA TAG TCA GGT CTT TGG AAA	PstI	Reverse primer for 5' fragment of *eveB* gene
P11	TAC GGT AAC CAG TTT ACT AGC CTT TGT TTT	KpnI	Forward primer for 3' fragment of *eveB* gene
P12	TGT CTG TTA TGA ATA GGG AAA TT		Reverse primer for 3' fragment of *eveB* gene
P22	TAC GGT ACC TTA CAA ACA AAT CGT TTA ACT TC	KpnI	Forward primer for *ermB* gene
P24	TTC GGA TCC GAA TTA TTT CCT CCC GTT AAA	BamHI	Reverse primer for *ermB* gene
P25	TAC GAG CTC ATG TCA AAT ATA ATA ACT TAT TTA AA	SacI	Forward primer for 5' fragment of *eveA* gene

P26	TAC GGT ACC GAC CTG AGA GAA CTA CTT TAT	KpnI	Reverse primer for 5' fragment of *eveA*
P27	TCC GGA TCC GTA CGC TGC AAT GTT TAC AAA	BamHI	Forward primer for 3' fragment of *eveA* gene
P28	TTC CTG CAG CTA CTT AGT CTC TCT GCT ATT	PstI	Reverse primer for 3' fragment of *eveA* gene
PF68	ACC ATG AAG GTC TCA GCA CCT AAT AT		Forward primer for *eveB* gene
PR69	ATT ACT GAT TTT AGA AGT GCT ATT		Reverse primer for *eveB* gene
PF70	ACC ATG CAA TCC TCC TTA AAG CAA AC		Forward primer for *eveB* gene
PR71	ATC TAA AGA ACG CAC AAC GG		Reverse primer for *eveB* gene
PR73	TAC GGT AAC CTA ATC TAC TGA TTT CAA GG		Reverse primer for *eveB* gene
PGK-pstF	TTC CTG CAG TTA TTT TCA GTC AAT GC	PstI	Forward primer for 3' fragment of *pgk* gene
PGK-hinR	TTC AAG CTT TTT TCA GTC AAT GCT GCC AAA CC	HindIII	Reverse primer for 3' fragment of *pgk* gene

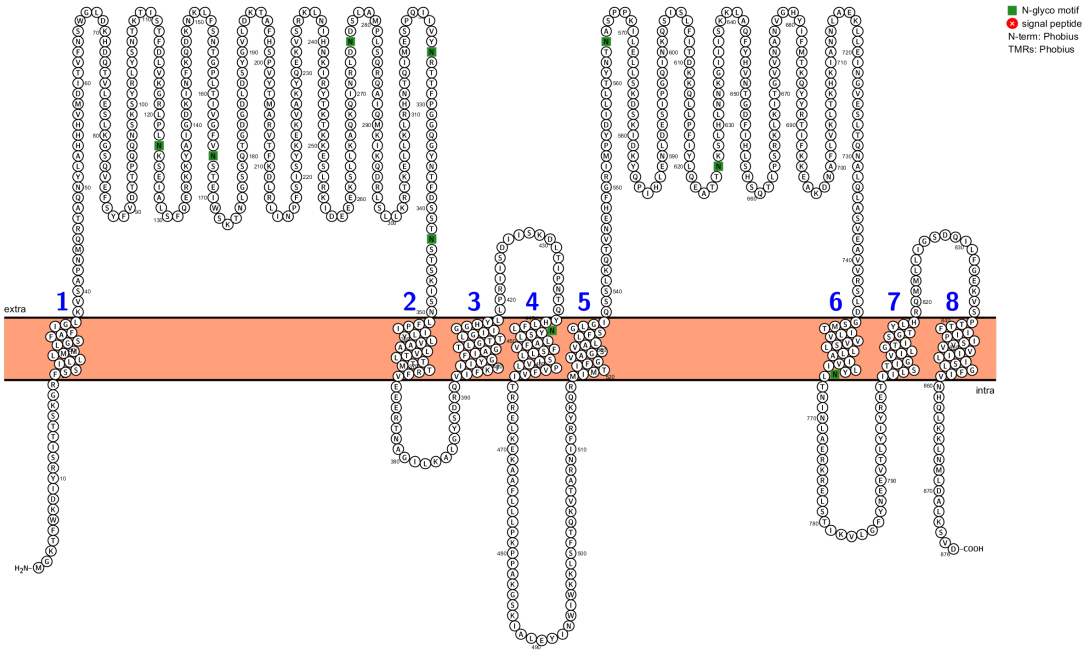

Figure 2.1. Predicted transmembrane topology for the GBS EveB (Sag1003) protein. The model was obtained by using the PROTTER program (48).

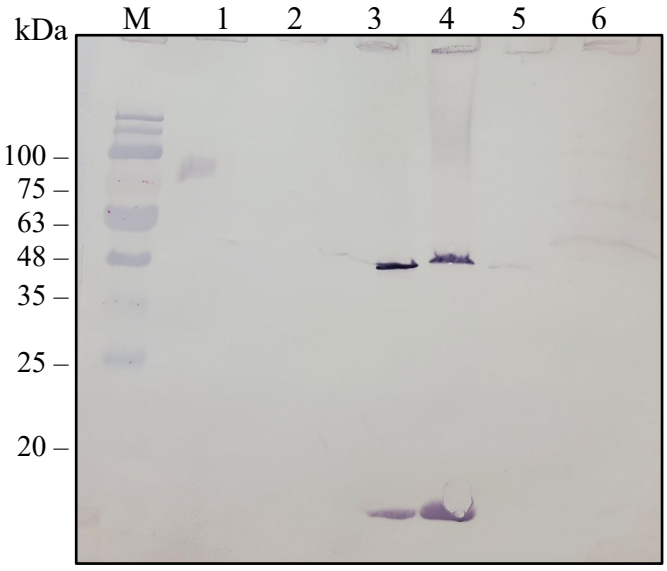

Figure 2.2. Far Western blot analysis of GBS incubated with r-GBS-PGK and probed with anti-GBS-PGK. Whole-cell lysates and cell fractions of GBS were separated by 10% SDS-PAGE and western transferred to a nitrocellulose membrane. The membrane was blocked first with 5% skim milk for 1 h at room temperature, incubated for 16 h at 4°C with r-GBS-PGK (25 µg/mL) and probed with anti-GBS-PGK antibodies (1:1500 in blocking buffer) for 3 h at 4°C, followed by alkaline phosphatase conjugate anti-rabbit-IgG (1:10 000 in blocking buffer) for 1 h at room temperature. The membrane was developed using BCIP/NBT for approximately 15 minutes before stopping with 3 changes of distilled water. Lane M: Protein marker BLUelf prestained protein ladder (FroggaBio, Toronto, ON., Canada), lane 1: plasminogen, lane 2: TH broth, lane 3: whole cell lysate, lane 4: cell wall fraction, lane 5: cytoplasmic fraction, lane 6: extracellular fraction. The antibody was bound to a 43 kDa protein and a 15 kDa protein.

```
  1 msniitylkn nsnltfdela lndvdilcln efgyisfekl inttemksvl vcelyheylq
 61 tmaksysfmf tsqrhdlcql mmtskrfknl tlsyyraeis lefekqfaam vftipninyh
121 qvvfrgtdan ligwkedfkl tymreisahr saikylntil pyfdkvvlsg hskggnlaly
181 aamftkpdlk akidliwlid spglqktllp tteykttkqk cirllpeesi vgmmlysdie
241 pliissnarg ilqhdvttwe iqepailktg tglslksicf ektfqqwmae lksqerklff
301 dllfdsflss gvssllddfnl asrakmmkaf hsfreldddk krlfnkslkl lvtifwgayh
361 dnsretk
```

Figure 2.3. Peptide sequences identified from the mass spectrometric (MS) analysis of the 15 kDa protein. Peptides identified by MS are underlined in the protein sequence.

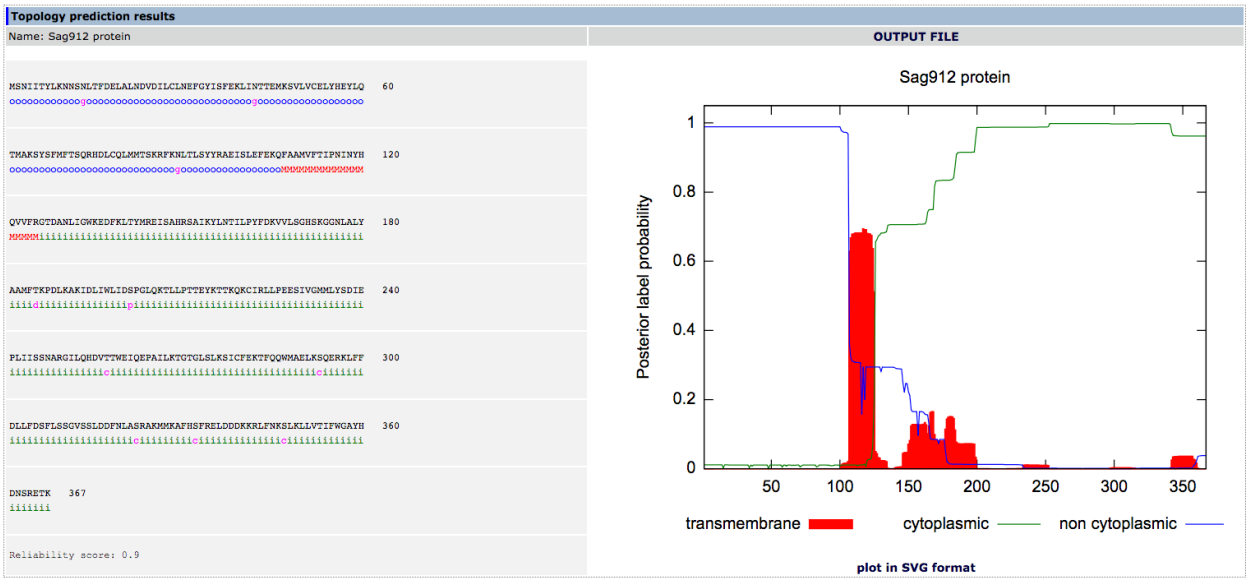

Figure 2.4. The HMMpTM server predicted residues #106 to #125 as the transmembrane region in the EveA (Sag912) protein.

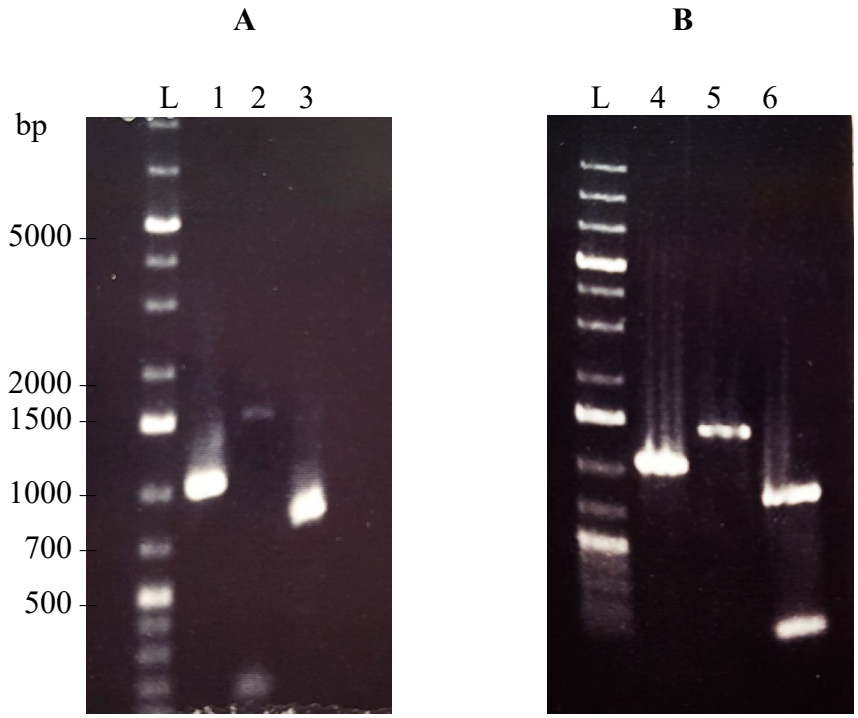

Figure 2.5. Genetic confirmation of the inactivation of the *eveA* gene (A) and *eveB* gene (B) in NCS13 *eveA::erm* and NCS13 *eveB::erm*, respectively. PCR results are as follows: Lane 1: *eveA* gene from the NCS13 parent strain, Lane 2: *eveA* gene from the NCS13 *eveA::erm* mutant containing the *erm* gene, Lane 3: Erythromycin gene from the NCS13 *eveA::erm* mutant, Lane 4: *eveB* gene from the NCS13 parent strain, Lane 5: *eveB* gene from the *eveB::erm* mutant containing the *erm* gene, Lane 6: Erythromycin gene from the NCS13 *eveB::erm* mutant. L: GeneRuler 1 Kb plus DNA ladder standard (Invitrogen).

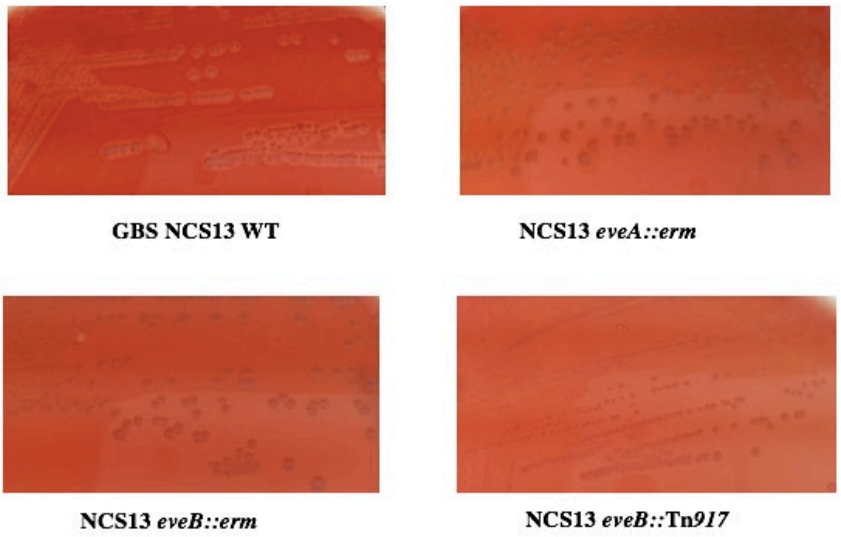

Figure 2.6. Close-up photographs of the loss zones of ß-hemolysis surrounding the colonies of NCS13 *eveA::erm*, NCS13 *eveB::erm* and NCS13 *eveB::*Tn*917* compared to the wild type GBS NCS13 on blood agar plates.

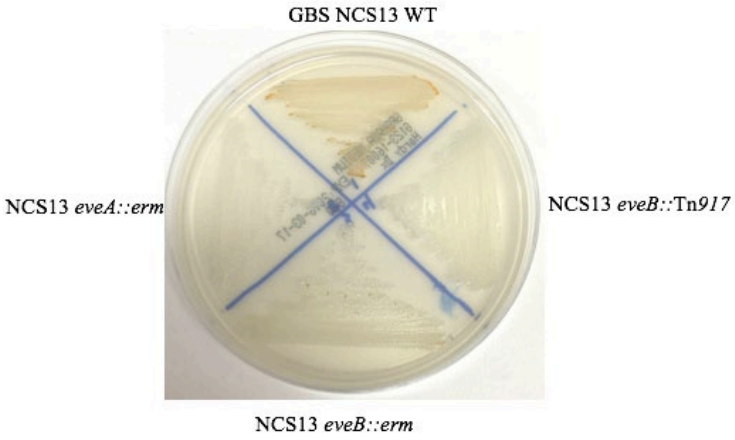

Figure 2.7. Orange pigmentation is present in the wild type strain, yet is absent in the mutant strains.

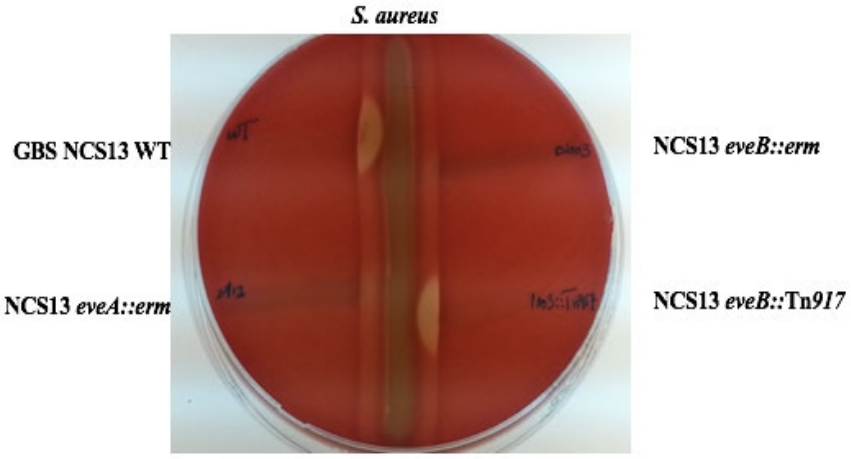

Figure 2.8. CAMP reactions for different GBS strains. Vertical streak: *Staphylococcus aureus*. Horizontal streaks: NCS13 *eveA::erm,* NCS13 *eveB::erm,* NCS13 *eveB::*Tn*917* and wild type GBS NCS13. A CAMP test revealed reduced synergistic hemolysis at the intersection of the streaks of NCS13 *eveA::erm* and NCS13 *eveB::erm,* with a perpendicular streak of beta-lysin-producing *S. aureus.* However, no reduction was seen with the NCS13 *eveB*::Tn*917* mutant strain.

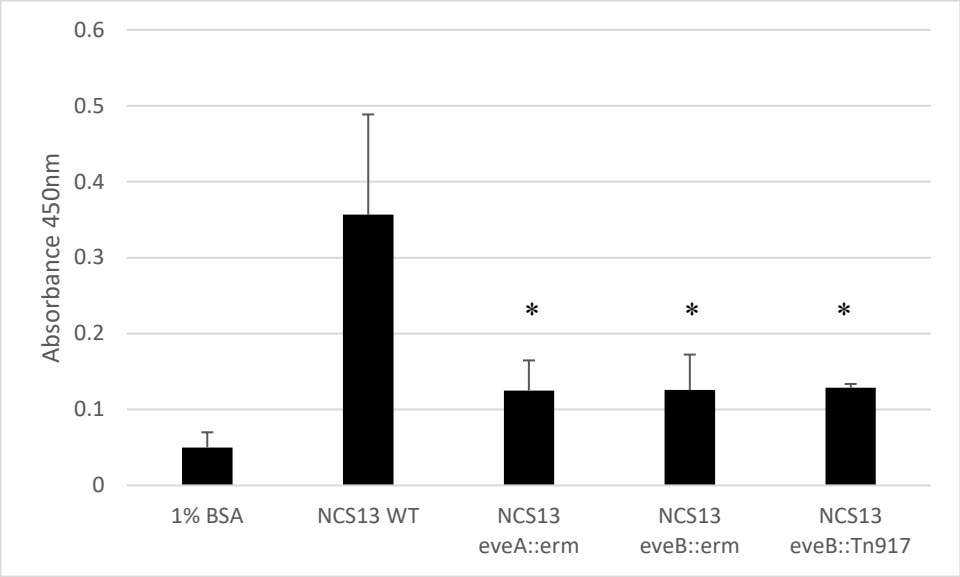

Figure 2.9. Expression of phosphoglycerate kinase (PGK) on the surface of NCS13 mutant strains. Colonies of NCS13 *eveA::erm*, NCS13 *eveB::erm*, NCS13 *eveB::Tn917* and NCS13 wild type were assayed for surface expression of GBS-PGK using an ELISA. The A_{450} values from the wells containing the mutant strains were compared with measurements from the wells containing the wild type NCS13 strain to determine the relative A_{450}. The experiments were performed in triplicate. The bars represent the average value of experiments run in triplicate; error bars represent standard deviation. Asterisks (*) indicate statistical significance ($p < 0.05$).

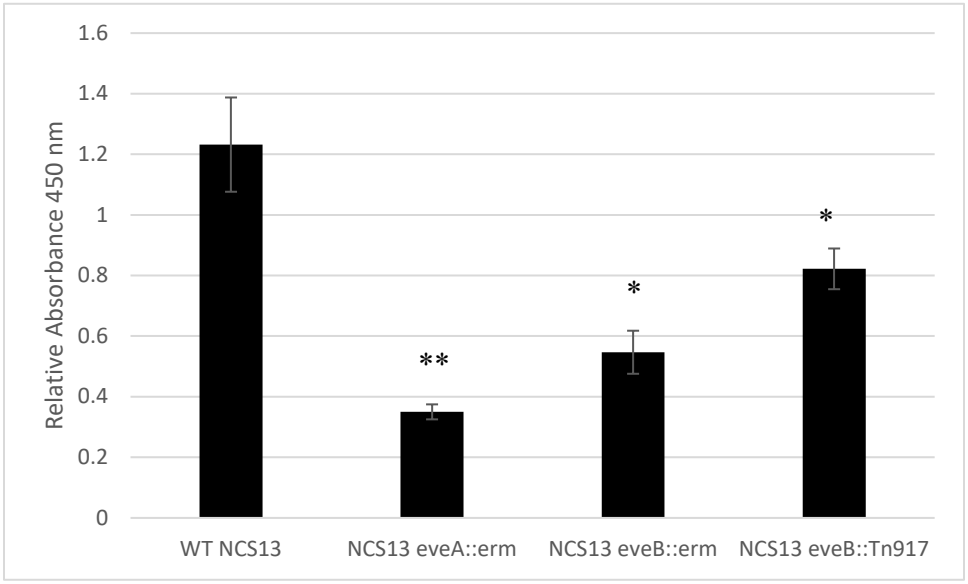

Figure 2.10. Plasminogen binding to the surface of GBS NCS13 strains. Binding of plasminogen (0.1µg/ml) to whole cells of NCS13 WT, NCS13 *eveA::erm*, NCS13 *eveB::erm*, NCS13 *eveB::Tn917* were analyzed by ELISA, with bound plasminogen detected using a monoclonal anti-plasminogen antibody (SBF1). The data represents the means and standard errors from two separate experiments. The bars represent the average value of experiments run in duplicate; error bars represent standard deviation. Asterisks (*) indicate statistical significance ($p < 0.05$), asterisks (**) indicate statistical significance ($p < 0.01$).

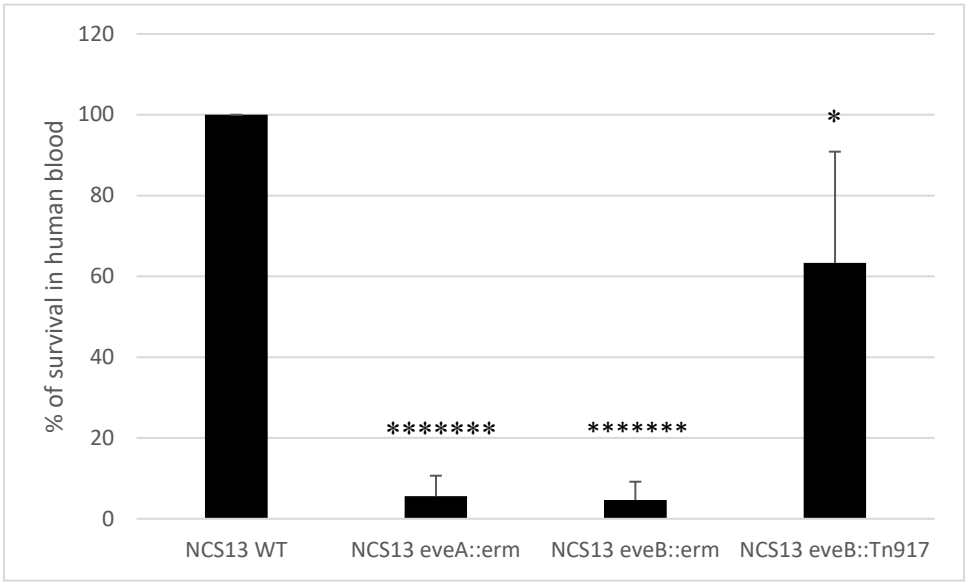

Figure 2.11. Phagocytosis assay of NCS13 strains. Phagocytosis assays were performed as described previously. Diluted cultures of NCS13 WT, NCS13 *eveA::erm*, NCS13 *eveB::erm*, and NCS13 *eveB::Tn917* (100 µl, ~ 1.5 X 10^3 CFU/ml) were combined with fresh human blood (1 ml), and the mixtures were rotated at 37°C for 3 hours. Viable cell counts were determined by plating diluted samples onto BAP. The percentage of viable bacteria in human blood was calculated as follows: The number of CFU obtained at the end of the incubation period divided by the number of CFU in the original inoculum, then multiplied by 100%. The data represent the means and standard error of three independent experiments. The bars represent the average value of experiments run in triplicate; error bars represent standard deviation. Asterisks (*) indicate statistical significance ($P < 0.05$), asterisks (*******) indicate statistical significance ($P < 0.0000001$).

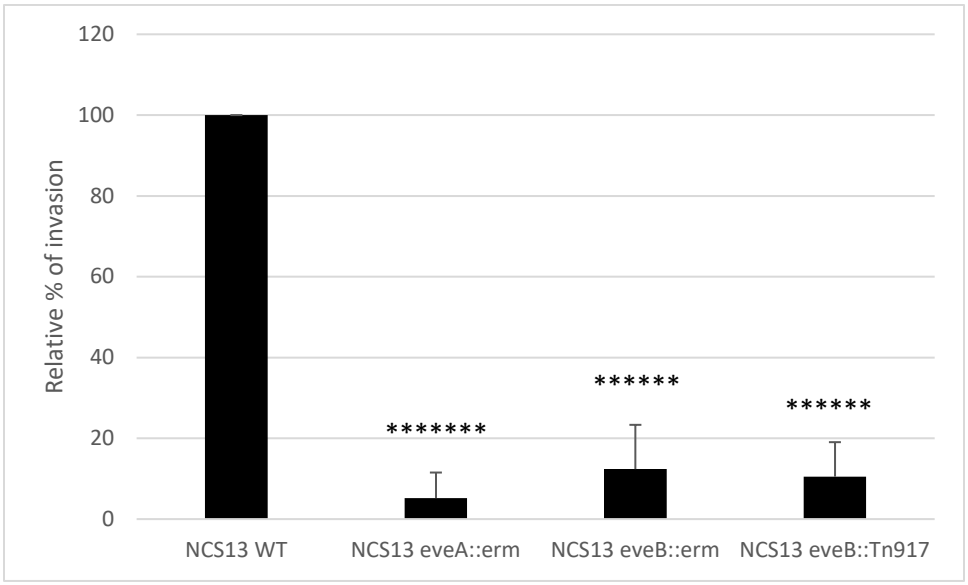

Figure 2.12. Streptococcal invasion assay into HeLa cells by the mutant strains NCS13 *eveA::erm,* NCS13 *eveB::erm* and NCS13 *eveB*::Tn*917*, and the NCS13 wild type strain. The HeLa cells were grown in 24-well tissue culture plates to full confluency. The mutant strains and wild type strain were grown overnight, diluted to 0.5 Mcfarland and further diluted to 1:100. One hundred microliters of 1:100 bacterial suspensions were used to inoculate each well of HeLa cells, and the invasion was allowed for 2h at 37°C in 5% CO_2 atmosphere. Non-invaded bacteria were washed away 3X with PBS, and 100 µg/ml gentamicin and 5µg/ml penicillin was added for 2h to kill any remaining cell-associated bacteria. The infected cells were trypsinised and lysed with 0.1% triton X-100, and the number of invaded bacteria was determined by the counting CFUs after overnight incubation on BAP. Bacterial invasion was expressed as a comparison to the wild type NCS13 strain, which was set as 100%. Each bar represents an average of results from three independent experiments, each from triplicate wells ± standard errors. Asterisks (******) indicate statistical significance ($P < 0.000001$), asterisks (*******) indicate statistical significance ($P < 0.0000001$).

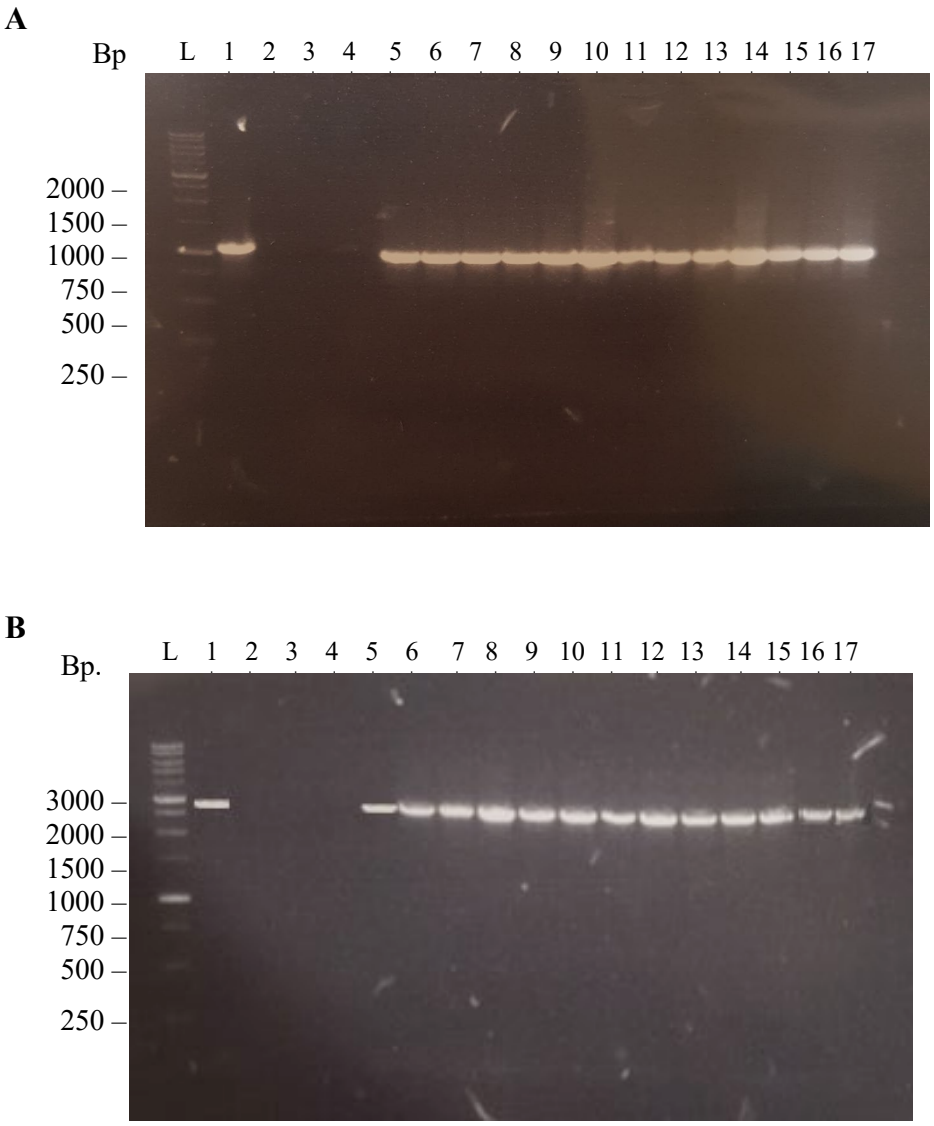

Figure 2.13. PCR amplification of the GBS *eveA* (A) and *eveB* (B) genes from other strains of bacteria. DNA templates were as follows: GBS NCS13, *S. pyogenes* (ATCC19615), *S. pneumoniae* (ATCC49619), *E. coli* DH5a, GBS non-capsulated strain (COH-13), GBS non-typeable strain (18SR431), GBS non-typeable strain (17SR409), GBS ST-Ia (97SR400), GBS ST-Ib (97SR570), GBS ST-II (97SR138), GBS ST-III (COH-I), GBS ST-IV (97SR331), GBS ST-V (97SR384), GBS ST-VI (9842), GBS ST-VII (7271), GBS ST-VIII (JM9), and GBS ST-IX (ATCC 27412) (Lanes 1 to 17, respectively). L: 1 Kb DNA ladder (size standard) (FroggaBio, Ontario, Canada).

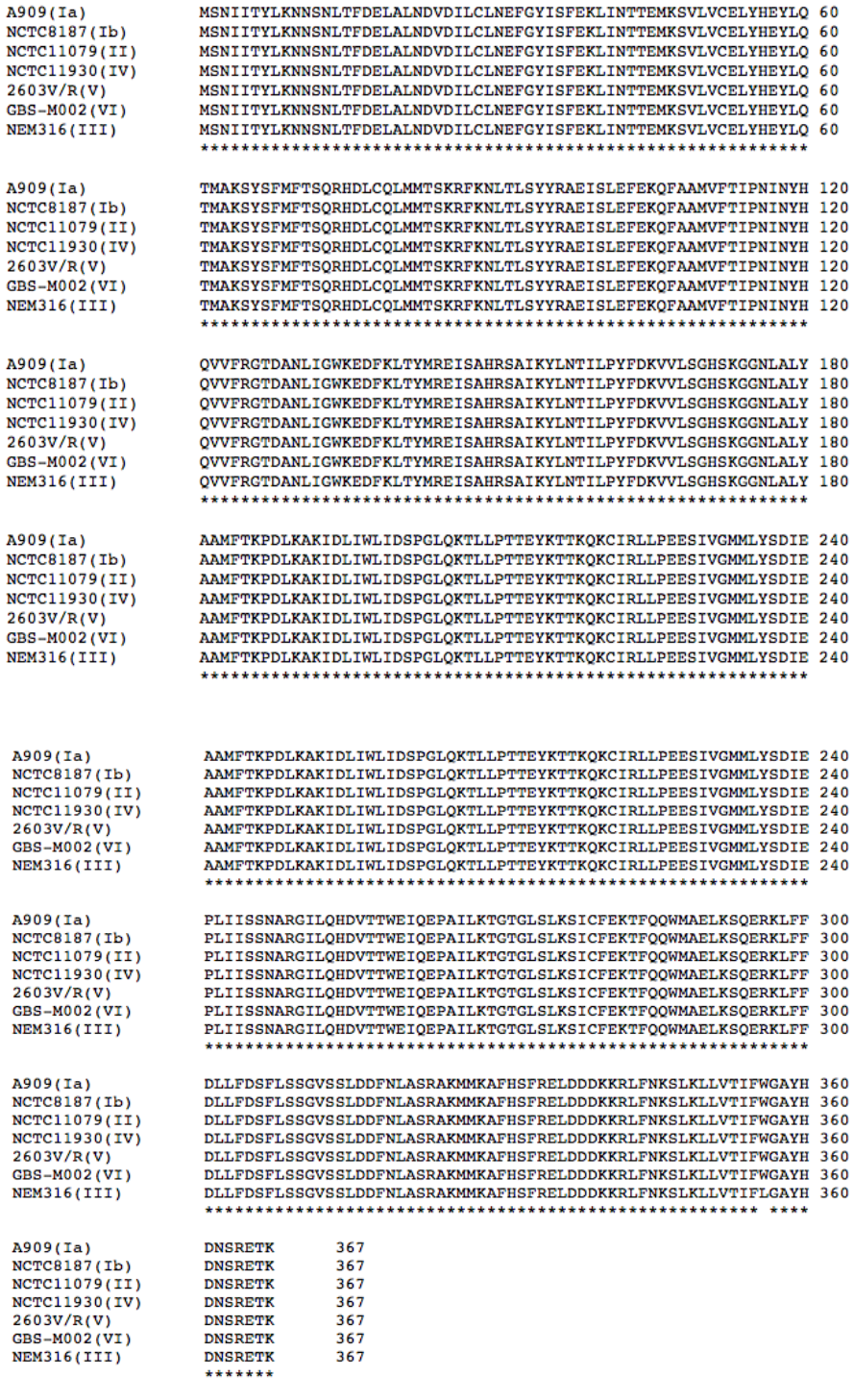

Figure 2.14. Comparison of the predicted amino acid sequences of EveA proteins (367 aa) from each of the following: serotype Ia strain (A909), serotype Ib strain (NCTC8187), serotype II strain (NCTC11079), serotype III strains (NEM316), serotype IV strain (NCTC11930), serotype V strain (2603 V/R), and serotype VI strain (GBS-M002).

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A909(Ia)          MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
NCTC8187(Ib)      MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
NCTC11079(II)     MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
COH-I(III)        MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
NCTC11930(IV)     MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
2603V/R(V)        MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
GBS-M002(VI)      MGKTFWKDIYRSITTSKGRFSSILLLMMLGSFAFIGLKVSAPNMQRTAQNYLAHHHVMDI 60
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A909(Ia)          TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
NCTC8187(Ib)      TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
NCTC11079(II)     TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
COH-I(III)        TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
NCTC11930(IV)     TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
2603V/R(V)        TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
GBS-M002(VI)      TVFNSWGLDKHDQTVLESLKGSQVEFSYFVDTTPQQNSKSYRLYSNTKTISTFDLVKGRL 120
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A909(Ia)          PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
NCTC8187(Ib)      PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
NCTC11079(II)     PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
COH-I(III)        PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
NCTC11930(IV)     PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
2603V/R(V)        PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
GBS-M002(VI)      PLNKSEIALSFQERKKYAIGDKINFKQDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQ 180
                  ************************************************************

A909(Ia)          TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
NCTC8187(Ib)      TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
NCTC11079(II)     TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
COH-I(III)        TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
NCTC11930(IV)     TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
2603V/R(V)        TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
GBS-M002(VI)      TGDGDLDSYGVLDKTAFHSPVYTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIH 240
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A909(Ia)          NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
NCTC8187(Ib)      NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
NCTC11079(II)     NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
COH-I(III)        NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
NCTC11930(IV)     NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
2603V/R(V)        NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
GBS-M002(VI)      NKIRYTKTKKESLRKIDEEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLL 300
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A909(Ia)          KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
NCTC8187(Ib)      KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
NCTC11079(II)     KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
COH-I(III)        KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
NCTC11930(IV)     KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
2603V/R(V)        KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
GBS-M002(VI)      KRTKELLKLRHNTQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISNLFPIILYLVA 360
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A909(Ia)          ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
NCTC8187(Ib)      ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
NCTC11079(II)     ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
COH-I(III)        ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
NCTC11930(IV)     ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
2603V/R(V)        ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
GBS-M002(VI)      ALVTLTTMTRFVEEERTNAGILKALGYSDRQVIFKFIIYGFIAGTLGTTLGIIGGHYLLP 420
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A909(Ia)          RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
NCTC8187(Ib)      RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
NCTC11079(II)     RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
COH-I(III)        RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
NCTC11930(IV)     RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
2603V/R(V)        RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
GBS-M002(VI)      RIISDIISKDLTIPNTQYHLFLNYSLLAFVFSLLSIVLPVFVITRRELKEKAAFLLLPKP 480
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A909(Ia)          PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
NCTC8187(Ib)      PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
NCTC11079(II)     PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
COH-I(III)        PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
NCTC11930(IV)     PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
2603V/R(V)        PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
GBS-M002(VI)      PAKGSKIALEYINWIWKKLSFTQKVTARNIFRYKQRMIMTIFGVAGSVALLFSGLGIQSS 540
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A909(Ia)          LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
NCTC8187(Ib)      LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
NCTC11079(II)     LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
COH-I(III)        LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
NCTC11930(IV)     LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
2603V/R(V)        LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
GBS-M002(VI)      LKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDESIPGQIN 600
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A909(Ia)          KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
NCTC8187(Ib)      KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
NCTC11079(II)     KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
COH-I(III)        KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
NCTC11930(IV)     KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
2603V/R(V)        KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
GBS-M002(VI)      KQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGDFIHLSHSQ 660
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A909(Ia)          TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
NCTC8187(Ib)      TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
NCTC11079(II)     TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
COH-I(III)        TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
NCTC11930(IV)     TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
2603V/R(V)        TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
GBS-M002(VI)      TLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTKHKIANNLAEKLLE 720
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A909(Ia)          INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
NCTC8187(Ib)      INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
NCTC11079(II)     INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
COH-I(III)        INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
NCTC11930(IV)     INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
2603V/R(V)        INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
GBS-M002(VI)      INGVESLTQNALQLASVEAVVRSLDGSMTILVVVSLLLAIVILYNLTNINLAERKRELST 780
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A909(Ia)          IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
NCTC8187(Ib)      IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
NCTC11079(II)     IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
COH-I(III)        IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
NCTC11930(IV)     IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
2603V/R(V)        IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
GBS-M002(VI)      IKVLGFYNEEVTLYIYRETIILSTIGVILGTISGTYLHRQMMLLIGSDQILFGEKVSPTT 840
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A909(Ia)          FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
NCTC8187(Ib)      FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
NCTC11079(II)     FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
COH-I(III)        FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
NCTC11930(IV)     FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
2603V/R(V)        FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
GBS-M002(VI)      FIIPISVVVIILISLGFIVNHQLKKLNMLDALKSVD 876
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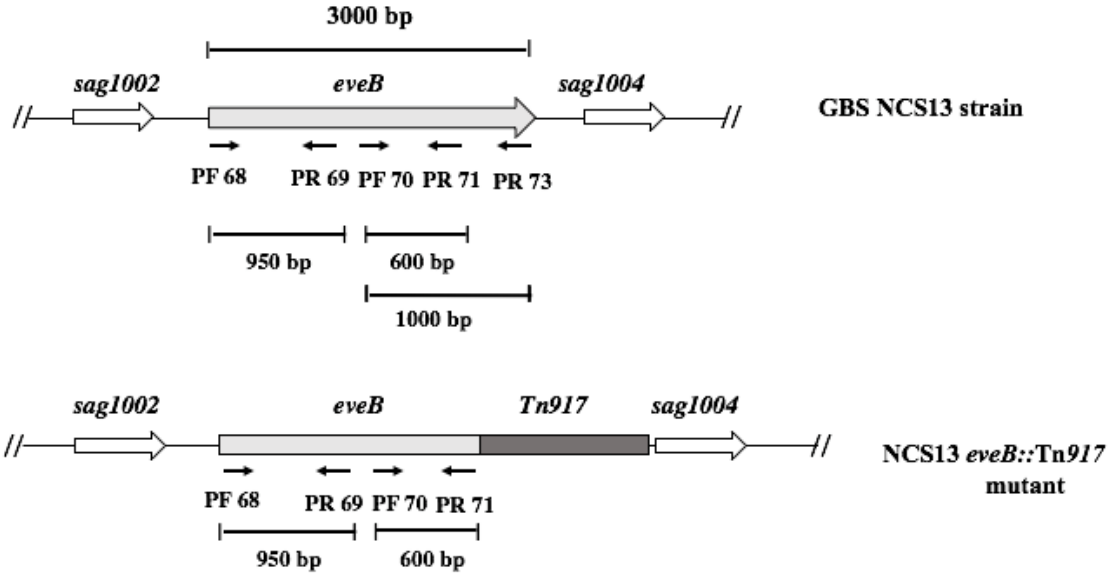
Figure 2.15. Comparison of the predicted amino acid sequences of EveB proteins (876 aa) from different GBS strains. Serotype Ia strain (A909), serotype Ib strain (NCTC8187), serotype II strain (NCTC11079), serotype III strains (COH-I), serotype IV strain (NCTC11930), serotype V strain (2603 V/R), and serotype VI strain (GBS-M002).

A



B

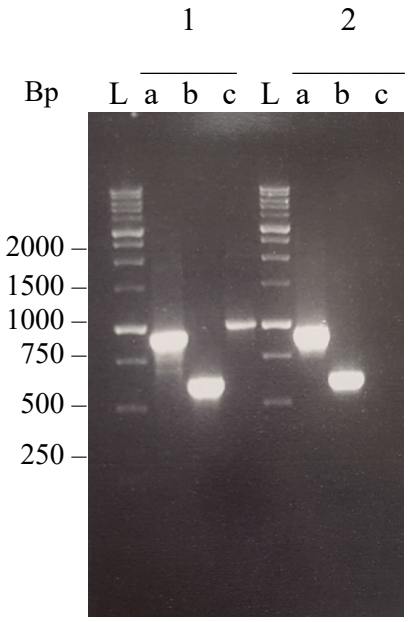


Figure 2.16. Genetic confirmation of the Tn*917* insertion site at the C-terminal domain of the *eveB* gene in the NCS13 *eveB*::Tn*917* mutant strain. (A) Primer design for detection of the Tn*917* insertion site. Arrows indicate primers and lines indicate the size of the amplicons. (B) Agarose gel electrophoresis of the PCR amplification of the *eveB* gene fragments using DNA templates of (1) NCS13 wild type strain and (2) NCS13 *eveB*::Tn*917* mutant strain. The fragments were PCR amplified using the primer pairs, lane a: PF68/PR69, lane b: PF70/PR71, lane c: PF70/PR73. L: 1 Kb DNA ladder (size standard) (FroggaBio).

2.5. References:

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Chapter 3

The External virulence effectors EveA and EveB, of Group B streptococcus are Immunogenic

3.1. Introduction:

Group B streptococcus (GBS), also known as *Streptococcus agalactiae*, is a commensal organism that colonizes the urogenital and gastrointestinal tracts of up to 50% of healthy adults (1). Pregnant women who are vaginally colonized with GBS are at risk for ascending infections or transmission of GBS to the newborn during delivery which can lead to severe neonatal infection, such as septicemia and meningitis (2–4). GBS infection in infants is classified as either early-onset disease (EOD) or late-onset disease (LOD). In EOD, infections occur within the first 6 days of life, while in LOD, the infection occurs after the first week of life and within the first 90 days (5). Prenatal screening for GBS and intrapartum antimicrobial prophylaxis are recommended to prevent EOD that is caused by vertical transmission of GBS to the neonate during delivery. However, these strategies are unlikely to prevent LOD in neonates, where mode of acquisition is not entirely clear as well as other GBS-related disease in non-pregnant adults. GBS has also been associated with high rates of invasive disease in the elderly and in patients with underlying medical conditions, such as patients with diabetes, cancer, cirrhosis and HIV infection (6–8). It is likely vaccination against GBS infection would be the most effective intervention for GBS disease prevention. Furthermore, promising vaccines should be able to induce functionally active antibodies that could cross the placenta and provide protection against neonatal GBS infection.

GBS is serotyped into ten structurally and antigenically unique serotypes based on the capsular polysaccharide (CPS) (9–11). The CPS represents the major virulence factor in GBS: it interferes with phagocytic clearance and helps GBS avoid host defense mechanisms (12). Baker and Kasper (13) proved that transplacental transmission of

maternal GBS antibodies specific to the GBS CPS were able to confer protection to the neonate against GBS infections. Furthermore, many studies have successfully conjugated GBS CPS to carrier proteins for maternal immunization during pregnancy (14–16). However, the occurrence of capsular switching or the presence of non-typeable GBS strains may impact the efficacy of serotype-specific polysaccharide-based vaccines (17,18). Consequently, other surface-exposed and conserved GBS virulence factors may potentially serve as candidates for vaccine development. Of these proteins, the α and β components of the C protein complex, the Rib protein, the surface-immunogenic protein (Sip) and the C5a peptidase have been identified as potential vaccine candidates for their ability to elicit protective immunity in animal models and to induce complement dependent opsonophagocytic killing of bacteria *in vitro* (19–22).

We recently identified two proteins thought to be expressed on the surface of GBS which we designated external virulence effector A (EveA) and external virulence effector B (EveB). Comparison of the predicted amino acid sequences of EveA and EveB proteins from eight different GBS strains clearly indicated that these proteins are highly conserved.

In this chapter, I present data regarding the expression and purification of EveA and EveB. EveB (876 amino acids) is a transmembrane protein with eight transmembrane domains making the protein quite large and potentially difficult to express. Therefore, EveB was designed to be expressed as two fragments designated EveB (up) (amino terminal half of protein that is predicted to extend outside of GBS cell) and EveB (down) (carboxy terminal half that is predicted to extend outside of the GBS cell). EveB(up) and EveB(down) contain 312 and 207 amino acids, respectively. We also determined the

immunogenicity of EveA and EveB proteins using the sera of GBS-infected patients. Western immunoblotting was used to determine if naturally induced antibodies to EveA and EveB could recognize the purified EveA and EveB proteins.

3.2. Material and methods:

3.2.1. Bacterial strains and media:

The GBS NCS13 (23) was cultured in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, MI) at 35°C. *E. coli* BL21 (DE3) {pEXP5-CT/*eveA*}, *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(up)*} or *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(down)*} were grown aerobically with agitation at 200 rpm and 37°C in Luria Bertani (LB) agar or broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, w/v) in the presence of 100 µg/ml of ampicillin.

3.2.2. Designing primers and cloning *eveA* and *eveB* genes:

The DNA sequences of *eveA* and *eveB* were amplified by PCR from GBS NCS13 genomic DNA. EveB is a protein with eight transmembrane domains (Fig. 1.3). Due to the long sequence of the *eveB* gene, with an approximate molecular mass of 99 kDa, only the long external peptide sequences were designed to be expressed as two fragments, EveB(up) (between transmembrane (TM) domains 1 and 2) and EveB(down) (between TM 5 and 6) (Fig. 1.3). The DNA sequence of *eveA*, *eveB(up)* and *eveB(Down)*, including the start codon and excluding the stop codon (recombinant EveA, EveB(up) and EveB(down) were expressed as C-terminal hexahestidyl tagged proteins), were amplified using the primer pairs P64/P65, P68/P69, P70/P71, respectively (Table 3.1). The DNA sequences of *eveA, eveB(up) and eveB(down)* were then ligated into the pEXP5-CT/TOPO cloning vector using a TOPO cloning reaction (ThermoFisher) to produce recombinant vectors: pEXP5-CT/*eveA*, pEXP5-CT/*eveB(up)* and pEXP5-CT/*eveB(down)*, respectively. Recombinant plasmids were transformed into one shot

Topo *E. coli* by heat shock. The transformants were recovered on LB agar containing 100 µg/mL of ampicillin. This was followed by plasmid DNA extraction using Qiagen mini plasmid prep purification kit (Qiagen, Mississauga, ON, Canada). The resulting clones were screened by PCR amplification of *eveA*, *eveB(up)* and *eveB(Down)* genes from the plasmids to confirm the presence of the *eveA*, *eveB(up)* and *eveB(Down)* insert. To confirm the correct orientations of *eveA*, *eveB(up)* and *eveB(Down)* genes and to confirm that each gene was cloned in-frame with the poly-histidine tag, PCR amplifications using the forward primers of each gene and the reverse primer pT7 Term Reverse was performed.

3.2.3. EveA, EveB(up) and EveB(Down) protein expression:

Recombinant plasmids pEXP5-CT/*eveA*, pEXP5-CT/*eveB(up)* and pEXP5-CT/*eveB(down)* were then transformed into *E. coli* BL21 DE3 (ThermoFisher) following the manufacturer's recommendations. The cells were grown for 16 h in 37°C at 250 rpm in 5 mL LB medium containing ampicillin (100 µg/mL). After overnight incubation, 100 mL of LB medium containing ampicillin (100 µg /mL) was added to the initial culture and then incubated in the same growth conditions as those mentioned above. Once a 0.5–0.7 OD_{600} had been reached, the cultures were induced with IPTG (1 mM final concentration) and incubated for 4 h (37°C, 250 rpm) to express the cloned proteins.

3.2.4. Solubilizing and purification of EveA, EveB(up) and EveB(down) proteins:

Bacterial cells were harvested by centrifugation at 5000 x g for 20 min at 4°C. Extraction of the soluble recombinant proteins, r-EveA, r-EveB(up) and r-EveB(down),

from *E. coli* BL21 (DE3) {pEXP5-CT/*eveA*}, *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(up)*} or *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(down)*} cells, respectively, was performed using 1% sarkosyl (Sigma). Recombinant proteins r-EveA, r-EveB(up) and r-EveB(down) were expressed inside the bacterial cells as insoluble proteins. Cell pellets from 100 mL bacterial cultures were suspended with 10 mL of lysis buffer A (50 mM NaH_2PO_4, 500 mM NaCl, pH 7.0) and with 1g of autoclaved 0.1 mm glass beads, vortexed for 2 minutes and then the lysed bacterial suspension was collected, without the glass beads, into clean tubes. The lysed bacterial suspensions were then centrifuged to pellet the cellular debris and the bacteria supernatants were removed. The cell pellets then were re-suspended with 5 mL of buffer A containing 1% sarkosyl. Cell debris was removed by centrifugation at 12,000 x g for 20 min at 4°C, and the collected supernatants were incubated with 1 mL Ni-NTA (Qiagen) agarose beads with some agitation at 4°C to allow the His-tagged protein to bind to the beads. The beads was transferred to a gravity flow column (Bio-Rad, Hercules, California, USA) with an end-cap in place and allowed to settle out of suspension. The end-cap was removed and the buffer allowed to drain until it reached the top of the beads. The columns were washed with wash buffer (50 mM NaH_2PO_4,500 mM NaCl, 5mM Immidazole pH 7) and the His-tagged proteins were eluted by adding elution buffer (50 mM NaH_2PO_4, 500 mM NaCl and 250 mM imidazole, pH 7.0) to the column.

3.2.5. Serum samples:

Sera of patients previously naturally infected with GBS and stored at -70 °C was used to determine if antibodies to EveA and EveB occurred in patients with invasive GBS

disease. Fifteen serum samples were used in total. Control sera (*n*=3) was used from healthy adult donors without a history of GBS infection, and 11 serum samples from adult patients with a history of GBS infection were assayed.

3.2.6. SDS-PAGE and Western immunoblotting:

To prepare the whole-cell (WC) lysates used for SDS-PAGE, a pellet of 1 mL volume of IPTG induced or un-induced *E. coli* cultures were added to 100 µl of SDS buffer (0.2 M Tris-HCl [pH 6.8], 1% SDS, 2% mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and then boiled for 5 min. After a brief centrifugation, 10 µl of supernatant was applied to a 10% SDS–PAGE gel. For the purified proteins, 10 µl (0.2 µg/µl) of eluted proteins was added to 10 µl of 5X SDS buffer, which was then applied to 10% SDS-PAGE without boiling. After electrophoresis, the proteins were stained with Comassie brilliant blue or transferred to nitrocellulose membranes for Western immunoblotting. The membranes were blocked in PBS containing 5% skim milk for 1 h. After washing with PBS containing 0.1% Tween 20 (PBS-T), the membranes were incubated either with anti-histidine antibody produced in rabbit (1:500) (GenScript) or with human sera (1:200) diluted in 5% skim milk for 18-24 h at 4 °C. For the Far Western blot, the membrane was first incubated with 25 µg/mL of r-GBS-PGK (expressed as previously discussed in chapter 2) diluted in PBS-T for 18-24 h at 4°C and then probed with anti-GBS-PGK antibodies (1:1500 in PBS-T) for 3 h at 4°C. Next, the membranes were washed three times with PBS-T and then incubated for 1 h in alkaline phosphatase-conjugated goat-anti-rabbit antibodies (Sigma) diluted 1:10,000 or alkaline phosphatase-conjugated goat-anti-human IgG antibodies (Abcam) diluted 1:5000.

Finally, the membranes were washed three times with PBS-T and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) commercial liquid substrate system (Promega) to visualize reaction.

3.2.7. Bioinformatics analysis:

Bioinformatics analysis was performed on the DNA and protein sequences using the BLAST program on the National Center for Biotechnology Information (NCBI) website. BLAST searches with the genome database of GBS 2603 V/R was also performed using the genomic BLAST program on the NCBI website.

3.3. Results:

3.3.1. Expression of EveA, EveB(up) and EveB(Down):

To facilitate the efficient expression of recombinant EveB protein, the protein was designed to be expressed as two fragments: EveB(up) and EveB(down) (Fig. 3.1). The sequences encoding EveA, EveB(up) and EveB(down) lacking the stop codon were PCR amplified and cloned into an expression vector pEXP5-CT creating pEXP5-CT/*eveA*, pEXP5-CT/*eveB(up)* and pEXP5-CT/*eveB(down)*, respectively, for protein expression as C-terminal hexahistidyl (6XHis) (Fig. 3.2). Small scale protein expression analysis was performed and SDS-PAGE analysis of the expression cultures revealed r-EveA, r-EveB(up) and r-EveB(down) expression in *E. coli* BL21 (DE3) {pEXP5-CT/*eveA*}, *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(up)*} or *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(down)*} cell lysates, respectively, after overnight growth (Fig. 3.3). In contrast, no protein expression was observed in *E. coli* BL21 (DE3) alone (Fig. 3.3). It was observed that induction after 4 h with IPTG (1 mM final concentration) gave the same yield of expressed proteins from non-induced culture (Fig. 3.4; data shown for EveA only). Therefore, protein extraction was performed from overnight cultures growing without induction. The basal level of protein expression is likely due to a leaky plasmid. As it is common in all BL21 (DE3) cell lines, there is always some basal level expression of T7 RNA polymerase.

3.3.2. Solubilization of r-EveA, r-EveB(up) and r-EveB(down) by 1% Sarkosyl:

To test the solubilization method of sarkosyl, overnight growth cultures of *E. coli* BL21 (DE3) {pEXP5-CT/*eveA*}, *E. coli* BL21 (DE3) {pEXP5-CT/*eveB(up)*} or *E. coli*

BL21 (DE3) {pEXP5-CT/*eveB(down)*} cells were harvested and the cell pellets were collected for the analysis. Pellets were lysed mechanically with glass beads suspended in lysis buffer. After the centrifugation, supernatants were collected to analyze whether or not the expressed proteins were expressed as a soluble protein. Cell pellets were then re-suspended in lysis buffer containing 1% of sarkosyl. Both supernatant and solubilized pellets with sarkosyl were loaded into 10% SDS-PAGE. The r-EveA, r-EveB(up) and r-EveB(down) were determined to be expressed as insoluble proteins (Fig. 3.5).

3.3.3. Purification of r-EveA, r-EveB(up) and r-EveB(down):

The r-EveA, r-EveB(up) and r-EveB(Down) were purified by affinity chromatography using Qiagen systems Ni-NTA resin and analyzed by SDS-PAGE. As shown by Comassie-blue stained SDS-PAGE, the purified EveA, EveB(up) and EveB(down) migrated at the expected locations, 43 kDa, 30 kDa and 20 kDa, respectively (Fig. 3.6 A). Aliquots of r-EveA, r-EveB(up) and r-EveB(down) from elution fractions were analyzed by Western blot for reactivity with anti-polyhistidine antibody. In addition to the r-EveA, r-EveB(up) and r-EveB(down), smaller protein bands showed reactivity with the anti-polyhistidine antibody (Fig. 3.6 B), indicating that these were likely degradation fragments of r-EveA, r-EveB(up) and r-EveB(down), and do not represent other protein species.

3.3.4. EveA, EveB(up) and EveB(down) are immunogenic:

The immunogenicity of EveA, EveB(up) and EveB(down) proteins in humans with GBS infection was investigated. To confirm that anti-EveA, anti-EveB(up) and anti-

EveB(down) antibodies are detectable in patients previously infected with GBS, Western blot of purified r-EveA, r-EveB(up) and r-EveB(down) proteins probed with human patient sera confirmed the presence of protein bands at 43kDa, 30 kDa and 20 kDa, respectively, in the purified proteins preparation (Fig. 3.7). Antibodies that recognized the 43kDa, 30 kDa and 20 kDa of r-EveA, r-EveB(up) and r-EveB(down) proteins, respectively, were present only in the sera from GBS infected patients and not in the sera from the healthy control individuals (data not shown). These results confirmed that EveA, EveB(up) and EveB(Down) of GBS are immunogenic in humans.

3.3.5. Binding of r-GBS-PGK to EveA and EveB:

The surface expression of GBS-PGK has previously been shown to be significantly reduced on the surface of NCS13 *eveA::erm*, NCS13 *eveB::erm* and NCS13 *eveB::*Tn*917* mutant strains (discussed in chapter 2). In order to determine whether EveA and EveB on the surface of GBS act as a binding ligand for surface-expressed GBS-PGK, the recombinant GBS-PGK was assayed for the ability to bind to r-EveA, r-EveB(up) and r-EveB(down) using a Far Western blot. A 30 kDa band corresponding to rGBS-PGK binding to EveB(up) was clearly visible, while only a faint 43 kDa band corresponding to rGBS-PGK binding to EveA could be seen (Fig. 3.8). It was not possible to confirm the interaction of rGBS-PGK with EveB(down) using a Far Western as no visible band corresponding to the interaction was observed (Fig 3.8). Plasminogen was used as a positive control since the interaction of rGBS-PGK with plasminogen was previously investigated (24). A 100 kDa band corresponding to rGBS-PGK binding to plasminogen was clearly visible (Fig 3.8).

3.4. Discussion:

Despite the introduction of prenatal prophylaxis, GBS still constitutes the main cause of neonatal morbidity and mortality (25). In addition, the use of intrapartum prophylaxis led to increased emergence of antimicrobial resistance (26). Consequently, it was important to either determine ways of preventing GBS infection or develop efficient vaccines.

Conjugate-capsular polysaccharide vaccines are now being tested in clinical trials (27,28). However, challenges to the eradication of GBS disease will still exist due to the presence of non-typeable GBS strains that can cause disease (18) and the occurrence of capsular switching in GBS strains (17), thereby enabling evasion of host immunity conferred by vaccination. Thus, development of vaccines targeting immunogenic proteins may prove to be vital.

Previously, we identified two proteins, external virulence effector A (EveA) and external virulence effector B (EveB), which are involved in GBS virulence expression, as well as GBS invasion into HeLa cells *in vitro.* We determined that the GBS *eveA::erm* and GBS *eveB::erm* mutants have lost the typical antiphagocytic activity associated with the wild type strain. Comparison of the predicted amino acid sequences of EveA and EveB proteins from eight different GBS strains indicated that these proteins are highly conserved.

In this study, we were able to express, solubilize and purify EveA and the two fragments of EveB, EveB(up) and EveB(down), proteins. The proteins were extracted from the insoluble pellets as natively folded proteins using 1% sarkosyl. Sarkosyl or *N*-lauroylsarcosine is a mild and non-denaturing detergent typically used for protein

purification (29,30). We were also able to demonstrate the immunogenicity of EveA, EveB(up) and EveB(down) proteins. Antibodies from GBS infected patients' sera were able to recognize purified protein bands belong to EveA, EveB(up) and EveB(down) in a western blot assay.

Generally, identification of immunogenic proteins is usually based on animal immunization using cell surface components, followed by immune testing. However, in our study, we used human sera containing naturally induced antibodies after GBS infection. Results of our study showed that immunogenic proteins in GBS are not limited only to the cell surface proteins, such as Sip, Rib or C5a (20,21,31), but that there are other potential immunogenic proteins. We used the natural human infections by GBS to show that the GBS EveA and EveB protein antigens are involved in the immune response.

Additionally, in this study, we took advantages of having purified recombinants proteins of EveA, EveB(up) and EveB(down) to investigate the direct interactions between the purified proteins and rGBS-PGK. In chapter 2, we showed that surface expressed GBS-PGK was significantly reduced on the surface of *eveA* and *eveB* mutants of GBS NCS13 strain. It was not confirmed whether EveA and EveB on the surface of GBS could act as a binding ligand for surface expression GBS-PGK. Here, we determined that rGBS-PGK bound strongly to r-EveB(up) with some binding to r-EveA. There was no visible band corresponding to rGBS-PGK interaction with EveB(down). This identification suggests that GBS-PGK after secretion is attach to the bacterial surface through binding to EveA and EveB proteins on GBS.

In conclusion, our preliminary results suggested that the GBS proteins involved in GBS virulence could potentially be considered as candidate's vaccine components against the GBS infection. However, further work is required to show if the human antibodies are protective in a human infected with invasive GBS disease.

Table 3.1. Primers used in this study. The underlined sequences indicate the start codon.

Primer	**Sequence (5'—3')**	**Description**
P64	ACC ATG TCA AAT ATA ATA ACT TA	Forward primer *eveA*
P65	CTT AGT CTC TCT GCT ATT ATC	Reverse primer for *eveA*
P68	ACC ATG AAG GTC TCA GCA CCT AAT AT	Forward primer for *eveB(up)*
P69	ATT ACT GAT TTT AGA AGT GCT ATT	Reverse primer for *eveB(up)*
P70	ACC ATG CAA TCC TCC TTA AAG CAA AC	Forward primer for *eveB(Down)*
P71	ATC TAA AGA ACG CAC AAC GG	Reverse primer for *eveB(Down)*
PT7	ATC CGG ATA TAG TTC CTC CTT TC	Reverse primer for T7 promoter on pEXP5-CT

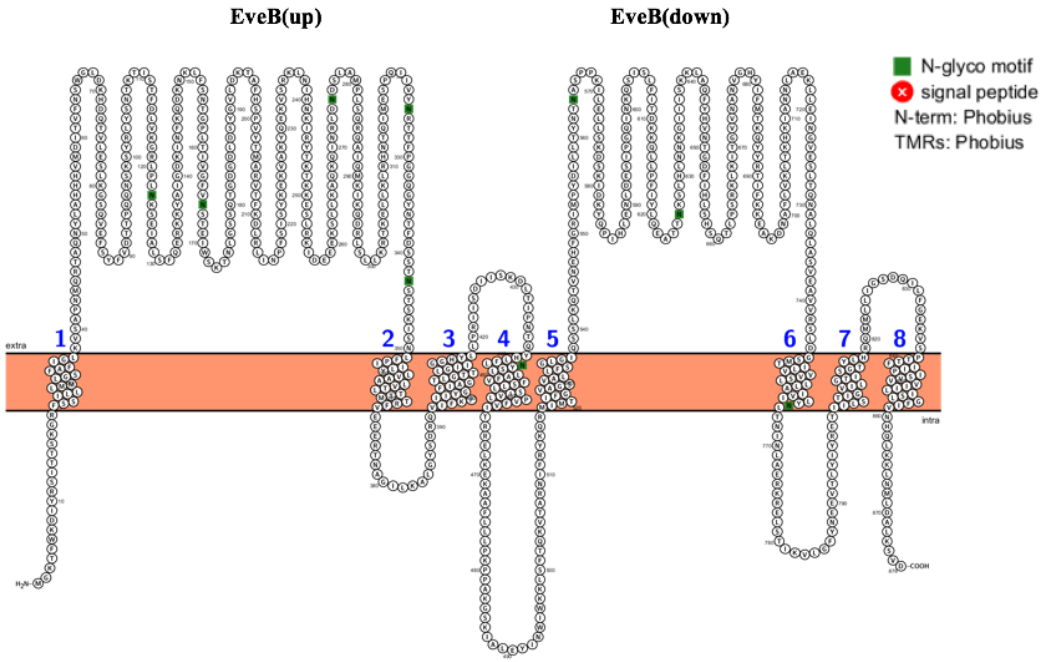

Figure 3.1. Predicted transmembrane topology for the GBS EveB protein. The model was obtained by using the PROTTER program (32). The EveB (up) and EveB(down) fragments are located outside the cytoplasmic membrane and were targeted for protein expression. The EveB (up) contains 312 aa (30 kDa) and EveB (down) contains 207 aa (20 kDa).

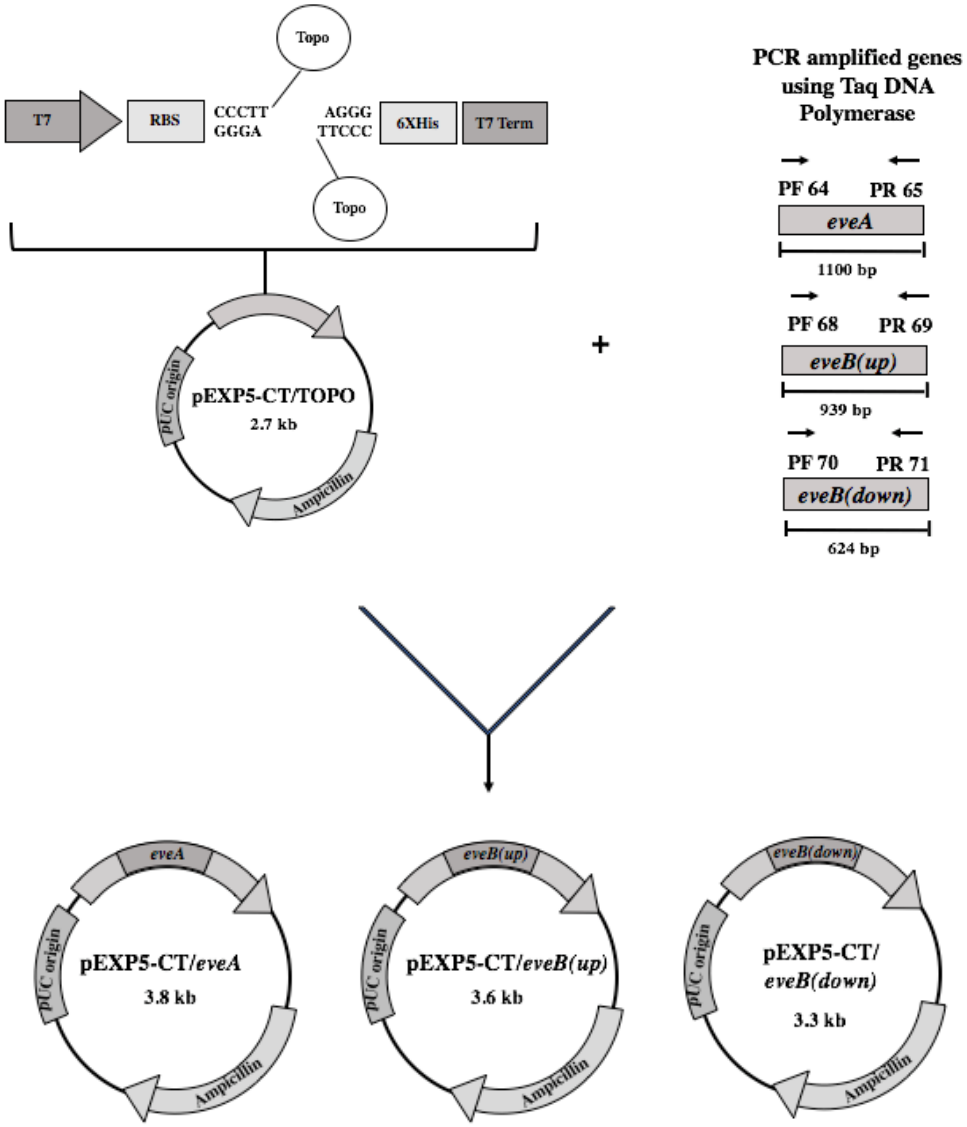

Figure 3.2. Diagram representing the construction of pEXP5-CT/*eveA*, pEXP5-CT/*eveB(up)* and pEXP5-CT/*eveB(down).*

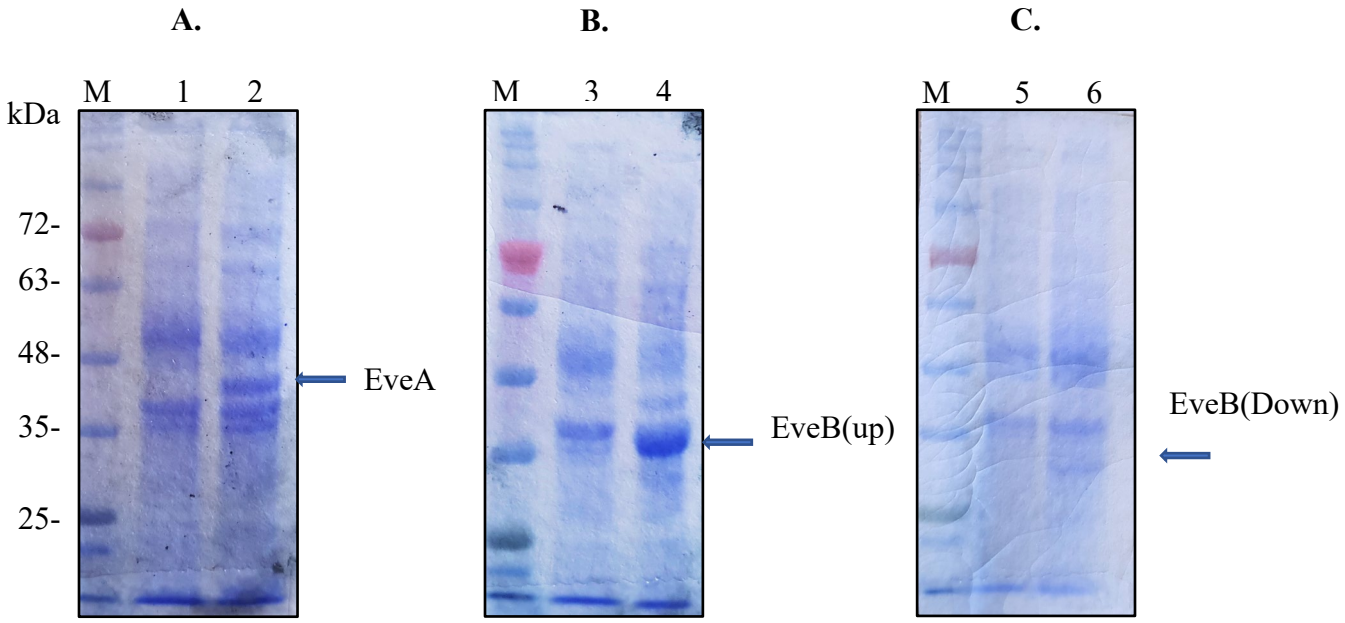

Figure 3.3 Expression of recombinant EveA, EveB(up) and EveB(down) fusion proteins from the bacterial expression system. SDS-PAGE (10%) with comassie-blue stained gels of recombinant EveA (A), recombinant EveB(up) (B) and recombinant EveB(down) (C) proteins. Lanes 1, 3, 5: cell lysates of BL21 (DE3) *E. coli*, lane 2: cell lysates of BL21(DE3) with pEXP5-CT/*eveA* vector, lane 4: cell lysates of BL21(DE3) with pEXP5-CT/*eveB(up)* vector, Lane 6: cell lysates of BL21(DE3) with pEXP5-CT/*eveB(down)* vector. M: protein marker (BLUelf prestained protein ladder (FroggaBio, Toronto, ON., Canada).

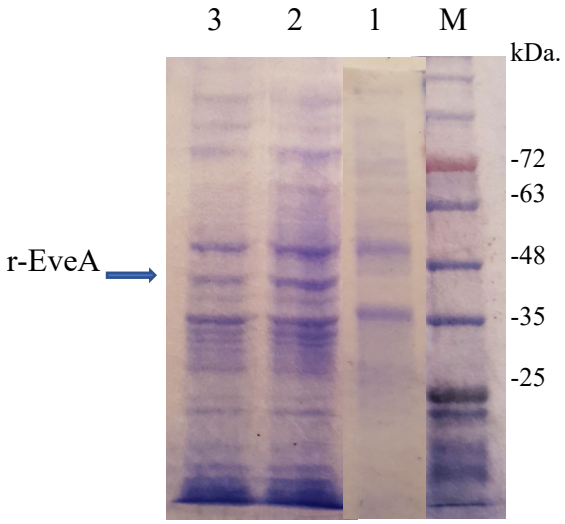

Figure 3.4. Optimizing test for the expression of EveA. Expression of transformed *E. coli* BL21 (DE3) cells with pEXP5-CT/*eveA* were un-induced or induced for 4 h at 37°C with IPTG. Comassie blue stained SDS-PAGE shows that the induced and un-induced transformed cells have the same yield of r-EveA expression. Lane 1: whole cell lysates of *E. coli* BL21 (DE3) without expression vector, lane 2: whole cell lysates of un-induced *E. coli* BL21 (DE3) cells with pEXP5-CT/*eveA*, lane 3: whole cell lysates of induced *E. coli* BL21 (DE3) cells with pEXP5-CT/*eveA*. Lane M: Protein marker.

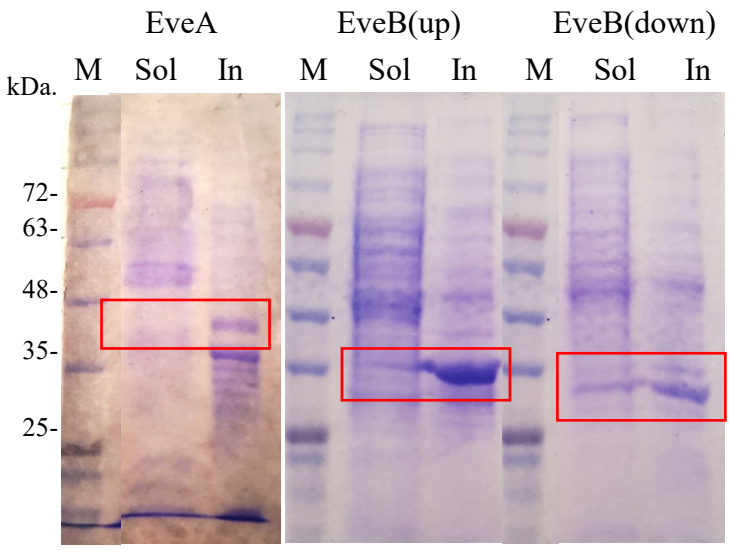

Figure 3.5. Solubilization of EveA, EveB(up) and EveB(down) proteins from pellets using 1% sarkosyl. The proteins expressed by the transformed cells were grown overnight at 37°C. The SDS-PAGE shows that the EveA (43 kDa), EveB(up) (30 kDa) and EveB(down) (20 kDa) fusion proteins are expressed in the insoluble fraction. Lane M: protein marker, Sol: soluble, In: insoluble.

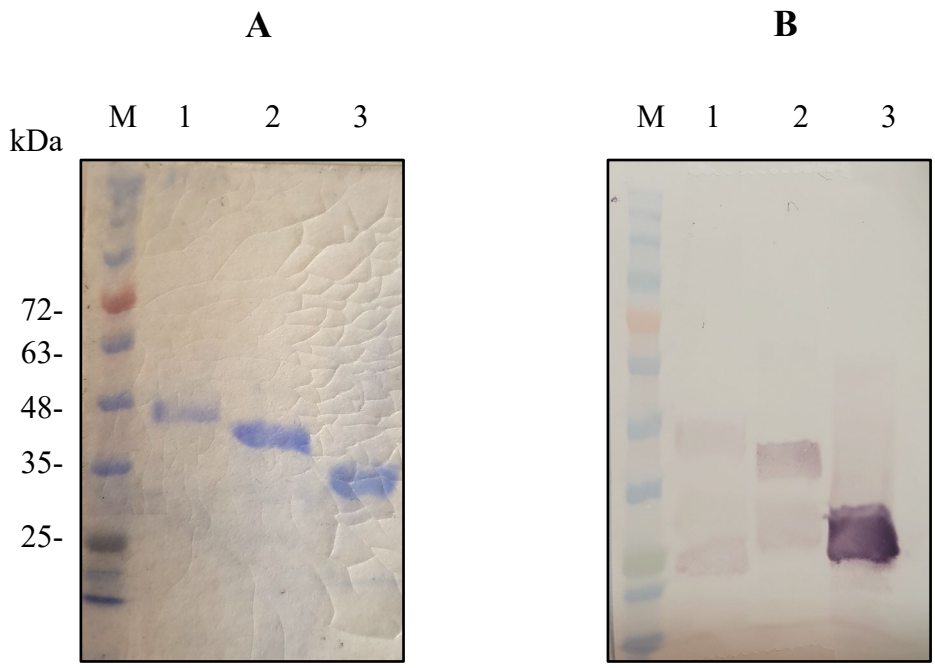

Figure 3.6. Purification of recombinant EveA, EveB(up) and EveB(down). (A) Comassie-blue stained SDS-PAGE (10%) gel of purified EveA, EveB(up) and EveB(down) proteins. (B) Western Blot analysis of EveA, EveB(up) and EveB(down) using anti-Histidine antibody. Lane 1: r-EveA, lane 2: r-EveB(up), lane 3: r-EveB(down), lane M: protein marker.

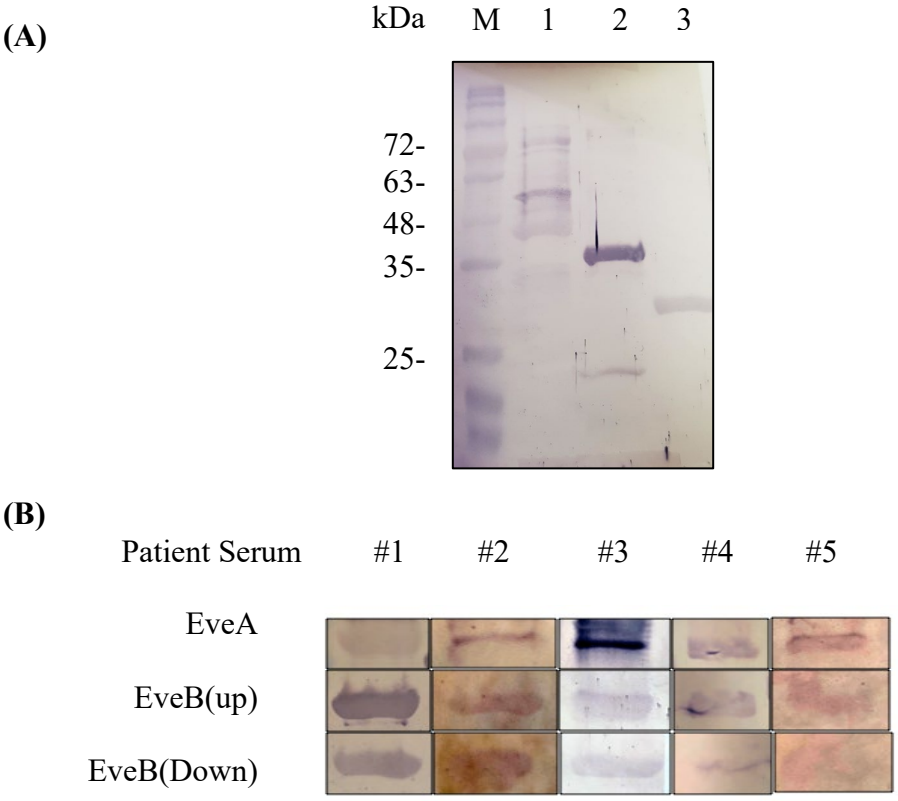

Figure 3.7. Western blot analysis of the recombinant from EveA, EveB(up) and EveB(down) with patients' human anti-sera. (A) Blot probed with human serum from one patient. Lane 1: r-EveA, lane 2: r- EveB(up), lane 3: r-EveB(down), lane M: protein marker. (B) Cropped bands for EveA, EveB (up) and EveB(down) from each blot probed with a specific patient serum.

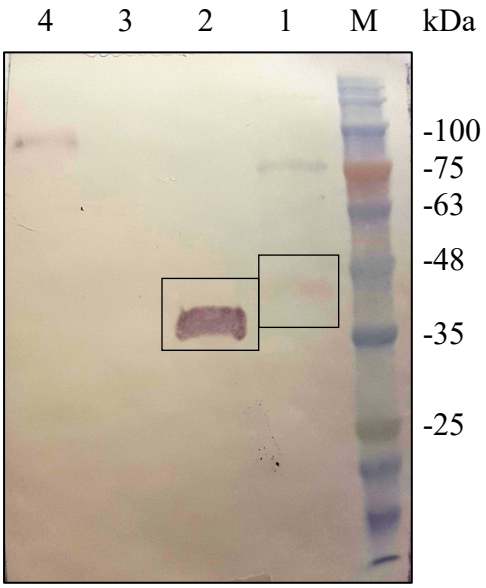

Figure 3.8. Interaction of r-GBS-PGK with r-EveA and r-EveB(up) visualized using a Far Western blot. The r-EveA (lane 1), r-EveB(up) (lane 2), r-EveB(down) (lane 3) and plasminogen (0.5 µg) (lane 4) (positive control) were separated by 10% SDS-PAGE and western transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 h at room temperature, incubated for 16 h at 4°C with r-GBS-PGK (25 µg/mL) and probed with anti-GBS-PGK antibodies (1:1500 in blocking buffer) for 3 h at 4°C, followed by alkaline phosphatase conjugate anti-rabbit-IgG (1:10 000 in blocking buffer) for 1 h at room temperature. The membrane was developed using BCIP/NBT for approximately 15 minutes before stopping with 3 changes of distilled water. Lane M is a protein marker.

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Chapter 4:

EveA and EveB, external virulence effectors of GBS, play roles in invasion of HeLa cells

4.1. Introduction:

GBS are opportunistic commensal pathogens that can colonize the intestinal and urogenital tract of healthy women (1). GBS remains a significant cause of bovine mastitis and human neonatal mortality and morbidity (2–4). Transmission of GBS from colonized mothers to their newborn can occur either by ascending infection *in utero* or during the birthing process through neonatal aspiration by the baby of contaminated vaginal fluid (1). GBS colonization and invasion of host barriers are dependent on their ability to adhere to host cells. Numerous GBS virulence factors facilitating adherence or invasion within the host have been characterized. These virulence factors include fibrinogen-binding proteins (Fbs), the laminin-binding protein (Lmb), the group B streptococcal C5a peptidase (ScpB), the streptococcal fibronectin-binding protein A (SfbA), the GBS immunogenic bacterial adhesin (BibA), and pili (5–12).

Recently, we identified two genes, external virulence effector A (*eveA*) and external virulence effector B (*eveB*) that are involved in GBS virulence expression and GBS invasion into HeLa cells *in vitro*. In addition, GBS *eveA::erm* and GBS *eveB::erm* mutants lost the typical anti-phagocytic activity associated with the wild type strain. The *eveA* and *eveB* genes are highly conserved in GBS sequenced genomes, and the EveA and EveB proteins are surface-localized on GBS. We also observed that EveA and EveB proteins are immunogenic, and in Western blot analyses, the antibodies from GBS-exposed patients could recognize the purified EveA and EveB proteins.

In this study, polyclonal rabbit antibodies against EveA and EveB purified proteins were produced. We studied the neutralizing activities of produced antibodies in GBS (i) invasion into HeLa cells and (ii) antiphagocytic activity in human blood. We also

investigated the adherence properties of these two isolated proteins, EveA and EveB, to HeLa cells and their effect in inhibiting the bacterial invasion. I also explored the bactericidal effect of EveA and B on GBS.

4.2. Material and Methods:

4.2.1. Cell cultures and growth condition:

GBS NCS13 strain was cultured in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, MI) at 35°C.

The human epithelial cell line HeLa 229 (ATCC CCL-2.1), obtained from the American Type Culture Collection (ATCC; Manassas, VA), was grown in OPTI-MEM I reduced serum medium supplemented with 4% fetal bovine serum (FBS; Gibco BRL, Burlington, ON). HeLa cell monolayers placed in 96 well plates (Corning Costar TC-Treated multiple well plates, Sigma-Aldrich, USA) were maintained in OPTI-MEM media supplemented with 4% FBS. Cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere to approximately 80-100% confluency for adhesion or invasion experiments.

4.2.2. EveA and EveB purified proteins and antibodies:

Purified recombinant proteins and polyclonal antibodies were ordered from Genscript (Piscataway, New Jersy, USA). The peptide sequences for the proteins are as follows:

EveA:

MSNIITYLKNNSNLTFDELALNDVDILCLNEFGYISFEKLINTTEMKSVLVCELYH
EYLQTMAKSYSFMFTSQRHDLCQLMMTSKRFKNLTLSYYRAEISLEFEKQFAAM
VFTIPNINYHQVVFRGTDANLIGWKEDFKLTYMREISAHRSAIKYLNTILPYFDKV
VLSGHSKGGNLALYAAMFTKPDLKAKIDLIWLIDSPGLQKTLLPTTEYKTTKQKC

IRLLPEESIVGMMLYSDIEPLIISSNARGILQHDVTTWEIQEPAILKTGTGLSLKSICF
EKTFQQWMAELKSQERKLFFDLLFDSFLSSGVSSLDDFNLASRAKMMKAFHSFR
ELDDDKKRLFNKSLKLLVTIFWGAYHDNSRETK

EveB(up):

KVSAPNMQRTAQNYLAHHHVMDITVFNSWGLDKHDQTVLESLKGSQVEFSYFV
DTTPQQNSKSYRLYSNTKTISTFDLVKGRLPLNKSEIALSFQERKKYAIGDKINFK
QDKNKLFSNTGPLTIVGFVNSTEIWSKTNLGSSQTGDGDLDSYGVLDKTAFHSPV
YTMARVTFKDLRLINPFSISYKEKVAKYQEKVSRKLNIHNKIRYTKTKKESLRKID
EEEKSLLKAQKQINRLDNDSLAMPLSQRQAIQMKIKQDRLSLLKRTKELLKLRHN
TQIMESPQIIVYNRTTFPGGQGYNTFDSSTNSTSKISN

EveB(down):

QSSLKQTVNEHFGRIMPYDILLTYNTNASPPKILELLSKDSKIDKYQPIHLENLDES
IPGQINKQSISLFITDKKQLLPFIYLQEATTNKSLHLNNKGIIISKKLAQFYHVNTGD
FIHLSHSQTLPSRKLKITGVVNANVGHYIFMTKQYYRTIFKKEAKDNAFLVKLTK
HKIANNLAEKLLEINGVESLTQNALQLASVEAVVRSLD

These peptide sequences obtained from GBS NCS13 strains were sent to Genscript to generate purified 6XHis-tag proteins. The purified proteins were used to immunize New Zealand rabbits to produce polyclonal antibodies. The EveB protein is a transmembrane protein with 8 transmembrane domains and a molecular mass of 99.4 kDa. EveB was designed to be expressed as EveB(up) and EveB(down), with the long external peptide sequences facing outward on the cell membrane (Fig.1). The binding of polyclonal rabbit antibodies to the purified proteins was confirmed by Western blot (immunoblot). The

purified EveA, EveB(up) and EveB(down) proteins were resolved by SDS-PAGE, electro-transferred to nitrocellulose and then Western blotting was performed.

4.2.3. Invasion assay:

Using invasion assay modified from previous study (13), the ability of EveA and EveB to inhibit the invasion of GBS NCS13 to HeLa cells was determined. After incubating the monolayer of HeLa cells with the purified EveA or EveB(up) or EveB(down) for 1h at 37°C using concentrations 2μg, 5μg and 10μg, the cells were washed once with 1X PBS. One ml of GBS culture grown overnight in TH broth was washed once with 1X PBS, and a 0.5 McFarland standard of GBS cultures was made and then diluted 1:100 in TH broth. One hundred microliters (1.5 X 10^5 cfu) of 1:100 dilutions were added to the HeLa cell monolayers. The 96-well plate was centrifuged at 100 g for 5 minutes at room temperature and incubated at 37°C in 5% CO_2 for 2 h. The monolayers were washed 3 times with 1X PBS and incubated for 2 h with fresh media containing 5 μg/ml of penicillin and 100 μg/ml of gentamicin to kill any remaining extracellular organisms. The monolayers were washed again with PBS, trypsinized, lysed with 0.2% Triton X-100, and plated on TH agars for quantitation of intracellular CFU. The relative percent invasion was calculated as: Number of CFU invaded into HeLa cells (treated with purified proteins) divided by number of CFU invaded into HeLa cells (non-treated with purified proteins), multiplied by 100.

For testing the inhibition of GBS invasion by anti-serum, 100 μl of 1:100 bacterial dilution of a 0.5 McFarland standard was incubated with different dilutions (1:100, 1:1000, 1:10000) each of anti-EveA, anti-EveB(up) and anti-EveB(down)

antibodies. Serum from an unimmunized rabbit was used as a negative control. These suspensions were incubated for 1 h at 37°C with shaking, and then the bacterial suspensions were added to the HeLa cell monolayers and incubated at 37°C for 2 h. The subsequent steps were done as described above.

4.2.4. Adhesion assay:

To determine whether the addition of purified EveA, EveB(up) or EveB(down) proteins to HeLa cells would inhibit bacterial adhesion, adhesion assays were performed. HeLa cell monolayers in tissue cultures within 96-well plates were incubated for 1h at 37°C with 10 μg/ml of each of the purified proteins diluted in OPTI-MEM/FBS. As a control, 10 μg/ml of bovine serum albumin (BSA) (Sigma) or no protein was used as a non-inhibiting control protein. After incubation, the wells were washed three times with pre-warmed 1X PBS. GBS cultures grown overnight in TH broth were washed once with 1X PBS and a 0.5 McFarland standard of GBS cultures was made and then diluted 1:100 in TH broth. 10 μl (1.5 X 10^4 cfu) of 1:100 dilutions were added to the monolayer. The plates were centrifuged at 100 g for 5 minutes at room temperature and incubated at 37°C in 5% CO_2 for 1 h. The monolayers were washed five times with 1X PBS and trypsinized for five minutes at 37°C. 100 μl of 1X PBS was added to each well to re-suspend the cells with PBS, and an aliquot of 50 μl of each well was plated on TH agars for quantitation of extracellular CFU. The relative percent adhesion was calculated as: Number of CFU bound onto HeLa cells treated with purified proteins divided by number of CFU bound onto HeLa cells not treated with purified proteins, multiplied by 100.

4.2.5. Opsonophagocytic killing assay:

To examine the neutralization activity of polyclonal antibodies produced against EveA, EveB(up) and EveB(down) in inhibiting the killing of the GBS NCS13 parent strain by fresh human blood, a protocol using freshly heparinized human whole blood was used. One ml of overnight GBS culture grown in TH broth was washed once with 1X PBS and a 0.5 McFarland standard of GBS cultures was made and then diluted 1:10,000 in TH broth. In 1.5 ml Eppendorf tubes, 100 µl of 1:10,000 bacterial dilution, containing approximately 10^3 CFU, was pre-incubated with different dilutions (1:100, 1:1000, 1:10000) each of anti-EveA, anti-EveB(up) and anti-EveB(down) antibodies. Serum from an unimmunized rabbit was used as a negative control. These suspensions were incubated for 1 h at room temperature with shaking. After incubation, 500 µl of freshly heparinized whole blood was added to each Eppendorf tube and incubated at 37°C under gentle rotation. Following the 3 h of incubation, 300 µl aliquots of the mixture were plated on blood agar plates (BAP) (Dalyn Biologicals, Calgary, Canada) to determine the number of CFU after growth in human blood. Growth of GBS in human blood is expressed as a relative percent of survival at the end of the incubation period by calculating the number of CFU obtained from blood incubated in the presence of antibodies divided by the number of CFU obtained from blood in the absence of antibodies addition, multiplied by 100. Whole blood in this experiment was collected from healthy volunteers in 6 ml Sodium Heparinized Vacutainer tubes (BD, Oakville, Ontario, Canada).

4.2.6. EveA, EveB(up) and EveB(down) binding by HeLa cells:

To assess EveA, EveB(up) and EveB(down) protein binding to HeLa cells, the following two binding assays were used:

4.2.6.1. Purified proteins binding to HeLa cells immobilized in microtiter plate wells:

HeLa cell monolayers were grown in tissue culture 96-well plates and incubated for 1h at 37°C with 2 µg/ml of EveA, EveB(up) and EveB(down) proteins diluted in OPTI-MEM/FBS. BSA (2 µg/ml) was used as a negative control. Following the incubation, the immobilized cells were washed three times with 1X PBS and incubated for 1 h at 37°C with each corresponding antibody, anti-EveA, anti-EveB(up) or anti-EveB (down) diluted (1:1000) in OPTI-MEM/FBS. After washing with PBS, a further 1 h incubation was performed in the presence of goat anti-rabbit IgG conjugated to Horseradish Peroxidase (Bio-Rad, Hercules, California, USA) (1:3000) diluted in OPTI-MEM/FBS. Following another wash, the plates were then treated with 100 µL of TMP (Sigma-Aldrich). After a 5-10 minute incubation at room temperature, the color reaction was stopped with 100 µL of 2M sulphuric acid (H_2SO_4), and the absorbance was measured at 450 nm with a Revelation 4.25 microplate reader.

4.2.6.2. Purified proteins binding to HeLa cells in suspensions:

One milliliter of detached HeLa cell suspensions (~ 2 X 10^5 cells), each treated with purified EveA, EveB(up) or EveB(down) proteins (2 µg/ml) or with BSA (2 µg/ml) as a control, were incubated for 1 h at 37°C with gentle shaking. The cells were then centrifuged and the unbound proteins present in the supernatant were removed. The cell

pellets were washed with PBS, centrifuged, re-suspended with 100 µl of SDS buffer (250 mM Tris. HCl, pH 6.8, 10% SDS, 30% Glycerol, 10 mM Dithiolthreitol, 0.05% Bromophenol Blue), and boiled for 5 minutes. Following a brief centrifugation to pellet insoluble material, 10 µl of each sample was loaded onto a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes for Western immunoblotting. The membranes were blocked in PBS containing 5% skim milk for 1 hour. After washing with PBS containing 0.1% Tween 20 (PBS-T), the membranes were incubated with each corresponding antibody, anti-EveA, anti-EveB(up) or anti-EveB (down) diluted (1:1000) in 5% skim milk. One of the membranes was incubated with pre-immune serum from rabbit diluted (1:1000) in 5% skim milk. All membranes were incubated for 18-24 h at 4°C. Following incubation, the membranes were washed three times with PBS-T, and then incubated for 1 h in alkaline phosphatase-conjugated goat-anti-rabbit IgG antibodies (sigma) diluted (1:10,000) in 5% skim milk. Finally, the membranes were washed three times with PBS-T and developed using the 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP/NBT) commercial liquid substrate system (Promega) to visualize the reaction.

4.2.7. Phagocytosis assay:

To investigate whether the addition of exogenous purified proteins would increase the anti-phagocytic activity of the parent strain of GBS NCS13 cells, the phagocytic assay was performed as previously described (14) with minor modifications. One milliliter of the GBS culture that was incubated overnight was washed once with 1X PBS buffer. A 0.5 McFarland standard of GBS culture was made and then diluted in TH broth

(1:10,000). One hundred microliters of 1:10,000 bacterial dilution was pre-incubated with 0.8 µg (total volume of proteins used in the whole reaction is 2 µg/ml) of each purified protein of EveA, EveB(up) or EveB(down) for 1h at room temperature. As a control, 0.8 µg of BSA or no addition of protein was used. After incubation, 300 µl of freshly heparinized human blood was added to each Eppendorf tube and incubated at 37°C under gentle rotation. Following 3 hours of incubation, 200 µl aliquots of the mixture were plated on BAP to determine the number of CFU after growth in human blood. As a control, 100 µl of the bacterial cell suspension (1:100) containing 0.8 µg of each purified protein, EveA, EveB(up) or EveB(down) was plated on BAP. The plates were incubated overnight at 37°C. Growth of GBS in human blood was expressed as a relative percent of survival at the end of the incubation period by calculating the number of CFU obtained from blood incubated in the presence of purified proteins divided by the number of CFU obtained from blood in the absence of the added purified proteins, multiplied by 100.

4.2.8. Bactericidal assay:

Bactericidal activity of the purified EveA and EveB(down) proteins against GBS NCS13 bacteria were determined by a microdilution method. The bacterial culture was grown in TH broth overnight at 37°C. The cultures were diluted in TH broth to obtain a 0.5 McFarland standard. The proteins were serially two-fold diluted in TH broth in sterile 96-well microtiter plates. The serially diluted proteins were inoculated with 2.5 µl of the 0.5 McFarland bacterial culture in sterile 96-well microtiter plates. Each tray included a growth control well and a sterility (un-inoculated) well. A penicillin antibiotic (Sigma) was used as the positive control. The inoculated microdilution trays were incubated at 37

°C for 16-20 h. The proteins and penicillin were assayed for antimicrobial activity in the range from 0.25 to 128 µg/ml. In each set of tests when determining the growth end points, the minimal inhibitory concentration (MIC) of the proteins was determined by comparing the amount of growth in the wells or tubes containing the proteins with the amount of growth in the growth-control wells or wells containing no antimicrobial agents.

4.2.9. Subcellular localization of EveA:

A 20 ml culture of GBS NCS13 was grown overnight on TH broth at 37ºC. A one ml aliquot of the overnight culture was transferred to a micro-centrifuge tube and centrifuged at 14000 x g for 5 minutes at 4ºC to generate a whole cell lysate. The supernatant was removed and the cell pellet was washed one time with 1 ml of PBS, resuspended in 200 µl of 1X SDS buffer (0.2 M Tris-HCl [pH 6.8], 1% SDS, 2% mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) with 70 mg of 100 µm-glass beads, vortexed vigorously for 1 minute and boiled for 5 minutes to generate the whole cell lysate. The remaining 19 ml of the culture was centrifuged at 5000 x g for 20 minutes at 4ºC and the supernatant was filtered through a 0.22 µm filter and collected as the extracellular fraction. The supernatant fraction and the sterile TH broth (negative control) were precipitated using 70% ammonium sulfate and resuspended in 100 µl of PBS. To prepare the GBS cell wall fraction, a method was performed as described by Cole *et al*. (15) with some modifications. Briefly, the pellet of 19 ml of the culture was washed twice with 1 ml of ice-cold PBS and after centrifugation (8000 x g, 20 minutes), the pellet was resuspended with 200 microliters of ice-cold mutanolysin mix (1 ml of spheroplast

forming buffer (20% sucrose, 1 mM MgCl2, 33 mM Tris, pH 8.0) mixed with 50 µl mutanolysin (5,000 U/ml)(Sigma)), incubated at 37°C for 2 h and centrifuged at 14000 x g for 5 minutes. The supernatant was resuspended in 1/5 volume of 1X SDS buffer and collected as the cell wall fraction. The cell pellet was then resuspended in 1 ml of PBS with 70 mg of 100 µm-glass beads, vortexed vigorously for 1 minute and boiled for 5 minutes to generate the cytoplasmic fraction. The GBS fractions were separated by 10% SDS–PAGE gel and transferred onto a nitrocellulose membrane for Western immunoblotting. For Western Blot, the membrane was blocked with 5% skim milk diluted in PBS for 1 h at room temperature and then the blot was probed with 1:200 dilution of polyclonal rabbit anti-EveA diluted in blocking buffer and incubated overnight at 4°C. The membrane was washed 3X with 1X PBST (PBS with 0.1 % Tween 20) and then incubated in 1:10,000 goat anti-rabbit IgG Alkaline Phosphatase conjugate (Sigma) for 1 h at room temperature. The blot washed again with PBST and developed using 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) commercial liquid substrate system (Promega).

For the identification of the EveA protein in GBS strains, GBS cell wall fractions were isolated from the GBS non-typeable (NT) strain (18SR431), GBS NT strain (17SR409), GBS non-capsulated strain (COH-13), GBS serotype (ST) Ia (97SR400), GBS ST-Ib (97SR570), GBS ST-II (97SR138), GBS ST-III (COH-I), GBS ST-IV (97SR331), GBS ST-V (97SR384), GBS ST-VI (9842), GBS ST-VII (7271), GBS ST-VIII (JM9), and GBS ST-IX (ATCC 27412) using a mutanolysin mix as described above. The GBS cell wall fractions from the different GBS strains were subjected to 10% SDS–PAGE gel and the subsequent steps were performed as described above.

4.2.10. Statistical analysis:

Data was analyzed using the Students t test and a p-value <0.05 was considered statistically significant. Data points correspond to the average value of all replicates and error bars represent the standard deviation.

4.3. Results:

4.3.1. Analysis of purification of proteins and specificity of anti-sera:

To test the purity of the purified EveA, EveB(up) and EveB(down) and the specificity of rabbit anti-sera raised against the purified proteins, Coomassie-blue stained SDS-PAGE and western blot were performed. As shown by Coomassie-blue stained SDS-PAGE, the purified EveA, EveB(up) and EveB(down) migrated at the expected locations, 43 kDa, 30 kDa and 20 kDa, respectively (Fig. 4.2A). The anti-sera to EveA, EveB(up) and EveB(down) assayed by Western blot against the homologous purified proteins recognized a clear band at the expected location (Fig. 4.2B).

4.3.2. Adhesion of purified proteins to HeLa cells inhibits GBS invasion:

To determine whether the purified EveA, EveB(up) and EveB(down) proteins could competitively inhibit GBS invasion, HeLa cell monolayers were incubated with 2, 5 and 10 µg/ml of each purified EveA, EveB(up) and EveB(down) protein before the invasion assay. Pre-incubation of the cells with 10 µg/ml of BSA had no effect, however, pre-incubation with 10 µg/ml of EveA and EveB(down) substantially diminished the invasion of GBS, (P= 0.000000028 and P= 0.00002.2), respectively (Fig. 4.3). Inhibition by 10 µg/ml of EveB(up) was not significant (P= 0.075).

4.3.3. Adhesion of purified proteins to HeLa cells do not inhibit GBS adherence:

To determine whether the purified EveA, EveB(up) and EveB(down) proteins could inhibit GBS adhesion, HeLa cell monolayers were incubated with the proteins before the adherence assay. Pre-incubation of HeLa cells with 10 µg/ml of EveA,

EveB(up) or EveB(down) did not inhibit the adherence of GBS (P= 0.291, P= 0.251 and P= 0.084, respectively) (Fig.4.4). This was similar to the result of the pre-incubation cells with BSA (P= 0.269) compared with Hela cells not treated with any protein (Fig. 4.4).

4.3.4. Binding of purified EveA, EveB(up) and EveB(down) to epithelial cell membranes:

Addition of purified EveA and EveB(down) proteins to HeLa cells prior to GBS infection was found to block the invasion of GBS into the HeLa cells. To demonstrate that this observation was specifically due to the physical binding between the purified proteins and the HeLa cells, and not due to unspecific effect of the purified proteins on the HeLa cells, additional assays were performed. Binding of the purified proteins to HeLa cells was determined by both ELISA assay and Western blot. A microassay of adherence to immobilized HeLa cell membranes was used to determine whether the purified EveA, EveB(up) and EveB(down) possess adherence properties. In this assay, the proteins that remained adherent on the HeLa cell membranes after washing with PBS were immunodetected with immune rabbit anti-serum to the homologous proteins. The purified proteins were highly adherent to HeLa cell membranes (Fig. 4.5A). The specific binding of the purified proteins to HeLa cell membranes was also confirmed by immunoblotting assay. The anti-EveA, anti-EveB(up) and anti-EveB(down) antibodies were able to recognize bands for the purified EveA, EveB(up) and EveB(down) proteins at the expected size (Fig. 4.5B).

4.3.5. Opsonizing antibodies did not inhibit GBS invasion:

To further evaluate whether EveA, EveB(up) or EveB(down) proteins from GBS have a role in the invasion of the whole bacteria, hyperimmune rabbit sera to these proteins were assayed for the ability to inhibit invasion of the GBS bacterial cells into viable HeLa cells. After incubation of the bacteria with appropriate serum dilutions (1:100, 1:1000 and 1:10,000), the invasion of bacterial whole cells into viable HeLa cells was evaluated. Three specific rabbit sera were studied: anti-EveA, anti-EveB(up) and anti-EveB(down). The non-inhibitory effect of these three sera on the invasion of GBS is shown in Fig. 6. The complete (100%) invasion level was established by incubating HeLa cell monolayers with GBS that was non-treated or treated with pre-immune rabbit serum. Anti-serum (1:100 dilutions) to EveA, EveB(up) and EveB(down) did not inhibit the invasion of GBS, P= 0.308, P=0.479, P=0.386, respectively (Fig. 6).

4.3.6. Opsonizing antibodies did not enhance bacterial killing in human blood

To determine the effect of opsonization with anti-EveA and anti-EveB antibodies against GBS by phagocytic cells in human blood, an opsonophagocytic killing assay was used. Most studies assaying the opsonophagocytic killing use either purified human-derived polymorphonuclear cells or cultured HL-60 cells (16–20). In this assay, fresh heparinized human whole blood was used. The un-opsonized or opsonized GBS with pre-immune rabbit serum were used as control. Following pre-incubation of GBS with 1:100 dilution of anti-EveA, anti-EveB(up) or anti-EveB(down) antibodies, over 100% of GBS survived after 3 hours of incubation in human blood, which was similar to the survival rate of GBS un-opsonized with sera. (Fig.7). The differences in the capacity of

phagocytic cells in the human blood to kill GBS that were pre-opsonized with anti-EveA, anti-EveB(up), anti-EveB(down) or pre-immune rabbit sera were not significant (*P*=0.168, *P*=0.116, *P*= 0.136 and *P*= 0.227; respectively). Antibodies against EveA and EveB did not enhance bacterial killing by human phagocytic cells.

4.3.7. Purified EveA, EveB(up) and EveB(Down) proteins enhance bactericidal activity:

To determine whether the addition of exogenous EveA, EveB(up) or EveB(down) will enhance bacterial resistance to phagocytic activity by phagocytic cells, the purified proteins were first incubated with GBS cells and then the suspension was inoculated into fresh heparinized human whole blood. The number of bacteria present after 3 h of incubation was determined by counting the CFU on BAP. The relative percent of survival was calculated as follows: the number of CFU in the presence of purified proteins divided by the number of CFU in the absence of purified proteins multiplied by 100. Surprisingly, the results revealed that the addition of a total volume of 2 µg/ml of purified EveA and EveB(down), but not EveB(up), enhanced bacterial killing by 92% (*P*= 0.02) and 99% (*P*= 0.0.014), respectively, compared to the absence of purified proteins (Fig. 8). To confirm whether the addition of these proteins enhances the phagocytic killing of GBS by the phagocytic cells themselves or if it is a direct effect of the proteins on GBS viability by acting as bactericidal agents, the same experiment was conducted without the addition of human whole blood cells. It was determined that the addition of purified EveA and EveB(down) proteins to bacterial cells significantly reduced bacterial viability by 84 % (*P*= 0.004) and 91% (*P*= 0.0.0012), respectively, compared to GBS viability without the

addition of EveA and EveB(down) proteins (Fig. 8). Bacterial viability in the presence of EveB(up) was not affected (P= 0.17).

4.3.8. Bactericidal activity of EveA and EveB(down):

Based on the results obtained from the previous experiment, the bactericidal activity of EveA and EveB(down) against GBS NCS13 was identified using a microdilution assay. GBS is known to have a high susceptibility to penicillin; thus, penicillin was used as the positive control. The minimal inhibitory concentration (MIC) of EveA and EveB(down) was determined to be 32 μg/ml and 16 μg/ml, respectively (Fig. 9). GBS is highly sensitive to penicillin and thus, no bacterial growth was identified in all wells (0.25-128 μg/ml), except in the growth control wells (Fig. 9).

4.3.9. Subcellular localization of EveA in GBS:

We took the advantage of having anti-EveA antibodies to determine the location of EveA protein within the GBS NCS13 strain. To do this, anti-EveA antibodies were used to probe the extracellular, cell wall and cytoplasmic contents of NCS13 using western blots. A 43 kDa protein band, the molecular weight of EveA, was visible in the cytoplasmic fraction (Fig. 4.11). However, the anti-EveA antibodies were also seen to have reacted with protein bands of about 48 kDa in the cell wall fraction and the whole cell lysate. This small addition of molecular weight (about 5 kDa) to the original size of the EveA protein could suggest that after secretion, the EveA protein might undergo post-translational modification, which adds a small amount to its molecular weight, so as to facilitate the protein binding to the surface of GBS. The anti-EveA antibodies were also

seen to react with a protein band of approximately 55 kDa in the extracellular fraction. It may be that the secreted EveA protein binds to other secreted proteins from GBS, resulting in a higher molecular weight for the EveA protein. Additional protein bands of lower molecular weight than the 43 kDa also reacted with the anti-EveA antibody in the whole cell lysate (cell wall and cytoplasmic) and cytoplasmic fraction, but were not visible in the cell wall fraction of GBS or the extracellular fraction. Since these bands did not occur in the cell wall fraction of GBS, it is likely that the extra bands seen below the typical band for the EveA protein in the whole cell lysate and cytoplasmic fraction of GBS might represent the cleavage of the EveA protein during the glass bead and boiling steps.

4.3.10. Identification of the EveA protein in GBS strains:

The anti-EveA antibody was also used to further evaluate the distribution of the EveA protein among GBS isolates from different serotypes. The GBS cell wall fractions from various GBS strains were prepared and subjected to SDS-PAGE and Western immunoblotting. The anti-EveA antibody recognized a protein band in all 14 GBS cell wall preparations tested and the results with representative strains are presented in Fig. 4.12. This identification demonstrated that EveA is not a serotype-specific protein and it is conserved in all GBS strains.

4.4. Discussion:

I previously demonstrated that mutants of the *eveA* and *eveB* genes, GBS *eveA::erm* and GBS *eveB::erm*, respectively, show reduction in bacterial invasion into HeLa cells and reduction in anti-phagocytic activities compared to the parent strain. This therefore suggested the possibility that the EveA and EveB proteins play significant roles in GBS invasion into HeLa cells and in anti-phagocytosis. I also previously demonstrated the immunogenicity of EveA and EveB using human sera containing naturally induced antibodies from GBS-infected patients.

While the previous chapter indicated that EveA and EveB potentiate immunogenicity, it is essential that the induced antibodies are able to react with the native bacterial antigens. Sera from immunized rabbits with EveA, EveB(up) and EveB(down) proteins were assayed to determine if the EveA and B antibodies could neutralize the ability of GBS to invade HeLa cells. At dilutions as low as 1:100 anti-EveA, anti-EveB(up) and anti-EveB(down) displayed no inhibition of GBS invasion into HeLa cells. The antibodies were also tested for functional activity in an opsonophagocytic assay. The results showed that anti-EveA, anti-EveB(up) and anti-EveB(down) antibodies at a concentration of 1:100 do not significantly reduce the number of CFU of the GBS NCS13 strain.

None of the rabbit polyclonal antibodies were able to prevent GBS from invading HeLa cells or reduce CFUs in the opsonophagocytosis assay. These finding suggest a complex nature of the target epitopes, which may impede optimal antibody affinity. One possible issue related to peptide-based antibody production is that all amino acids within the peptide are potentially available for antibody affinity maturation, while in native

proteins, the amino acids may be naturally buried within the protein structure. The native conformation of synthetic peptides is important for inducing conformation-specific antibodies that recognize α-helices in proteins (21,22). It could be that the peptides produced in this study to generate the corresponding antibodies were in random coil "non-native α-helical conformation," which were not sufficient to elicit a neutralizing response within a rabbit model. In other published studies, attempts for the induction of protective antibodies against immunogenic proteins from the respiratory syncytial virus and *Clostridioides difficile* resulted in the production of non-neutralizing antibodies; this was due to the weak recognition of the induced antibodies to the native proteins (23,24). In the work I presented here, it could also be that the non-neutralizing antibodies directed against EveA and B bind to other antigenic determinants on the intact GBS surface or bind the same antigenic site as neutralizing antibodies, but with low affinity/avidity and consequently, fail to neutralize the GBS. Yasmeen *et al*. (25) determined that the low binding affinity of neutralizing antibodies to trimeric HIV-1 envelope glycoproteins resulted in some persistent infectivity and did not result in full protection from viral infection. This may be a similar case in our study, thereby inhibiting the optimal antigen-antibodies binding interaction.

The role of EveA, EveB(up) and EveB(down) purified proteins in bacterial attachment to eukaryotic cells was investigated. The assay using HeLa cells suggested that EveA and EveB proteins are not involved in the attachment of GBS to epithelial cells. However, in a competitive internalization assay, it was determined that the addition of exogenous EveA and EveB(down) to HeLa cell monolayers, prior to the addition of the GBS inoculum inhibited GBS internalization at a concentration of 10 μg/ml. There

was no significant effect noticed on GBS invasion into HeLa cells pre-treated with 10 μg/ml of EveB(up). These results suggest that EveA and EveB(down) play role(s) in the internalization of GBS into eukaryotic cells, but not in the initial GBS attachment to epithelial cells.

In the previous chapter, the finding that GBS *eveA::erm* and GBS *eveB::erm* mutant strains lost the anti-phagocytic properties of the parent strain raises a hypothesis as to whether EveA and EveB are anti-phagocytic in nature. It was hypothesized that the addition of exogenous EveA and EveB proteins to GBS cultures would increase bacterial anti-phagocytic activity and allow the bacteria to multiply in human blood. To try and begin to understand the contribution of EveA and EveB towards GBS anti-phagocytic activity, the anti-phagocytic activity of the parent strain in the presence of exogenous EveA, EveB(up) and EveB(down) purified proteins was determined. Interestingly, the bacteria were killed more rapidly in human whole blood in the presence of EveA and EveB(down) proteins. The increased phagocytosis of the GBS parent strain in the presence of EveA and EveB(down) is likely due to their bactericidal activities, and not due to the enhanced phagocytosis by phagocytic cells. The bactericidal activities of EveA and EveB(down) were measured by determining the MIC of EveA and EveB(down) as 32 μg/ml and 16 μg/ml, respectively. While these MICs are somewhat high in comparison to antibiotics such as penicillin, they are potentially low enough to suggest a reason for the low numbers of CFUs in the assays.

Antimicrobial peptides (AMPs) are produced by many organisms to protect against microbial infection. AMPs have excellent therapeutic potential, especially against pathogenic organisms resistant to conventional antibiotics. AMPs, with the average

length of 30-50 amino acids, share some similarities, such as electrical charge and amphipathicity (26,27). The cationic peptides interact selectively with anionic bacterial membranes to form transmembrane pores that disrupt the bacterial bilayer, thereby causing rapid lysis and cell death (27–29). Most of the active peptides of AMPs are generated from larger precursor proteins through proteolytic cleavage (30).

In this study, we showed that EveA and EveB(down) proteins from the GBS strain have some antimicrobial activities against the bacterial's own self however, do they act as AMPs? The peptide sequences of EveA and EveB(down), 367 aa and 207 aa, respectively, are larger than the average size of AMPs. It may be possible that some of the peptides in these large proteins are acting as antimicrobial agents. Since most AMPs are derived from large proteins, it may be that the exogenous EveA and EveB(down) proteins that were added to the bacterial culture undergo degradation into smaller peptides by protease enzymes on the surface of GBS, which facilitates the active peptides to start the action of killing similar to AMPs. GBS surfaces have many proteases that contribute to its virulence, and one of these proteases is the cell-surface-associated protein (CspA) that has been known to degrade host proteins and chemokines (31,32).

The EveB protein is predicted to be a transmembrane protein with 8 transmembrane domains. However, a cell wall anchoring motif (LPXTG) in the C-terminal region of EveA was not identified. Thus, in this study we used the polyclonal rabbit anti-EveA antibody to perform subcellular localization of the EveA protein in GBS. Anti-EveA antibodies were found to react with protein bands in the cell wall, cytoplasmic and extracellular fractions, thereby suggesting that the EveA protein binds to the GBS surface after secretion. Immunoblot assays using the anti-EveA antibody

indicated that a protein band reacted with anti-EveA antibodies present in all cell wall fractions of the 13 GBS strains tested, which included representative isolates of all serotypes, suggesting that the EveA protein is conserved in all GBS isolates and it is not serotype-specific.

In summary, rabbit antibodies against EveA and the two peptides EveB, EveB(up) and EveB(down), were shown to be non-neutralizing antibodies. The antibodies did not protect HeLa cells from invasion by GBS and did not enhance phagocytic killing of GBS. It was also determined that, although the antibodies do not provide protection against GBS invasion, the EveA and EveB(down) proteins were shown to have minimal antimicrobial effects on GBS. The necessity of EveA and EveB(down) proteins for GBS invasion into HeLa cells suggest a mechanism by which EveA and EveB may contribute to pathogenesis. It is noticeable that the C-terminal of the EveB protein, from where EveB(down) is derived, is more active in the assays measuring virulence than the N-terminal region. To my knowledge, this is the first report of bacterial proteins that, when present in high volume, have antimicrobial effects against the bacteria itself.

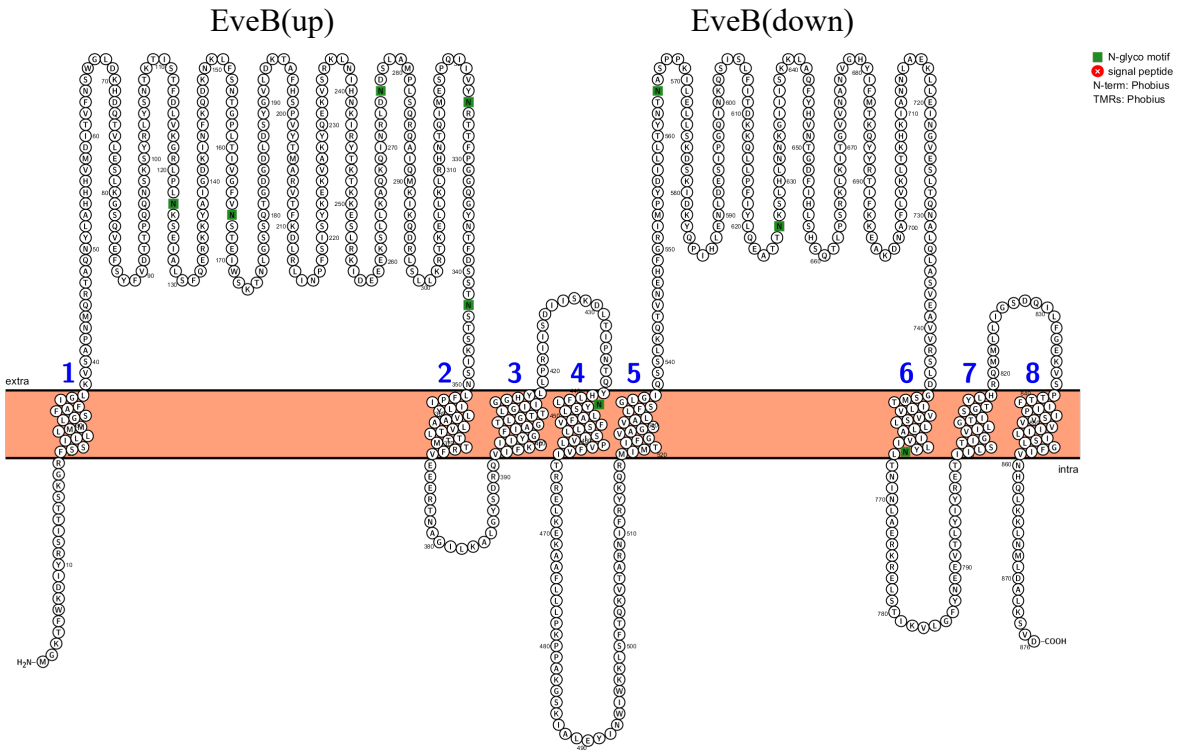


Figure 4.1. Predicted transmembrane topology for the GBS EveB protein. The model was obtained by using the PROTTER program (33). The EveB (up) and (down) fragments are located outside the cytoplasmic membrane and were targeted for protein expression. The EveB(up) contains 312 aa (30 kDa) and EveB(down) contains 207 aa (20 kDa).

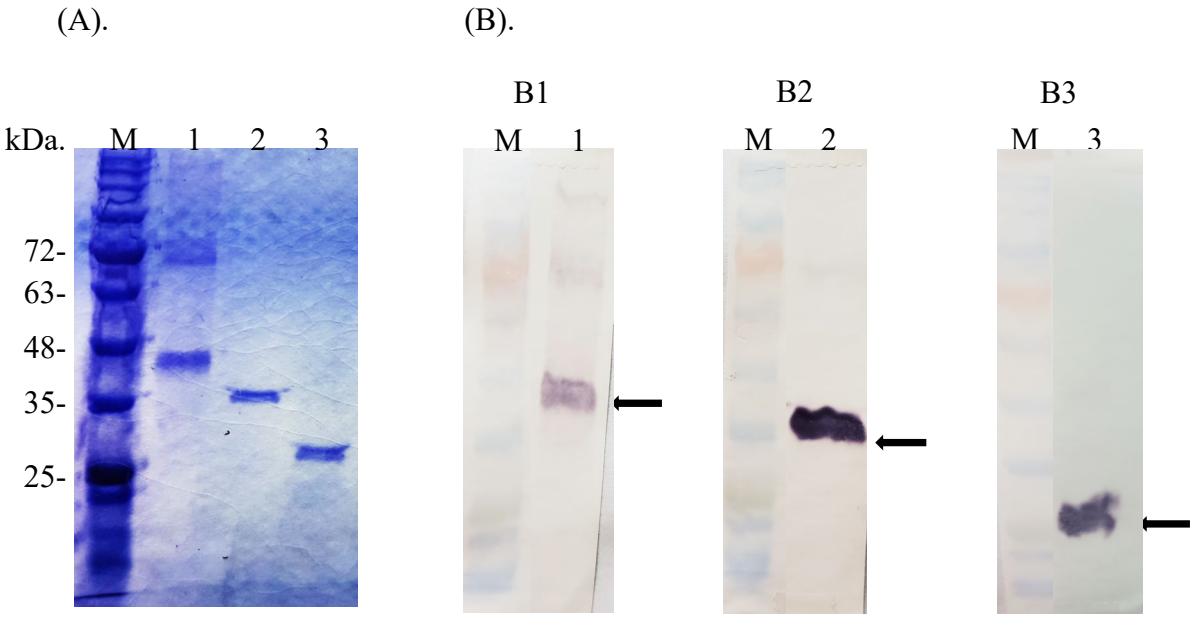


Figure 4.2. Analysis of purified EveA, EveB(up) and EveB(down) proteins. (A) Comassie-blue stained SDS-PAGE (10%) gel of EveA (Lane 1), EveB(up) (Lane 2) and EveB(down) (Lane 3). (B) Western blots of EveA (B1), EveB(up) (B2) and EveB(down) (B3) using anti-EveA, anti-EveB(up) and anti-EveB(down) antibodies, respectively. Lane M, protein marker. Twenty nanograms of each purified protein were loaded onto each lane. The blots were probed with the primary antibodies (1:1000) overnight at 4ºC. After three washes of the blots with 1X PBS/0.1% Tween-20, the blots were incubated with a secondary antibody (anti-rabbit antibodies conjugated with alkaline phosphatase) (1:10,000) for 1 h at room temperature. Following the incubation, the blots were washed 3 times and developed using BCIP/NBT.

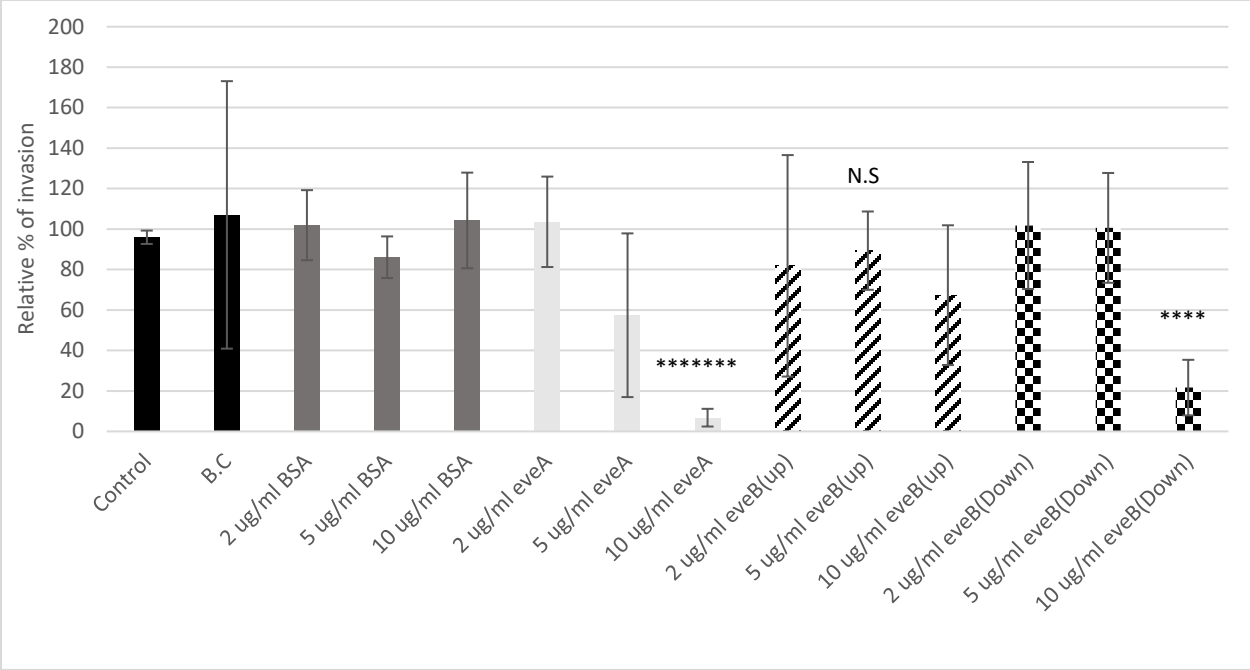


Figure 4.3. Invasion of treated HeLa cells with different concentrations of purified EveA, EveB(up) and EveB(down) proteins from the GBS NCS13 strain. Intracellular CFU recovered after 2 hours of infection was expressed as relative percent invasion. The bars represent the average values obtained from four independent experiments compared with the value obtained from the control (invasion of untreated HeLa cells by GBS) to determine the statistical significance. The bars represent the average value of experiments run in quadruplicate; error bars represent standard deviation. An asterisk (****) indicates statistical significance ($P < 0.0001$), (*******) indicates ($P < 0.00000001$) and N.S indicates not significant ($P > 0.05$).

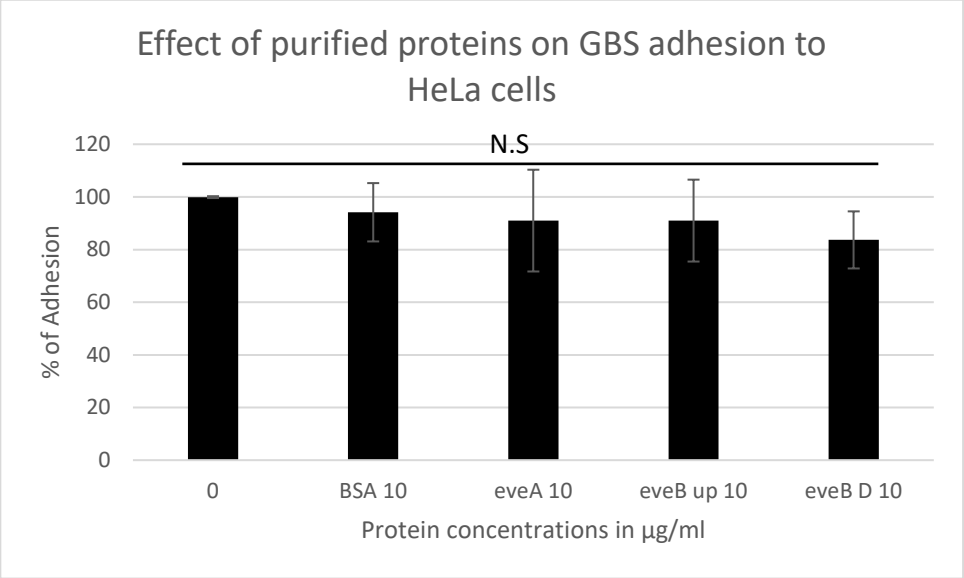
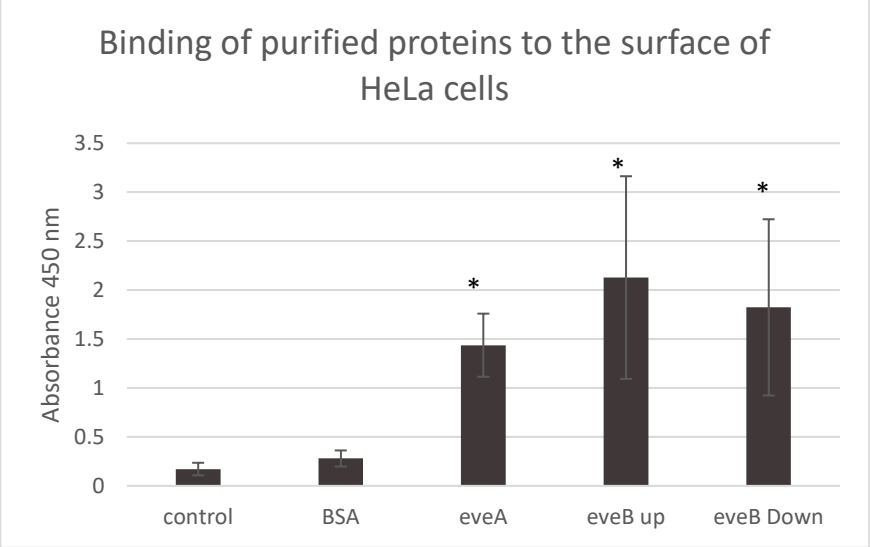
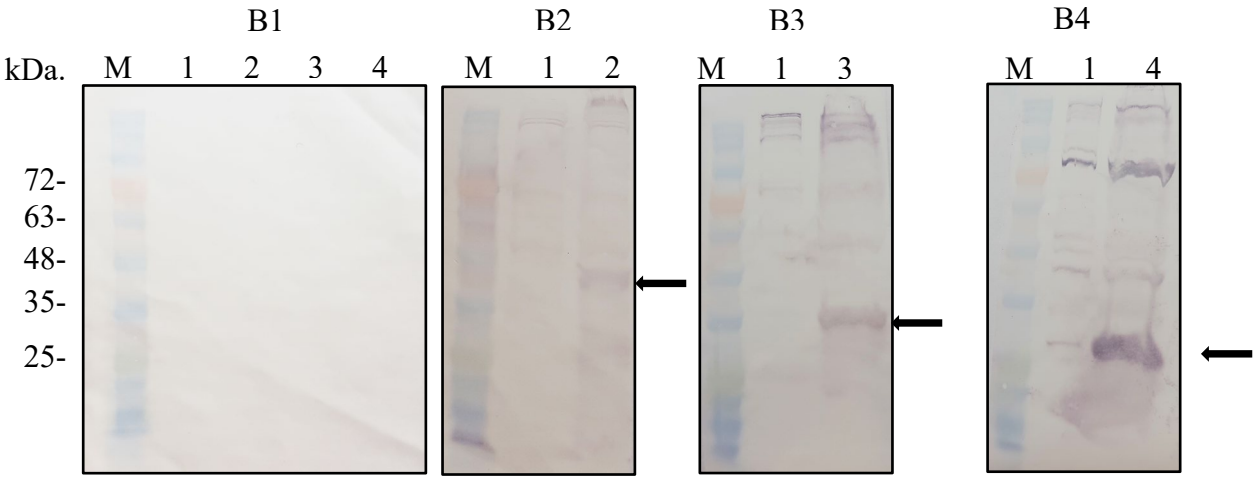


Figure 4.4. Adhesion of GBS to treated HeLa cells with purified EveA, EveB(up) and EveB(down) proteins. The attached CFU recovered after 1 hour of infection was expressed as relative percent adhesion. The bars represent the average values obtained from two independent experiments compared with the value obtained from the control (adhesion of GBS to untreated HeLa cells) to determine the statistical significance; error bars represent standard deviation; N.S indicates not significant ($P > 0.05$).

(A)


Binding of purified proteins to the surface of HeLa cells
Absorbance 450 nm
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
control
BSA
eveA
eveB up
eveB Down

(B)


B1
B2
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M 1 2 3 4
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Figure 4.5. Adherence of purified EveA, EveB(up) and EveB(down) proteins to HeLa cell membranes. (A) Adherence of purified EveA, EveB(up) and EveB(down) proteins was analyzed by using an ELISA assay. The adhering absorbances were determined by using rabbit polyclonal anti-EveA, anti-EveB(up) or anti-EveB(down) antibodies. As a control, BSA was used and incubated with HeLa cells. The results shown are the average of duplicate analysis to determine the statistical significance. The bars represent the average value of experiments run in duplicate; error bars represent standard deviation. An asterisk (*) indicates statistical significance ($P < 0.05$). (B) Adherence of purified EveA, EveB(up) and EveB(down) via immunoblotting assay. The purified proteins, and BSA as a control, were incubated with HeLa cells in cell suspensions for 1 h at 37ºC (see Materials and Methods). Cell lysates of HeLa cells were loaded onto SDS-PAGE (10%) and transferred to nitrocellulose membranes. The resulting bands belonging to EveA, EveB(up) and EveB(down) were detected by using rabbit polyclonal anti-EveA (B2), anti-EveB(up) (B3) or anti-EveB(down) (B4) antibodies. One blot was used to incubate with rabbit pre-immune serum (B1). M; protein marker, Lane 1: HeLa cells pre-incubated with BSA, Lane 2: HeLa cells pre-incubated with EveA, Lane 3: HeLa cells pre-incubated with EveB(up), Lane 4: HeLa cells pre-incubated with EveB(down).

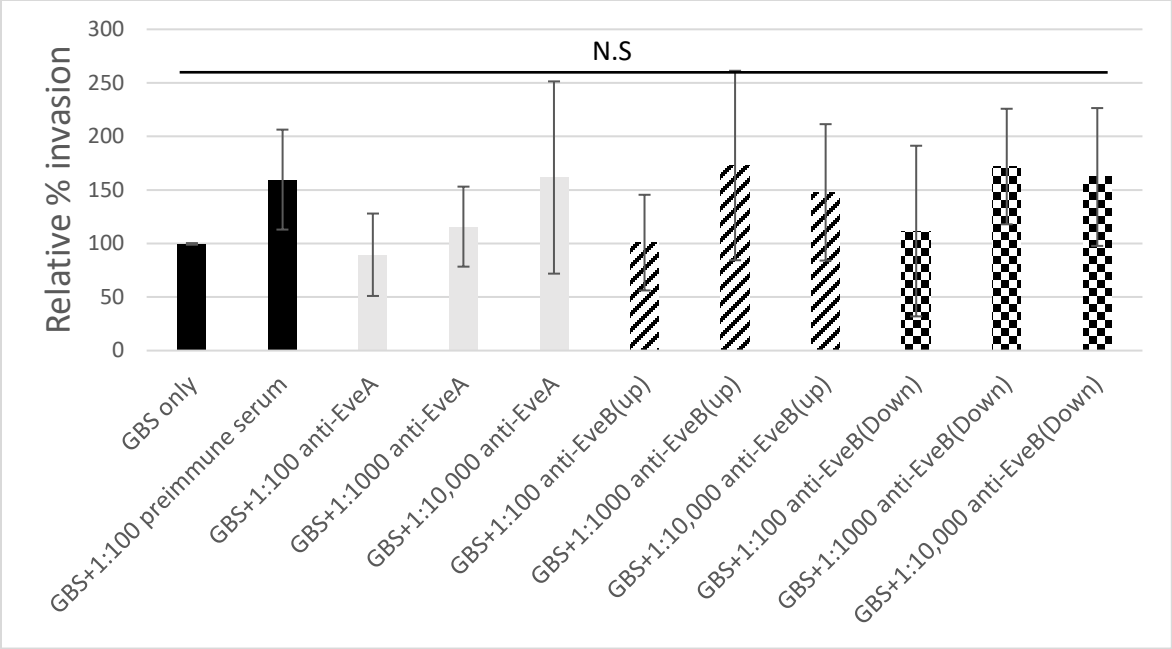

Figure 4.6. Invasion of HeLa cells by the GBS NCS13 strain treated with different dilutions of anti-EveA, anti-EveB(up) and anti-EveB(down) antibodies. Intracellular CFU recovered after 2 hours of infection was expressed as relative percent invasion. The bars represent the average values obtained from four independent experiments compared with the value obtained from the control (GBS without pre-incubation with antibodies) to determine the statistical significance; error bars represent standard deviation; N.S indicates not significant ($P > 0.05$).

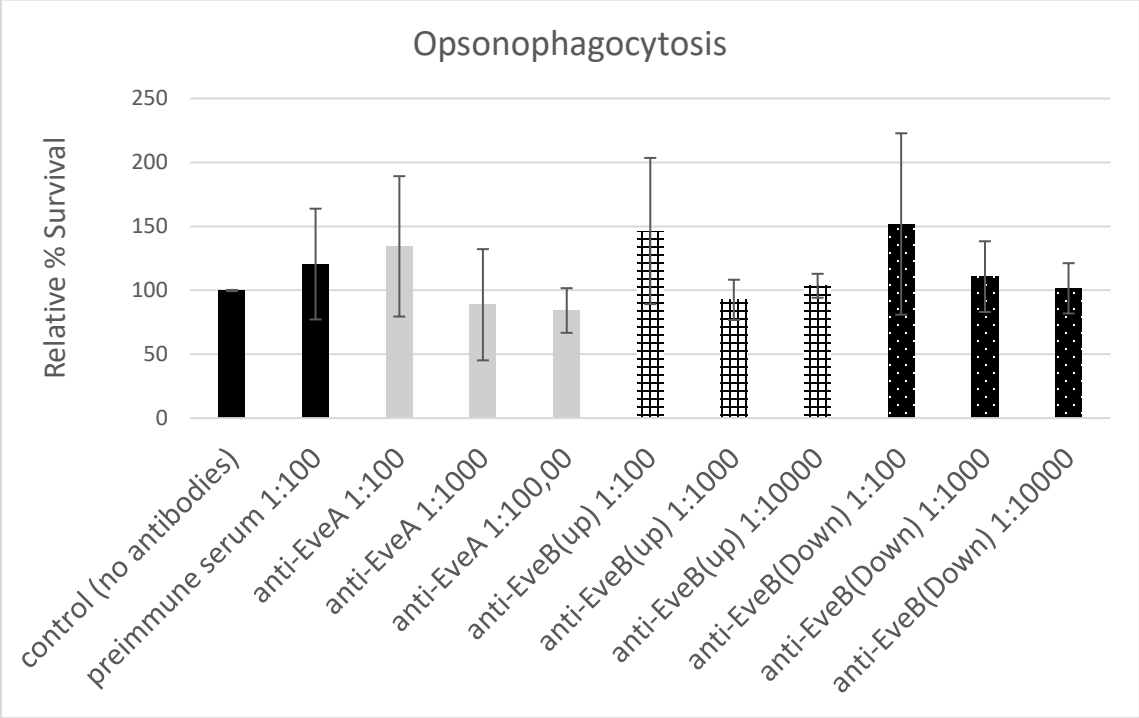

Figure 4.7. Killing of opsonized and non-opsonized GBS NCS13 by human blood. GBS were pre-incubated with various sera, including pre-immune rabbit serum, anti-EveA, anti-EveB(up) and anti-EveB(down), for 1 h at room temperature. Then, opsonized or non-opsonized GBS were added to fresh whole blood and incubated for 3 hours at 37°C. The data are from three independent experiments. The bars represent the average value of experiments run in triplicate; error bars represent standard deviation. The percentage of survival was calculated by using the number of non-opsonized GBS associated with human blood after 3 hours of incubation at 37°C as 100%.

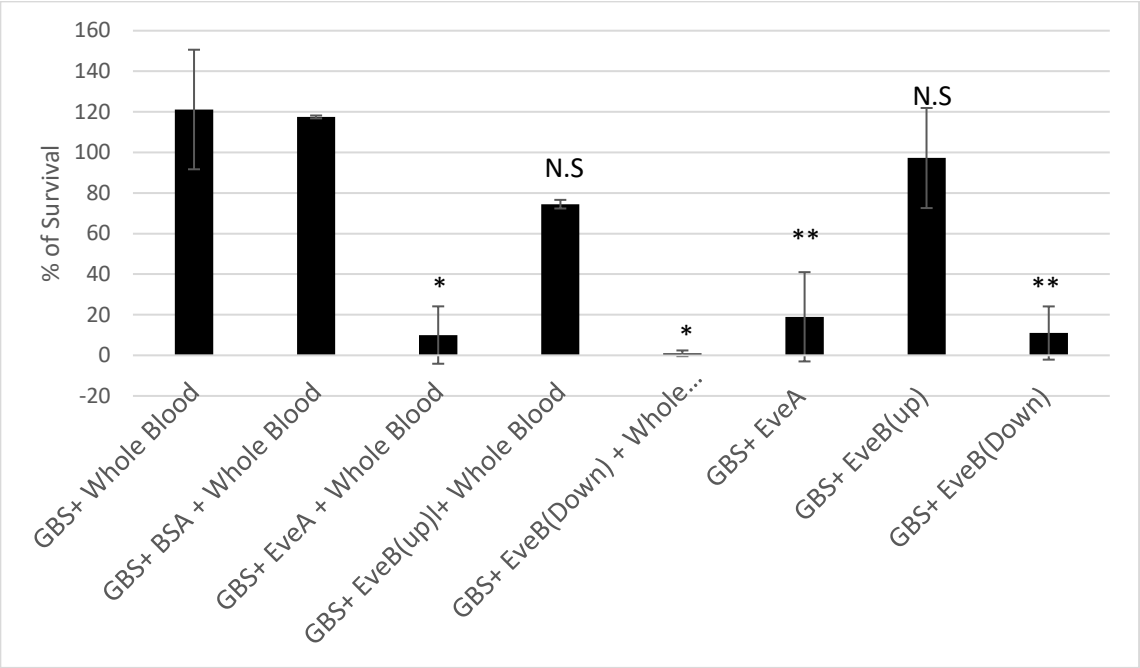

Figure 4.8. Killing of pre-incubated GBS with EveA, EveB(up) and EveB(down) proteins by human blood. The anti-phagocytic activity of GBS in the presence of purified proteins (EveA, EveB(up) and EveB(down)) or Bovine Serum Albumin (BSA) was tested *in vitro* using human blood. The relative percent of survival at the end of the incubation period is the number of CFU obtained from the sample incubated with purified proteins divided by the number of CFU obtained from the sample without the proteins, multiplied by 100. As a control, GBS incubated with purified proteins without the addition of whole blood was used to assess the effect of proteins on GBS viability. The bars represent the average values obtained from two independent experiments compared with the value obtained from the control to determine the statistical significance; error bars represent standard deviation; an asterisk (*) indicates statistical significance ($P < 0.05$), (**) indicates ($P < 0.01$) and N.S indicates not significant.

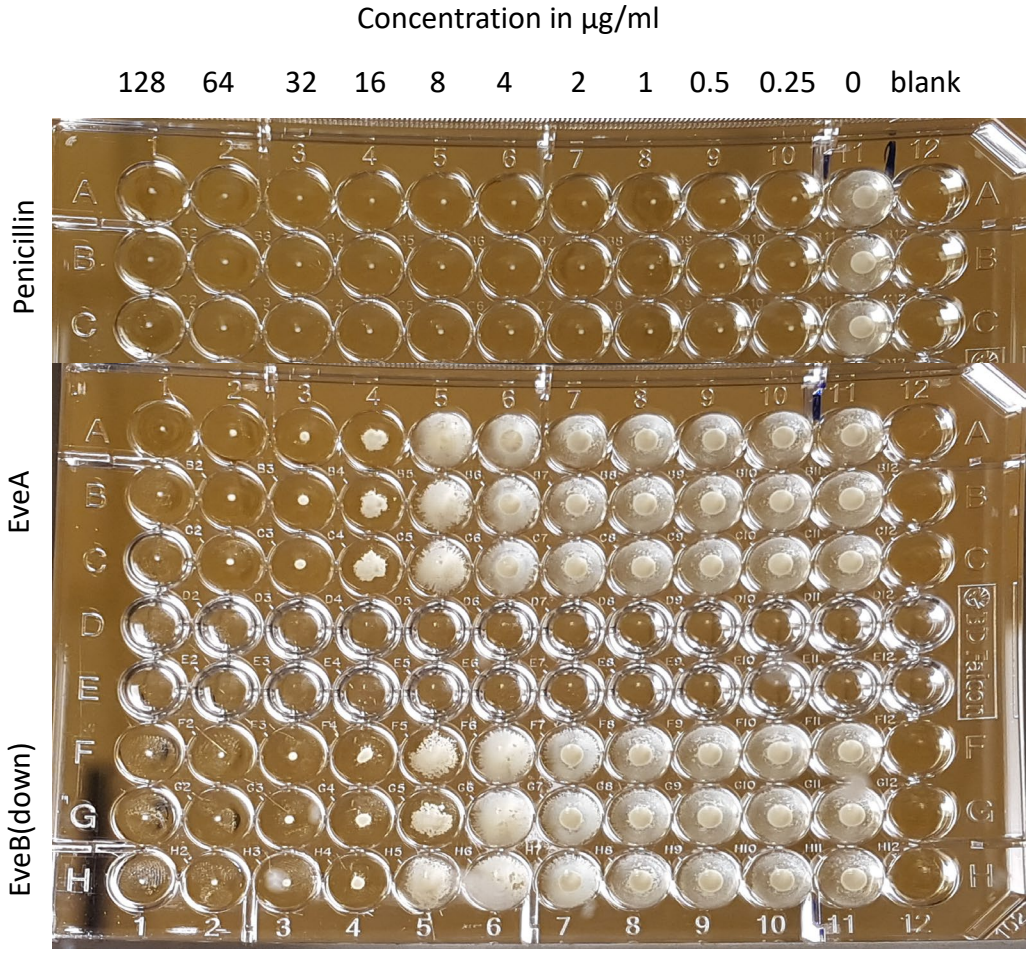

Figure 4.9. Determination of the minimum inhibitory concentration (MIC) for EveA, EveB(down) and penicillin against GBS NCS13. After 18 hours of incubation, photographs of the plates were taken using an inverted mirror. Column 12 (Blank) confirms no contamination occurred while preparing the plates. Column 11 (0 µg/ml), a negative control shows the highest growth. The highest concentration incorporated into the plate is 128 µg/ml and the lowest, achieved through double serial dilution, is 0.25 µg/ml. Column 3 containing EveA and column 4 containing EveB(down) showed no growth change, therefore the concentration of proteins in these columns were taken as the MIC values. Penicillin (128-0.25 µg/ml) was used as positive control.

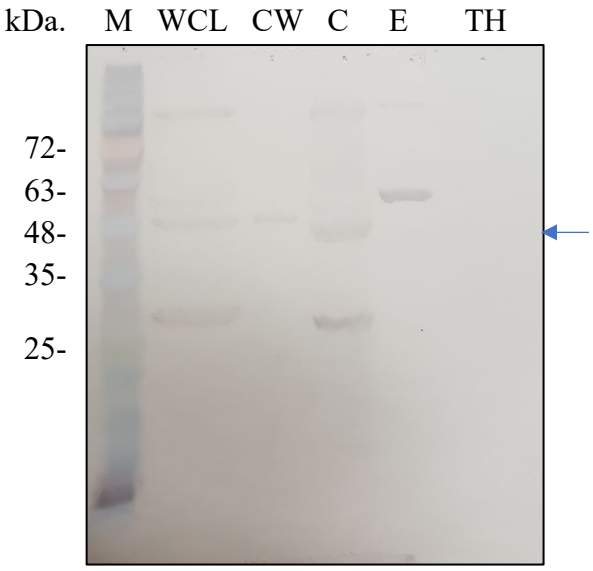

Figure 4.10. Subcellular localization of EveA. An overnight culture of GBS NCS13 strain was used to identify the location of the EveA protein. Bacterial cell fractions were subjected to a 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with rabbit anti-EveA antibodies overnight. The blot was then incubated with anti-rabbit-IgG alkaline phosphatase conjugate antibodies and developed using BCIP/NBT. Lane designations correspond to protein marker (M), whole cell lysate (WCL), cell wall (CW), cytoplasm (C), extracellular fraction (E) and sterile Todd Hewitt broth (TH), which was used as a negative control.

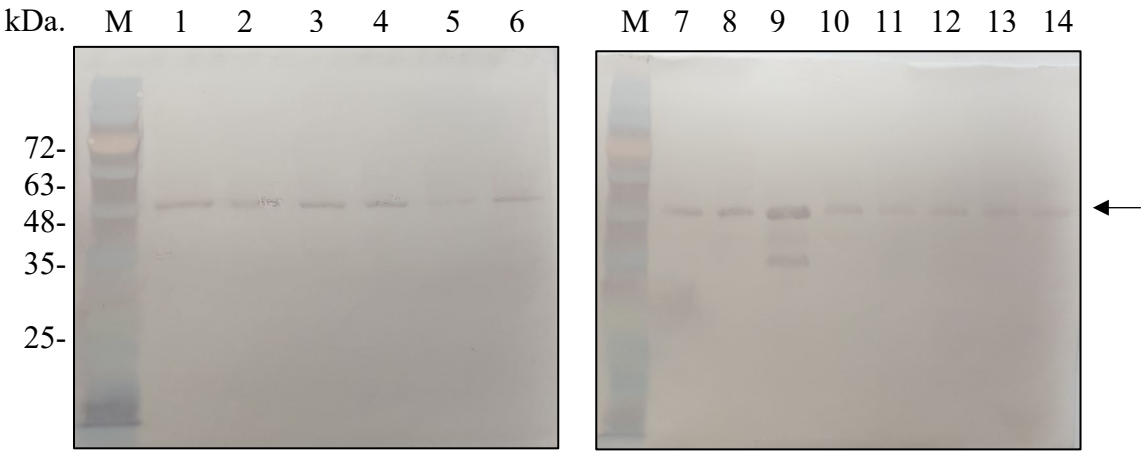

Figure 4.11. Immunoblots showing the reactivity of the EveA antibodies with GBS cell wall fraction preparations obtained from different GBS strains. The GBS strains used are: GBS NCS13, GBS non-typeable strain (18SR431), GBS non-typeable strain (17SR409), GBS non-capsulated strain (COH-13), GBS ST-Ia (97SR400), GBS ST-Ib (97SR570), GBS ST-II (97SR138), GBS ST-III (COH-I), GBS ST-IV (97SR331), GBS ST-V (97SR384), GBS ST-VI (9842), GBS ST-VII (7271), GBS ST-VIII (JM9), and GBS ST-IX (ATCC 27412) (Lanes 1 to 14, respectively). Lane M is a protein marker.

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Chapter 5

The effect of exogenous polyols (erythritol, sorbitol and mannitol) on Group B Streptococcus virulence.

5.1. Introduction:

The Group B Streptococcus (GBS), also known as *Streptococcus agalactiae*, is a Gram positive encapsulated bacteria which is invasive in adults as well as a major cause of pneumonia, sepsis and meningitis in the neonate (1–3). Of neonatal GBS infections, 10% become lethal, with 25- 35% of surviving infants with meningitis having permanent neurological sequelae (1). Interestingly, GBS is also a commensal bacteria of the adult gastro-intestinal tract and is present asymptomatically in the vaginal flora of 30% of healthy women (4,5). The signals controlling the switch from a commensal bacteria to a highly virulent bacteria is largely unknown. One factor that may be associated with the change to a virulent organism is exposure to polyols. Polyols (of which erythritol and mannitol are members) are sugar alcohols derived naturally from a variety of fruits and also can be commercially produced from other carbohydrates such as sucrose, glucose and starch (6–8). Polyols have been found also in the placentas of ruminants and amniotic fluids of human (9–12). The presence of erythritol in the placentas of ruminants has been proposed as an explanation for the accumulation of various species of virulent bacteria found at these sites, eventually leading to abortion (13,14). One such bacteria, *Brucella abortus,* was found to replicate to high numbers in the placenta due to the presence of erythritol (9,15). Work by Petersen *et al* studying the pathogenesis of *Brucella* abortion using a mouse model demonstrated that *Brucella* can localize to an artificial site containing erythritol within the mouse, resulting in a significant immune response (13). Microarray analysis of *Brucella* grown in media containing erythritol revealed an up-regulation of several virulence factors including genes related to carbohydrate metabolism such as *pgk* (phosphoglycerate kinase), *gapdh* (glyceraldehyde 3-phosphate

dehydrogenase) and *pgm* (phosphoglycerate mutase) (13,16). Other bacteria have also been found to be more virulent in the presence of erythritol. A study of *Chlamydia psittaci* showed that the addition of erythritol to the growth media significantly enhanced the growth of *C. psittaci* (14). A previous study investigated that mannitol (another polyol) promoted the adherence of *Burkholderia multivorans* by the upregulation of fimbrial and afimbrial adhesins genes (17,18). Based on these previous observations, we theorized that the presence of polyols may influence the way GBS interacts with its environment. The objective of this study was to investigate and compare the effect of three different exogenous polyols (erythritol, sorbitol and mannitol) on GBS growth, the surface expression of GBS phosphoglycerate kinase (GBS-PGK), bacterial survival in human blood and the ability to invade human cells.

5.2. Material and methods:

5.2.1. Bacterial strain, cell line, and growth conditions:

A previously described Group B streptococcus serotype III isolate collected from a case of severe LOD was used in this study (19). This strain was designated UAH150639. UAH150639 was grown in 2 ml of Todd-Hewitt (TH) broth overnight at 37°C and then transferred to 5 ml of fresh TH supplemented with varying concentrations of polyols the following day. The supplemented TH broth contained 1% (0.08 mol/L), 2% (0.16 mol/L) or 4% (0.32 mol/L) of erythritol (NowFood, USA), or 1% (0.055 mol/L), 2% (0.11 mol/L) or 4% (0.22 mol/L) of sorbitol (Sigma-Aldrich, St. Louis, USA), or 1% (0.055 mol/L), 2% (0.11 mol/L) or 4% (0.22 mol/L) of mannitol (Sigma-Aldrich, St. Louis, USA). The stocks of the polyols were prepared as 50% concentrations of each polyol by dilution in sterile distilled water and sterilized using filter sterilizations. In the control group, only TH broth was used, with no polyols added. The bacterial cells were cultured in a shaker incubator at 37 °C. Growth was assayed by measuring the absorbance at a wavelength of 600 nm at late log-phase of bacterial growth.

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) (ATCC, Manassas, VA, USA) was grown in OPTI-MEM I reduced serum medium supplemented with 4% fetal bovine serum (FBS) (Thermo Fisher Scientific, Toronto, Canada).

5.2.2. Assay for GBS-PGK surface expression in UAH150639:

The expression of GBS-PGK on the surface of GBS UAH150639 was assayed using an ELISA as previously described with some modifications (20). One ml of

UAH150639 was grown overnight in TH media supplemented with 1%, 2% or 4% of each of erythritol, mannitol or sorbitol. A 1 ml growth control with no polyols added was also used. This was then centrifuged to pellet the bacteria and washed once with 1 ml of Tris-buffered saline (TBS) and re-suspended in 1 ml of TBS. One hundred microlitres of washed bacterial cells were added to the wells of a 96-well polystyrene plate (Maxi-sorp; NUNC, Thermo Fischer Scientific, Nepean, Canada) and incubated for 2 h at 37°C to all bacterial cells to adhere to the plastic. Wells were washed once with TBS and blocked with 5% skim milk in TBS for 1 h. After blocking, the wells were washed 3 times with TBS and incubated for 1 h with anti-rGBS-PGK antibodies (1:300 in blocking buffer) (21). After 1 h, wells were further washed 3 times with TBS and incubated 1 h with anti-rabbit IgG-Horse-Radish Peroxidase then conjugate antibodies (1:1000 in blocking buffer; Sigma-Aldrich, St. Louis, USA). After 1 h, wells were washed 3 times with TBS and developed with 50 µl of TMP (Sigma-Aldrich, St. Louis, USA) for 30 min at room temperature before stopping the reaction with 50 µL of 2M sulphuric acid (H_2SO_4). The absorbance at 450 nm (A_{450}) was measured using a Revelation 4.25 microplate reader (Dynex Technologies, Chantilly, VA, USA). The A_{450} values obtained were compared with the average A_{450} measurement from UAH150639 bacterial cells grown without polyols to determine the relative A_{450}.

5.2.3. Anti-phagocytic activity of GBS grown in polyols:

To determine the ability of UAH150639 grown in polyols to resist phagocytosis and survive in heparinized human blood, the assay was performed as previously described with minor modifications (22). Overnight 1 ml cultures of UAH150639 was grown in the presence of different concentrations of 1%, 2% or 4% of either erythritol,

mannitol or sorbitol. The cultures were centrifuged to pellet the bacteria and washed once with 1X TBS buffer. A 0.5 McFarland standard of the bacterial cultures was made and then diluted in TH broth (1:10,000) to obtain 1.5 X 10^3 CFU/ml for the initial inoculum of the assay. Fifty microliters of the suspension containing approximately 75 CFU was added to 250 µl of freshly heparinized blood in sterile test tubes and was incubated at 37°C under gentle rotation (4 rpm). After 3 h of incubation, 150 µl aliquots of each of the suspensions were plated on blood agar plates (Dalyn Biologicals, Calgary, Canada) to determine the number of CFU after growth in human blood. Plates were incubated overnight at 37°C. Growth of UAH150639 strain in human blood was expressed by calculating the number of CFU obtained at the end of the incubation period over the number of CFU obtained at time zero (T_0). The growth of GBS culture in TH without polyols added to the media was used as a negative control for antiphagocytic activity. Blood was collected from healthy volunteers in 6 ml Sodium Heparinized Vacutainer tubes (BD, Oakville, Canada).

5.2.4. Invasion assay of HeLa cells by GBS grown in the presence of polyols:

To determine the effect of polyols on the invasion by GBS into a cultured cell line, a standard antibiotic protection assay was performed as previously described using HeLa cells (23). HeLa cell culture monolayers were grown to confluence in 24 well plates (Corning Costar TC-Treated multiple well plates, Sigma-Aldrich, St. Louis, USA). One ml of UAH150639 cultures grown overnight in TH broth with different concentrations of polyols (1%, 2% or 4%) (erythritol, mannitol, sorbitol), were washed once with 1X TBS and a 0.5 McFarland standard of each culture was made and then

diluted 1:100 in TH broth. Immediately, 100 µl (1.2 X 10^5 cfu) of 1:100 dilutions (giving a multiplicity of infection of 1:1) were added to the monolayer. The plates were centrifuged at 100 *g* for 5 min and incubated at 37°C in 5% CO_2 for 2 h. The HeLa cell monolayer was then washed 3 times with PBS and incubated for 2 h with fresh cell culture media containing 5 µg/ml of penicillin and 100 µg/ml of gentamicin to kill remaining extracellular bacteria. The HeLa cell monolayers were washed again with PBS, treated with trypsin, lysed with 0.1% Triton X-100, and plated on TH agar for quantitation of intracellular CFUs. Relative percent invasion was calculated as: Number of CFU (GBS treated with different concentrations of polyols) invaded into HeLa cells/ Number of CFU (GBS untreated with polyols) invaded into HeLa cells X 100.

5.2.5. Statistical analysis:

Data was analyzed using the Students t test and a p-value <0.05 was considered statistically significant. Data points correspond to the average value of all replicates and error bars represent the standard deviation.

5.3. Results:

5.3.1. Effect of erythritol, sorbitol and mannitol on the growth of GBS:

To determine if different concentrations (1%, 2% or 4%) of each polyol (erythritol, sorbitol or mannitol) has an effect on GBS growth, the absorbance (A_{600}) of 20 h liquid cultures of GBS UAH150639 grown in various concentrations of polyols or without polyols were measured (Fig. 5.1). None of the three polyols assayed displayed any significant enhancement or inhibition of GBS growth. This suggests that the three polyols assayed have no significant effect on GBS growth.

5.3.2. Expression of PGK on the surface of GBS treated with polyols:

We have previously shown that GBS expresses PGK on its surface in addition to intracellular expression (21). To determine if GBS grown in media containing polyols affects the surface expression of GBS-PGK, the amount of surface expressed GBS-PGK was assayed using antibodies targeting GBS-PGK after growth of UAH150639 in the presence of 1%, 2% or 4% of erythritol, sorbitol, or mannitol. UAH15039 grown in TH broth supplemented with 1%, 2%, or 4 % erythritol significantly increased surface expression of GBS-PGK compared with GBS grown in media without erythritol (P=0.003, P=0.012, P=0.014 respectively) (Fig. 5.2). Interestingly, UAH15039 grown in TH broth containing 1%, 2% or 4% sorbitol or mannitol had no significant effect on the surface expression of GBS-PGK, (P=0.37, P=0.298, P=0.093, respectively for sorbitol) (Fig. 5.2), (P=0.215, P=0.054, P=0.074, respectively for mannitol) (Fig. 5.2), compared

with GBS grown without addition of sorbitol or mannitol to media.

5.3.3. Contribution of erythritol, sorbitol and mannitol to the antiphagocytic activity of GBS:

In order for GBS to cause sepsis, and in some cases meningitis, GBS must evade such host defenses as phagocytosis. We therefore wanted to determine if GBS grown in the presence of polyols enhanced the survival of GBS in human blood. The anti-phagocytic ability of GBS UAH150639 grown in different concentrations of polyols was determined by measuring the bacterial ability to multiply in fresh human blood during a 3 h incubation period. UAH150639 grown in media supplemented with 1%, 2% or 4% of erythritol or sorbitol increased the number of bacteria able to survive in fresh human blood (erythritol: P=0.026, P=0.019 and P=0.0002, respectively; sorbitol: P= 0.036, P= 0.034 and P= 0.002, respectively) compared with GBS grown with 0% of polyols (Fig. 5.3). Interestingly, GBS grown in the presence of 1% mannitol did not have as significant a survival percentage (P=0.32) as erythritol or sorbitol, however, UAH150639 did show some enhanced survival when grown in 2% or 4% mannitol only (P=0.036 and P=0.035, respectively) (Fig. 5.3).

5.3.4. Invasion of HeLa cells by GBS in the presence of polyols:

GBS is a highly invasive organism being able to invade a variety of host cells. We were interested in determining if this phenotype could be enhanced when grown in the

presence of polyols. Similar to the previously described experiments, UAH150639 was grown in THB in the presence of different concentrations (1%, 2% or 4%) of polyols (erythritol, sorbitol, and mannitol) and then assayed for the bacteria's ability to invade HeLa cells. Interestingly, the number of intracellular CFU recovered after infection was significantly increased only in GBS grown in 1% erythritol (P= 0.041) and not with 1% sorbitol (P= 0.118) or 1% mannitol (P= 0.484) (Fig. 5.4). It was found that GBS treated with 2% or 4% of the polyols significantly reduced the number of CFUs indicating a reduced ability to invade in comparison to GBS grown in the presence of 1% polyols (erythritol: P= 0.011, P= 0.045, respectively; sorbitol: P= 0.002, P= 0.012, respectively; mannitol: P= 0.007, P= 0.011, respectively) compared to the invasion with GBS untreated (Fig. 5.4).

5.4. Discussion:

In the present study, I investigated the effect of erythritol, sorbitol and mannitol on GBS virulence. The GBS strain used in this study was serotype III clinical isolates (UAH150639). I showed that growing GBS in the presence of three different concentrations (1%, 2% and 4%) of erythritol, sorbitol or mannitol did not affect bacterial growth and was similar to the growth of GBS in the media without addition of polyols (Fig. 5.1). In contrast to our investigation, previous studies with oral streptococci demonstrated that the addition of polyols to the bacterial media significantly inhibited the growth of *Streptococcus mutans* and *Streptococcus gordonii* (24,25). Other studies in *Brucella* and *Chlamydia* showed different results in that there was significant increase in bacterial growth when the bacteria were grown in media supplemented with polyols (9,14,15). The explanation for the differences in bacteria growth in the presence of polyols may be due to the fact that different bacteria vary in their susceptibility to environmental changes.

The attachment and internalization of GBS into eukaryotic cells occurs through a number of surface-expressed and secreted proteins (26–29). One such protein in GBS is phosphoglycerate kinase (PGK), an important glycolytic enzyme that is expressed inside the cell, where it is involved in glycolysis, as well as expressed on the surface of the GBS cell (21). The surface localization of PGK appears to contribute to bacterial virulence through its ability to bind strongly to host plasminogen (21). The fact that PGK binds to plasminogen suggests a potential role for PGK in aiding dissemination of the bacteria within the host through the breakdown of host extracellular matrix proteins and endothelium. In this study, we tested the expression of PGK on the surface of GBS

treated with different concentrations of erythritol, sorbitol or mannitol. GBS UAH150639 grown in media supplemented with 1%, 2% or 4% of erythritol had a significantly increased GBS-PGK surface expression as compared to GBS grown without erythritol (Fig. 5.2). However, this increase in GBS-PGK surface expression was not seen when GBS was grown in the presence of sorbitol or mannitol (Fig. 5.2). The results suggest that the increased expression of GBS-PGK on the surface of GBS treated with erythritol was not due to increased bacterial growth, as the presence of different concentrations of erythritol was previously shown to not affect GBS bacterial growth (Fig. 5.1). These results instead suggest that erythritol plays a role in the upregulation of the *pgk* gene in GBS. This result was similar to previous results from studies on *Brucella* grown in media containing erythritol, which showed an up-regulation of the genes related to carbohydrate metabolism, including the *pgk* gene (13,16).

In order to understand the contribution of polyols to GBS antiphagocytic activity, we determined the antiphagocytic activity of GBS UAH150639 in the presence of different concentrations of erythritol, sorbitol and mannitol. We observed increased phagocytosis of GBS in the presence of 1%, 2% and 4% of erythritol and sorbitol, as well as in 2% and 4% of mannitol. This may be due to the upregulation of GBS genes that act in bacterial opsonic activities. Further evidence for the possible involvement of erythritol in virulence was investigated by García-Lobo *et al.,* who demonstrated that erythritol significantly upregulated the *virB* genes in *Brucella*, which are essential for virulence and intracellular replication within macrophages (16).

The unexpected finding in this study is that the invasion of human epithelial cells by the GBS (15SR0639) strain pre-incubated in media supplemented with 2% and 4% of

polyols (erythritol, sorbitol and mannitol) decreased significantly as compared to the GBS invasion without the addition of polyols (Fig. 5.4). The significant increase in the invasion of HeLa cells by GBS pre-incubated with 1% erythritol, but not with 1% of sorbitol or mannitol, raises an important question as to whether this phenomenon coincides with the increase in GBS-PGK surface expression in GBS treated with 1% erythritol. It appears that 1% of erythritol is the optimal concentration for GBS to enhance or upregulate genes important for GBS invasion. In contrast to 1% of erythritol, 1% of sorbitol and mannitol do not have any significant effect on GBS invasion, and this may be due to the insignificant increase of PGK surface expression on GBS treated with sorbitol and mannitol. It may be that erythritol, sorbitol and mannitol at concentrations higher than 1% have a reverse effect on the expression of genes involved in bacterial invasion, which would explain our findings that there was a significant decrease in GBS invasion by 2% and 4% of polyols (erythritol, sorbitol and mannitol). As it has been previously demonstrated with oral Streptococci, 2% and 4% of polyols decrease bacterial adherence and biofilm formation in *S. mutans* and *S. gordonii* (24,25,30).

The results in this study indicate that 1% of erythritol has a significant effect on GBS virulence. 1% of erythritol added to the media enhances GBS-PGK surface expression, GBS anti-phagocytic activity and GBS invasion into HeLa cells. It was hypothesized that GBS pathogenesis was enhanced by the synergistic effect of the expression of virulence genes. This study demonstrated that despite the increase in GBS-PGK surface expression in the presence of 2% and 4% of erythritol, its effect on GBS virulence is dependent on the expression of other virulence genes, for example the genes involved in GBS invasion. We also observed that, although the bacterial ability to resist

phagocytosis was increased in the presence of sorbitol and mannitol, the GBS-PGK surface expression was not significantly increased, which confirms the hypothesis about GBS pathogenesis.

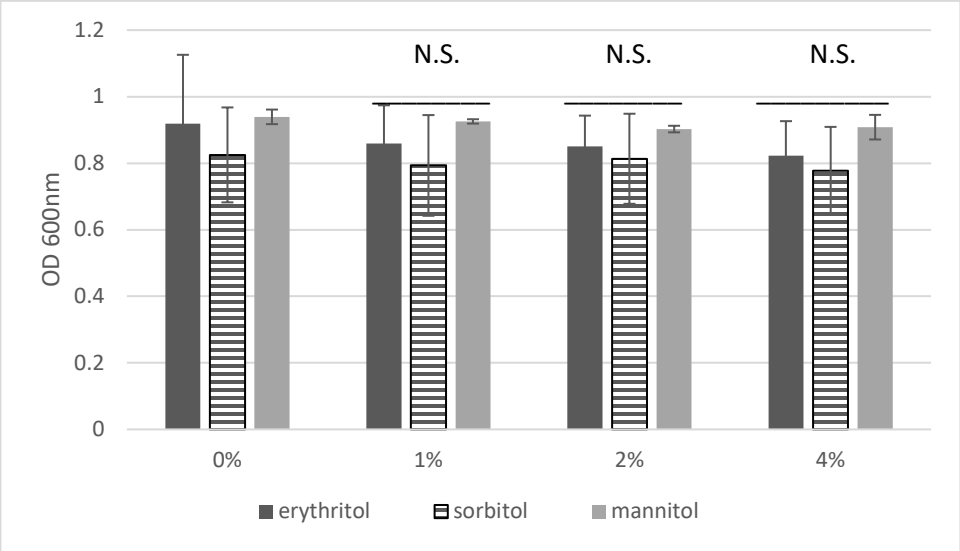

Figure 5.1. Growth of GBS UAH150639 in TH broth supplemented with 1%, 2% or 4% of erythritol, sorbitol or mannitol. All the cultures were grown for 20 h and their growth measured by optical density at 600 nm. Data presented is the average OD_{600} of three independent experiments; error bars represent standard deviation. OD_{600} values were compared with the value obtained from GBS UAH150639 grown without polyols to determine statistical significance; N.S indicates not significant.

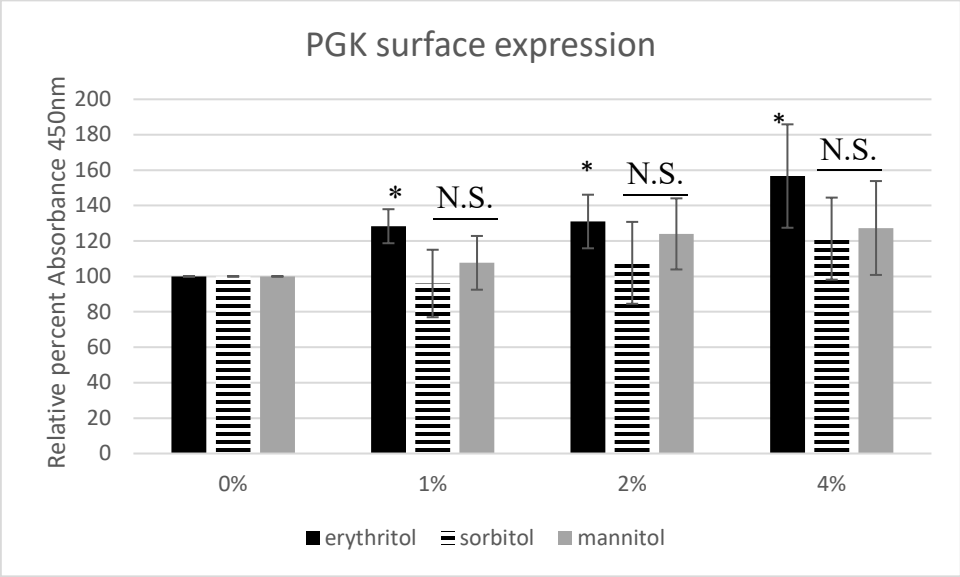

Figure 5.2. Expression of PGK on the surface of GBS grown in the presence of polyols. GBS UAH150639 was grown for 20h in TH broth supplemented with 1%, 2% or 4% of erythritol (A), sorbitol (B) or mannitol (C). The quantity of GBS-PGK expressed on the bacterial surface was measured using polyclonal antibodies against GBS-PGK. Triplicate experiments were performed in triplicate. The bars represent the average relative A_{450} values from all 9 replicates compared with GBS grown in THB without addition of polyols (0%); error bars represent standard deviation. A_{450} values were compared with the value obtained from (0%) to determine statistical significance; an asterisk (*) indicates statistical significance ($P < 0.05$) and N.S indicates not significant.

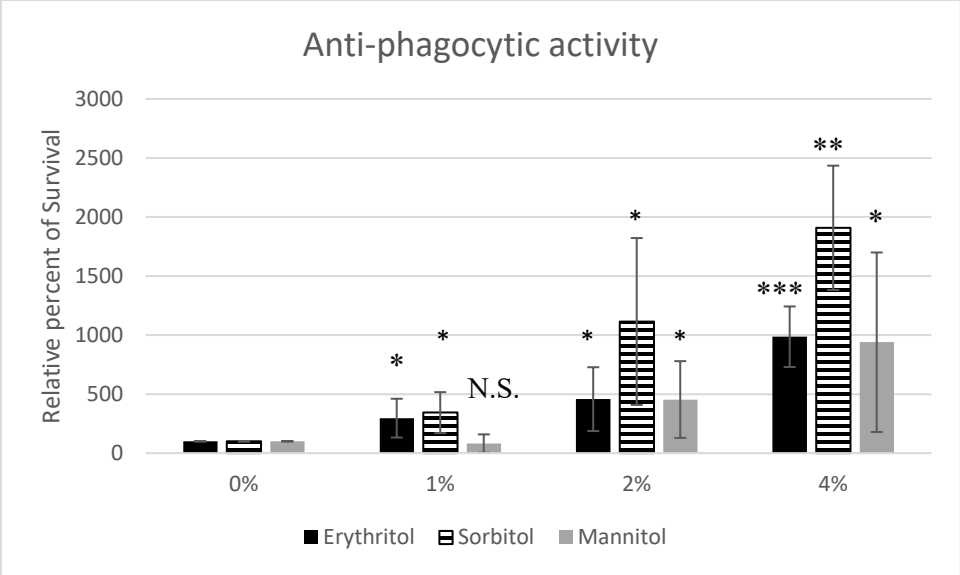

Figure 5.3. Survival of GBS in fresh human blood after 20 h growth in polyols. GBS was grown in 1%, 2% or 4% of erythritol, sorbitol or mannitol and then assayed for the bacteria's ability to survive in fresh human blood. Survival is expressed as a relative percent of survival in comparison to the control (UAH150639 grown in TH broth only). Relative percent of survival is the number of CFU obtained at the end of the incubation period over the number of CFU obtained at time zero multiplied by 100. Bars represent the average values obtained from three independent experiments compared with the value obtained from (0%) to determine statistical significance; error bars represent standard deviation; an asterisk (*) indicates statistical significance ($P < 0.05$), (**) indicates ($P < 0.01$), (***) indicates ($P < 0.001$) and N.S indicates not significant.

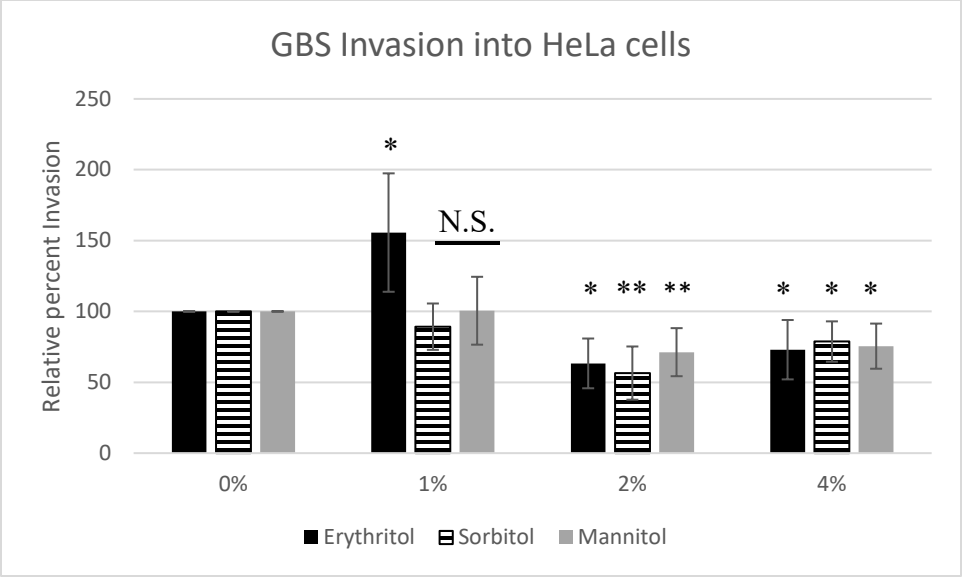

Figure 5.4. Invasion of HeLa cells by GBS UAH150639 grown in 1%, 2% or 4% erythritol, sorbitol, mannitol. Intracellular CFU were recovered after 2 hours of infection and was expressed as relative percent invasion. Bars represent the average values obtained from three independent experiments compared with the value obtained from (0%) to determine statistical significance; error bars represent standard deviation; an asterisk (*) indicates statistical significance ($P < 0.05$), (**) indicates ($P < 0.01$) and N.S indicates not significant.

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Chapter 6:

The Group B Streptococcus phosphoglycerate kinase mutant reduces plasminogen binding to the bacterial surface

6.1. Introduction:

Streptococcus agalactiae (Group B Streptococcus, GBS) is a commensal bacterium of the adult gastro-intestinal tract and is present asymptomatically in the vaginal flora of 10-30% of healthy women (1). Although GBS is a commensal bacterium, GBS is considered the leading cause of invasive infections in neonates causing pneumonia, septicemia and meningitis, as well as a serious cause of mortality or morbidity in adults with underlying diseases (2–4). As with most streptococci, the process of human infection by GBS is complex and multifactorial (5). Adhesion and invasion of GBS into the host cell involves a number of pathogen-host cell interactions. The first step in host cell invasion involves adhesion of GBS to the host cells, which is mediated by multiple interactions between bacterial surface proteins and related receptors, such as fibrinogen, laminin, fibronectin and plasminogen that are present on host cell surfaces (6–11). GBS surface-associated proteins are important in the host-pathogen relationship as they can provide the first contact of the bacteria with the host before internalization (12). Of these GBS proteins, laminin-binding proteins, C5a peptidase, glyceraldehyde 3 phosphate dehydrogenase and lipotechoic acid and many others were thought to mediate host-pathogen interaction (8,13–15).

While many GBS surface proteins have well characterized roles, the role of surface-expressed phosphoglycerate kinase (PGK) in GBS virulence is not clearly defined. PGK is one of the glycolytic enzymes that catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP producing 3-phosphoglycerate and ATP. Typically, PGK is a cytoplasmic protein lacking a signal sequence or a hydrophobic anchor. However, it has also been identified as being

expressed on the surface of GBS and *Streptococcus pneumoniae* (9,12,16). The observation that GBS-PGK can bind host plasminogen suggests a role of this protein in GBS virulence (9). Plasminogen is an inactive proenzyme that is present in plasma and can be converted to plasmin by host activators, such as tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (17). During infection, plasminogen binds to the GBS surface, and is then converted into plasmin by host tPA or uPA (18,19). Plasmin then contributes to the generation of active metalloproteases, degradation of the extracellular matrix and severe weakening of the blood brain barrier (18–20).

It is unclear how surface-expressed GBS-PGK participates in bacterial pathogenesis. The GBS-PGK is encoded by a single gene, and since PGK is essential for cell function, several attempts to create a GBS mutant lacking GBS were unsuccessful. Here, by exploiting a novel strategy called auxotrophic complementation, a GBS *pgk* mutant was created. Growth media of the GBS *pgk* mutant was supplemented exogenously with 10% fetal bovine serum to supplement the deficiency that the mutant strain had. The growth pattern of this mutant was less compared to the wild type and the complemented mutant strains. Plasminogen binding to the surface of the mutant strain was less, thereby confirming the role of surface-expressed GBS-PGK in binding to host plasminogen and contributing to bacterial pathogenesis. The present study, therefore, was carried out to address why PGK is exported onto the GBS surface and to elucidate the significance of plasminogen binding to GBS-PGK located on the surface of GBS, as well as the role of PGK in GBS pathogenesis. Although the results of this study are preliminary and did not reveal the role of surface expressed GBS-PGK in bacterial virulence, further work can be done using this GBS *pgk* mutant strain.

6.2. Materials and Methods:

6.2.1. Bacterial strains, plasmid and growth conditions:

The Group B streptococcus NCS13 strain was obtained from our own laboratory stock. The GBS NCS13 wild type strain, the GBS Δ*pgk* strain and GBS Δ*pgk* complemented with pUC19-*pgk* were grown in Todd Hewitt broth medium (Difco Laboratories, Detroit, Mich.) for 18-24 h at 37ºC. If necessary, the medium was supplemented with erythromycin (1 μg/ml) and with 10% fetal bovine serum (FBS) (Gibco BRL, Burlington, ON). The *Escherichia coli* DH5α with pUC19-*pgk* (21) was cultured at 37°C in Luria-Bertani medium (Gibco-BRL, Burlington, ON) containing ampicillin (100 μg/ml) as needed.

6.2.2. Generation of GBS *pgk* deletion mutant and complemented strain:

The GBS *pgk* gene was amplified by PCR from genomic DNA of NCS13 strain. Primers containing one artificial restriction site at each end were constructed according to the *pgk* nucleotide sequence. The primer sequences used in this study are listed in Table 6.1. Construction of GBS *pgk* mutant was accomplished via insertional inactivation with an erythromycin resistance gene via an allelic exchange strategy (Fig. 6.1). To achieve this, the upstream (5') and downstream (3') DNA fragments (500 bp each) of the *pgk* gene were amplified by PCR from GBS NCS13 genomic DNA, along with an erythromycin resistance cassette (*ermB*) (800 bp) from the pTV1-OK plasmid. The forward and reverse primers PF36/PR35 (upstream fragment), PF34/PR33 (downstream fragment) and PF40/PR2 (*ermB* gene) were used in this reaction. The upstream and downstream fragments of the *pgk* were digested with restriction enzymes KpnI and

BamHI for 1 h at 37ºC, respectively. The *ermB* gene was double digested with KpnI and BamHI. The DNA fragments were purified using a commercial DNA extraction and purification kit (Qiagen, Mississauga, Ontario, Canada). The purified DNA were ligated together at room temperature for 18 h with T4 DNA ligase (Invitrogen) and amplified by PCR with the primers PF36/PR33 to generate a 1.8 kb construct, *5'pgk(up)-ermB-5'pgk(down)*. The construct was then transformed into GBS electro-competent cells by electroporation and plated onto TH agar containing 1 μg/mL of erythromycin and 10% of FBS. PCR amplification was performed to confirm the presence of the desired mutation. For complementation of the mutant strain of *pgk*, a previously cloned 1.2 kb fragment, encompassing the WT *pgk* gene, incorporated into the pUC19 plasmid to create pUC19-*pgk* (21) was used to transform the electro-competent Δ*pgk* mutant. The transformants were selected on TH agar plate without addition of 10% FBS and erythromycin. PCR amplification was then performed on genomic DNA extracted from the transformants to confirm the replacement of the mutant *pgk* gene with the WT *pgk* gene.

6.2.3. Analysis of GBS-PGK surface expression:

Expression of GBS-PGK on the surface of the GBS Δ*pgk* mutant strain was assayed using an ELISA as previously described with some modifications (22). Overnight liquid growth culture of the GBS NCS13 WT strain, GBS Δ*pgk* mutant strain and complemented GBS Δ*pgk* mutant strain were washed once with 1 mL of PBS and re-suspended in 1 mL of PBS. The absorbance at 600 nm (A_{600}) was measured and used to determine the bacterial concentration. The bacterial cultures were adjusted to the same A_{600} as the GBS NCS13 wild type strain. One hundred microliters of washed bacterial

cultures were fixed to the wells of a 96-well polystyrene plate (Maxi-sorp; NUNC, Thermo Fischer Scientific, Nepean, Ontario, Canada) for overnight incubation at 37°C. Wells were washed once with PBST (PBS with 0.01% Tween-20) and blocked with 5% skim milk in PBST for 1 h. After blocking, the wells were washed 3X with PBST and incubated for 1 h with 100 µl anti-rGBS-PGK antibodies (0 or 1:3000) (23) diluted in blocking buffer. The wells were washed 3X with PBST and incubated 1 h with anti-rabbit IgG-Horseradish peroxidase conjugate antibodies (Sigma-Aldrich) (100 µl; 1:10,000) diluted in blocking buffer. The wells were then washed 3X with PBST and developed with 50 µL of 3,3',5,5'-Tetramethylbenzidine (TMP) (Sigma-Aldrich) for 30 minutes at room temperature before stopping the reaction with 50 µL of 2M sulphuric acid (H_2So_4). The absorbance at 450 nm (A_{450}) was measured using a Revelation 4.25 microplate reader (Dynex Technologies, Chantilly, Virginia, USA). The A_{450} values obtained were compared with the average A_{450} measurement from the GBS NCS13 wild type strain to determine the relative A_{450}.

The A_{450} measurements from the wells incubated without anti-rGBS-PGK were subtracted from the A_{450} measurements obtained from the wells incubated with 100 µl anti-rGBS-PGK antibodies (1:3000) to control for non-specific binding of the anti-rabbit IgG-Horseradish peroxidase conjugate antibodies to the bacterial surface.

6.2.4. Plasminogen binding assay to the surface of GBS strains:

Binding of plasminogen to the surface of the GBS Δ*pgk* mutant strain was analyzed using an ELISA assay. One milliliter of overnight growth cultures of the GBS strains were washed once with 1X PBS and re-suspended in 1 mL of 1X PBS to the same

A_{600} as the GBS NCS13 wild type strain. One hundred microliters of the bacterial suspensions were aliquoted onto a 96-well microtiter plate for overnight incubation at 4°C. The plate wells were washed 1X with PBS and blocked with 5% skim milk in PBST (PBS with 0.01% Tween-20) for 1 h at 37°C. After blocking, the immobilized cells were washed 3X with PBST and incubated for 1 h at 37°C with plasminogen (0 or 0.1 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) diluted in blocking buffer. After washing 3X with PBST, a further 1 h incubation was performed in the presence of mouse anti-human plasminogen (SBF1) (Thermo Scientific, Rockford, Illinois, USA) (100 μl; 1:300) diluted in blocking buffer. The wells were washed again and incubated for 1 h at 37°C in the presence of anti-mouse IgG conjugated with alkaline phosphatase (100 μl; 1:5000) (New England Biolabs, Ltd., Whitby, Ontario, Canada). The wells were washed 3X with PBST and developed with 100 μL of alkaline phosphatase yellow substrate (Sigma-Aldrich) for 20 minutes at room temperature. The absorbance at 405 nm (A_{405}) was measured using a Revelation 4.25 microplate reader (Dynex Technologies, Chantilly, Virginia, USA). The A_{405} values obtained were compared with the average A_{405} measurement from the GBS NCS13 wild type strain to determine the relative A_{405}.

The A_{405} measurements from the wells incubated without plasminogen were subtracted from the A_{405} measurements obtained from the plates incubated with 0.1 μg/ml plasminogen to control for non-specific binding of the antibodies to bacterial surface.

6.2.5. Statistical analysis:

Data was analyzed using the Students t test and a p-value <0.05 was considered statistically significant. Data points correspond to the average value of all replicates and error bars represent the standard deviation.

6.3. Results:

6.3.1. Characterization of the GBS *Δpgk* mutant:

A defined *erm*^{r} *Δpgk* mutant of the GBS NCS13 strain was constructed by using the auxotrophic complementation strategy. A GBS auxotrophic for the phosphoglycerate kinase (PGK), an enzyme crucial in the glycolysis cycle, was constructed by knocking out the *pgk* gene by insertional inactivation with an erythromycin resistance (*erm*) gene via homologous recombination (Fig. 6.1). To compensate for this mutation, the auxotrophic mutant strain must be grown by including the appropriate auxotrophic growth factor in the medium. In this study, the media was supplemented with 10% FBS, and the mutant was successfully selected from the media supplemented with 10% FBS and containing 1 μg/mL erythromycin. Chromosomal DNA was isolated from the mutant strain and from the wild type strain for PCR amplification of the *pgk* gene. The PCR product of the *pgk* gene from the mutant strain contained a higher molecular size (~1.8 Kb) compared to the product from the wild type strain (~1.2 Kb), which confirmed the insertion of the *erm* gene into the *pgk* gene by homologous recombination (Fig. 6.2B). Complementation of the *Δpgk* mutant with WT *pgk* gene was achieved using a suicide vector (pUC19) (Fig. 6.2A). A wild type copy of the *pgk* gene was cloned into the pUC19 plasmid (21) and the resulting plasmid, pUC19/*pgk,* was electroporated into the *Δpgk* mutant strain to generate the complemented *Δpgk* mutant strain. This suicide vector directs insertion of the complementing gene (WT *pgk* gene) into the chromosomal *pgk::erm* gene via homologous recombination. We verified insertion of the WT *pgk* gene into the *pgk::erm* gene by PCR with primers PF36/PR33. The PCR product of the *pgk* gene from the complemented *Δpgk* mutant strain had a similar molecular weight as the

PCR product from the WT strain (Fig. 6.2B). The complemented mutant was sensitive to erythromycin and that was confirmed phenotypically by replica plating the complemented mutant on TH agar with and without 1 μg/mL erythromycin (Fig. 6.3). The GBS *Δpgk* mutant is a phosphoglycerate kinase auxotroph and only grows on media supplemented with 10% FBS, in contrast to the wild type strain and complemented mutant, which both have the functional *pgk* gene (Fig. 6.3A). Similarly, the optical density of the overnight liquid culture of the GBS wild type, GBS *Δpgk* mutant and complemented GBS *Δpgk* mutant strains in TH broth with and without 10% FBS was measured at an OD_{600} and indicated that the growth rate of the mutant is much weaker, even with 10% FBS supplementation than wild type and complemented GBS *Δpgk* mutant strains (Fig. 6.3B).

6.3.2. GBS *Δpgk* mutant expresses less PGK on the bacterial surface:

To determine whether *pgk* expression was taking place in the *Δpgk* mutant and if we would be able to complement the mutant strain with pUC19/*pgk* plasmid, the amount of GBS-PGK expressed in these bacterial cultures was analyzed via an ELISA assay using anti-PGK polyclonal antibodies (23). The wild-type and complemented *Δpgk* strains showed the presence of surface-expressed PGK protein, whereas the mutant strain expresses significantly lower (P= 0.030) PGK on the mutant surface (Fig. 6.4).

6.3.3. GBS *Δpgk* mutant has less plasminogen binding to the bacterial surface:

Our lab has previously demonstrated that the GBS-PGK protein binds strongly to plasminogen (9). The previous assay showed that the GBS *Δpgk* mutant strain had

significantly reduced amounts of surface-expressed GBS-PGK compared with that of wild type NCS13 and the complemented GBS *Δpgk* mutant strain. To determine whether inactivating the *pgk* gene in GBS will result in decreased plasminogen binding to the surface of GBS, an ELISA assay was conducted. Plasminogen binding to the surface of bacterial strains, GBS NCS13 wild type, GBS *Δpgk* mutant and complemented GBS *Δpgk* mutant strains, was tested following incubation of the bacterial cells with 0.1 µg/ml of plasminogen. The mutant strain showed a significant reduction (P= 0.000045) in plasminogen binding, resulting in a reduction of plasminogen binding to the surface of the mutant compared to the GBS NCS13 wild type (Fig. 6.5). The complemented GBS *Δpgk* mutant strain showed non-significant reduction in the A_{405} reading of plasminogen binding to the surface of the bacteria, similar to the A_{405} reading from the GBS NCS13 wild type strain (Fig. 6.5).

6.4. Discussion:

PGK is one of the key cytoplasmic enzymes involved in glycolysis, which also belongs to a novel class of anchorless cell wall-associated surface proteins. Several proteins, including PGK, that belong to this class of surface proteins are not only multifunctional, but are also essential for bacterial survival. Therefore, the resulting non-feasibility of creating a knockout mutant has been a major limiting factor in understanding their roles in GBS pathogenesis. In the present investigation, this limiting factor has been addressed by exploiting a strategy called auxotrophic complementation, which involves making a traditional gene knockout and supplementing the media with essential nutrients to maintain the bacterial viability. This strategy has been used previously with other microorganisms to study the role of essential genes in biological processes (24–27).

The importance of the GBS-PGK enzyme in cellular function was clearly observed in this study. The growth rate of the GBS *pgk::erm* mutant was significantly less than the wild type strain, even when the media was supplemented with 10% FBS. Growing the mutant in the presence of FBS was much better than in the absence of FBS. This identification might indicate that 10% FBS is not adequate to maintain the mutant growth at a level similar to the growth rate of wild type stain or that the *pgk* mutant would need better nourished media in addition to FBS. Interestingly, the *pgk* mutant was observed to grow in TH broth, even without any auxotrophic supplementation of FBS, which suggests that some of the TH broths' components might be complementing the defect of the *pgk* mutation.

To confirm that the inactivation of the GBS *pgk* gene was achieved, an ELISA assay was performed. The ELISA assay analysis using polyclonal rabbit anti-PGK antibodies demonstrated a significant reduction of *pgk* gene expression in the GBS *pgk::erm* mutant strain and showed that the PGK production was restored when this mutant strain was complemented with the wild type *pgk* gene from the pUC19/*pgk* plasmid.

PGK and many of the other anchorless proteins are multifactorial. In addition to the involvement in glycolysis, our laboratory has previously shown that GBS-PGK binds host protein plasminogen (9). Binding of the GBS-PGK to plasminogen suggests a role of surface-expressed GBS-PGK in disease pathogenesis. The biological significance of plasminogen binding to surface-expressed GBS-PGK is unknown. In this study, plasminogen binding to the surface of the GBS *pgk* mutant was significantly less than the binding to the wild type strain. This result confirmed the role of surface-expressed GBS-PGK in host plasminogen binding.

Finally, the results reported here demonstrate that the *pgk* mutant of GBS was successfully constructed. Compared to the wild type and complemented mutant, the mutant strain showed less growth rate, less GBS-PGK surface expression and less plasminogen binding to the surface of the mutant. Although the pathogenicity of GBS due to the ability of the pathogen to bind to host plasminogen was not assessed here, the work presented in this chapter will help for future research identifying the role of PGK in GBS pathogenesis.

Table 6.1. Primers used in this chapter. The underlined sequences indicate the restriction sites.

Primer	Sequence (5'—3')	Restriction site	Description
P33	CTC CTG CAG TTA TTT TTC AGT CAA TGC TGC C	PstI	Reverse primer for 3' fragment of *pgk* gene
P34	TAC GGT ACC AGG TAT CGA AAT CGG TAA CTC	KpnI	Forward primer for 3' fragment of *pgk* gene
P35	TTC GGA TCC GAA ATA CCT ACG TTT GAT GCA T	BamHI	Reverse primer for 5' fragment of *pgk* gene
P36	ATG GCT AAA TTG ACT GTT AAA GA		Forward primer for 5' fragment of *pgk* gene
P40	TTC GGA TCC TTA CAA ACA AAT CGT TTA ACT TC	BamHI	Forward primer for *ermB* gene
P2	TAC GGT ACC GAA TTA TTT CCT CCC GTT AAA	KpnI	Reverse primer for *ermB* gene

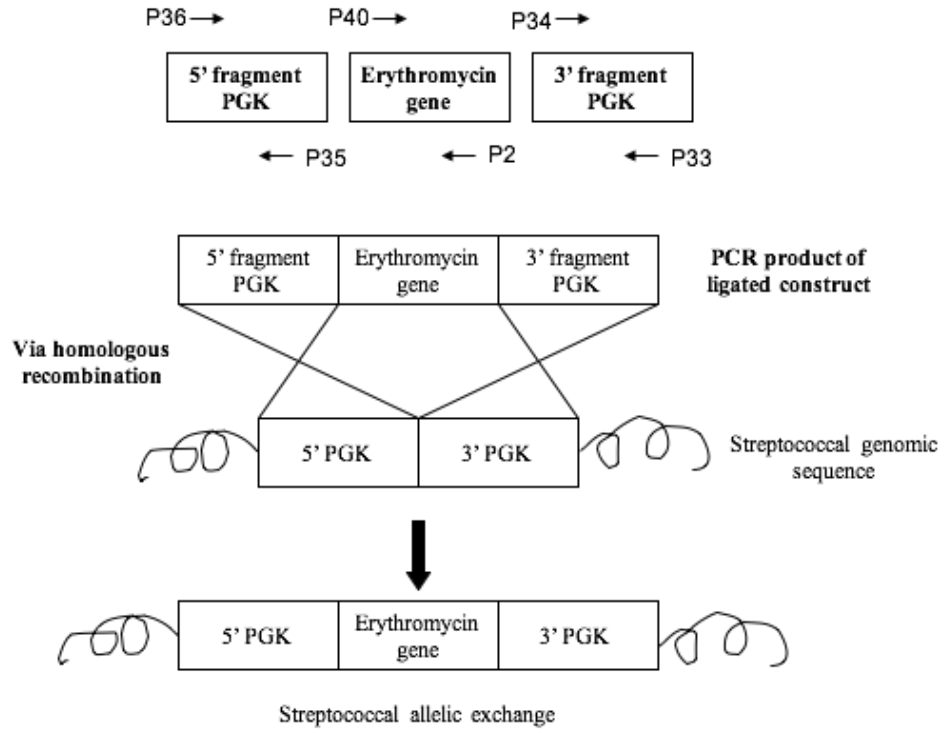


Figure 6.1. Strategy for construction of the GBS-PGK deletion mutant by homologous recombination. The upstream (5') and downstream (3') DNA fragments of the *pgk* gene were amplified by PCR from GBS NCS13 genomic DNA, along with an erythromycin resistance cassette (*ermB*) from pTV1-OK plasmid. The fragments were ligated together and amplified by PCR with the primers P36/P33 in order to generate a 1.8 kb construct that was used to transform GBS NCS13. The construct was transformed into GBS electro-competent cells and plated onto TH agar supplemented with 10% FBS.

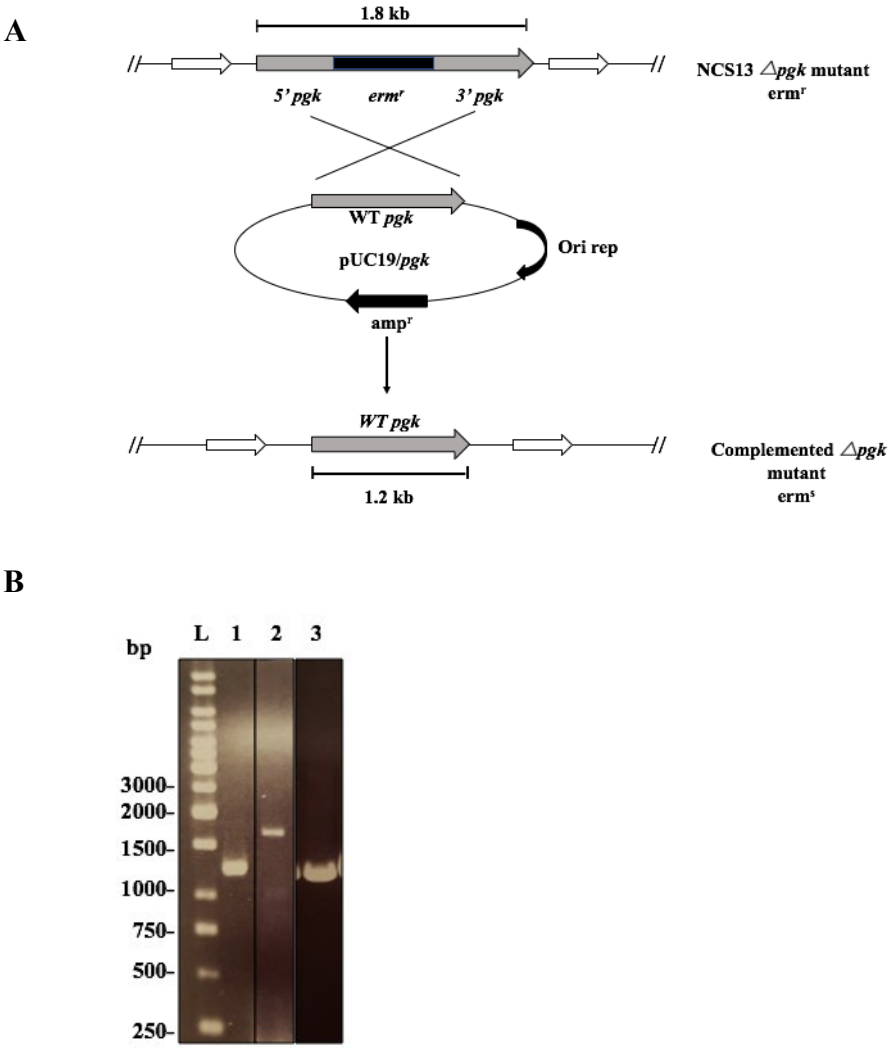
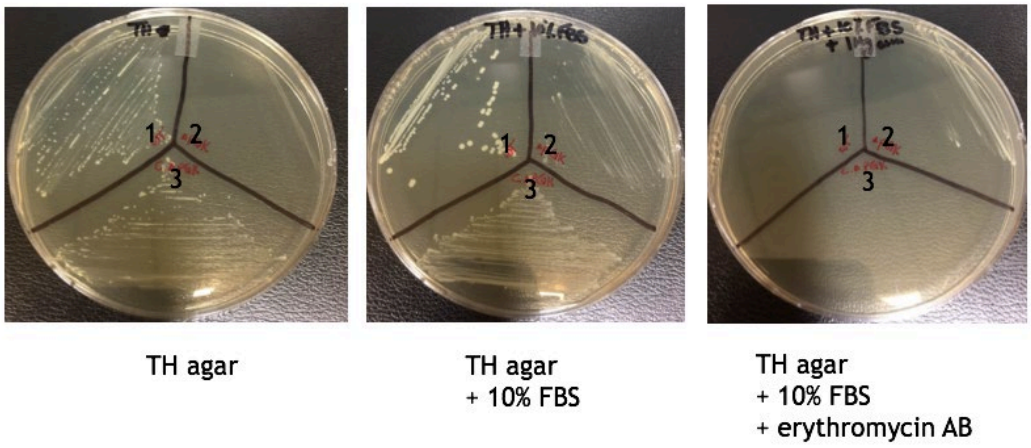


Figure 6.2. Genetic confirmation of the inactivation and complementation of the phosphoglycerate kinase gene (*pgk*) in GBS Δ*pgk* mutant. (A) diagram depicting construction of complemented Δ*pgk* mutant using a suicide vector pUC19/*pgk*. (B) agarose gel electrophoresis of the PCR amplification of the *pgk* gene from GBS NCS13 wild type strain (lane 1), GBS Δ*pgk* mutant (lane 2), and complemented GBS Δ*pgk* mutant (lane 3). L: 1 kb plus DNA ladder (Invitrogen).

A



B

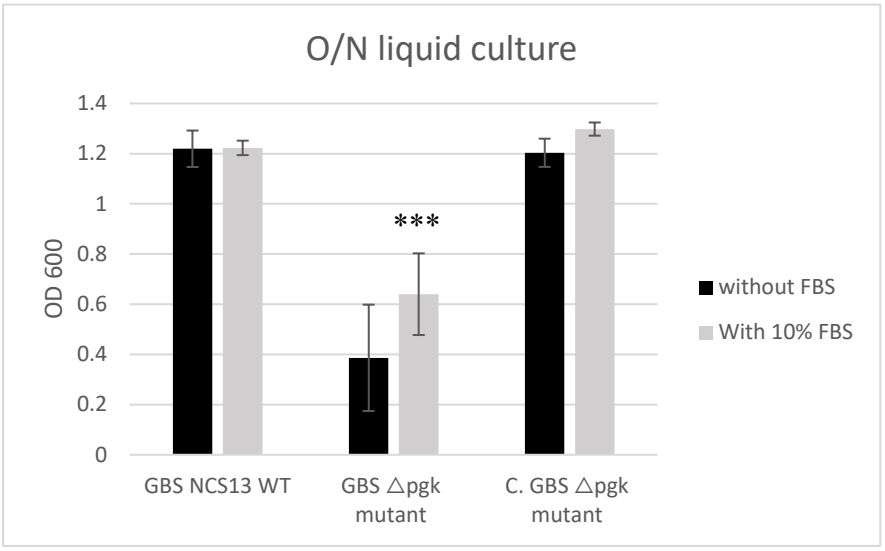


Figure 6.3. The GBS Δ*pgk* mutant is auxotrophic for phosphoglycerate kinase. (A) The wild type strain (1), the GBS Δ*pgk* mutant (2) and the complemented GBS Δ*pgk* mutant (3) were grown on TH agar plates with or without the addition of 10% FBS or 1 μg/mL erythromycin. (B) Optical density of overnight cultures grown in TH broth with or without FBS. Data presented is the average OD_{600} of three independent experiments; error bars represent standard deviation; (***) indicates statistical significance ($p<0.001$).

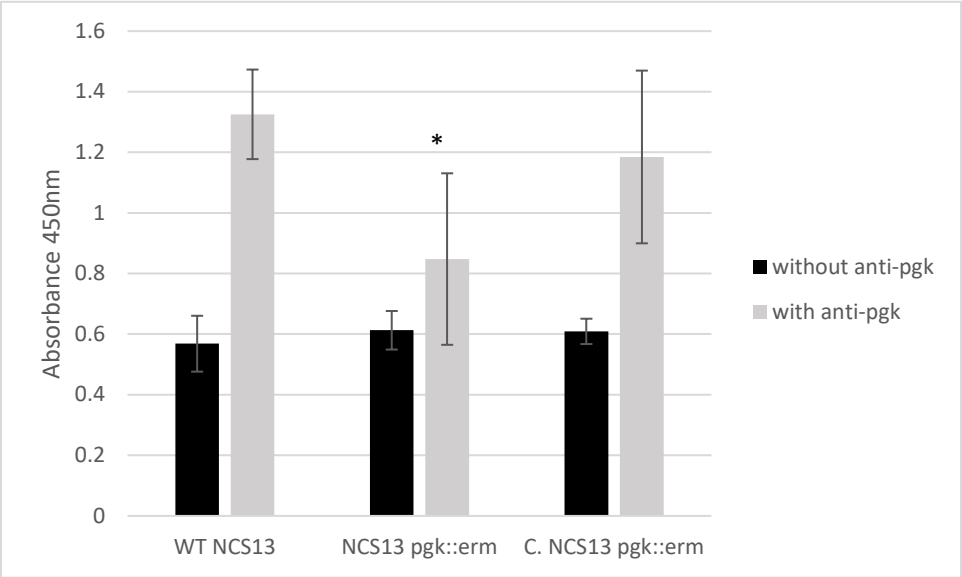


Figure 6.4. Expression of PGK on the surface of GBS NCS13 strains. Overnight growth cultures of GBS NCS13 wild type, GBS Δ*pgk* mutant and complemented GBS Δ*pgk* mutant strains were assayed for expression of PGK on their surface using anti-rGBS-PGK followed by anti-rabbit IgG-horse-radish peroxidase conjugate antibodies. Wells were developed using tetramethylbenzidine (TMP) and the A_{450} was measured. Experiment was performed duplicate in triplicate. A_{450} values from wells containing GBS Δ*pgk* mutant or complemented GBS Δ*pgk* mutant strains were compared to measurements from wells containing the wild type GBS NCS13 strain to determine the $\%A_{450}$. The bars represent the average relative A_{450} values from all 6 replicates; error bars represent standard deviation. (*) indicates statistical significance ($p<0.05$).

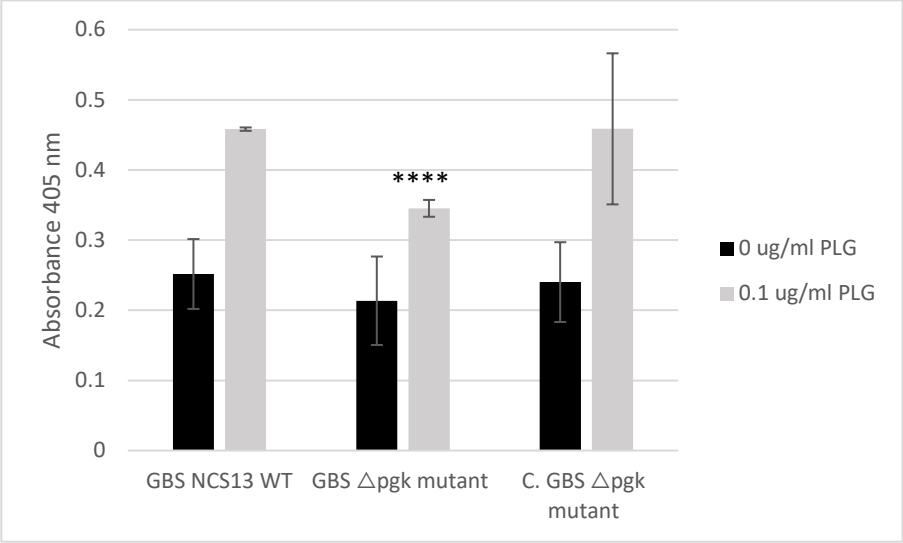


Figure 6.5. Plasminogen binding to the surface of GBS NCS13 strains. Binding of plasminogen (0.1µg/ml) to whole cells of GBS NCS13 WT, GBS Δ*pgk* mutant and complemented GBS Δ*pgk* mutant strains was analyzed by ELISA, with bound plasminogen detected using a monoclonal anti-plasminogen antibody (SBF1) followed by anti-mouse IgG conjugated with alkaline phosphatase. Wells were developed using alkaline phosphatase yellow substrate and the A_{405} was measured. A_{405} values from wells containing GBS Δ*pgk* mutant or complemented GBS Δ*pgk* mutant strains were compared to measurements from wells containing the wild type GBS NCS13 strain to determine the average A_{405}. Data represent the means from two separate experiments and error bars are standard deviation. Asterisks (****) indicate statistical significance ($p < 0.0001$).

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Chapter 7:

Summary and Future directions

Despite the introduction of antimicrobial prophylaxis to prevent infections caused by GBS, this bacteria remains the leading cause of neonatal septicemia and meningitis, as well as an increasingly common cause of invasive disease in adults (1–3). The implementation of an intrapartum antibiotic prophylaxis (IAP) has resulted in a significant decrease in the incidence of GBS disease in the countries with IAP programs. In the United States, invasive EOD GBS infections has declined from a high of 1.8 cases/1000 live births in the early 1990s to 0.26 cases/1000 live births in 2010 (4). Unfortunately, IAP has had no effect on the occurrence of GBS LOD, and thus LOD continues to be a major cause of neonatal morbidity and mortality (1,4). GBS has also emerged as a frequent cause of invasive infection in non-pregnant adults with underlying medical conditions. The incidence of GBS disease among these patients has increased. In 2014, the Centers for Disease Control and Prevention Active Bacterial Core surveillance system in the United States estimated that 93% of cases of invasive GBS disease occurred beyond infancy, particularly in non-pregnant adults (3,5). Moreover, GBS has exhibited increased resistance to macrolides, lincosamides, and streptogramins B, which significantly reduces the effectiveness of antibiotics (6–10). With the steady rates of invasive GBS disease still present in neonates in the era of IAP, the increasing infection rates among the elderly and immunocompromised, as well as the problems with growing resistance of GBS strains to antibiotics, the need for improved therapies to prevent GBS infections is becoming more evident.

In order to cause invasive disease, GBS needs to first accomplish several basic requirements, such as adhering to a tissue surface and competing with the normal flora present on the surface. Once attached, GBS needs to invade the host and replicate. To

survive in the hostile environment of the host, GBS must avoid the host immune system that is activated by the presence of the invading GBS. In each of these steps, there are several GBS virulence factors that are involved in the disease process. One such factor is the moonlighting glycolytic enzyme, phosphoglycerate kinase (PGK), a surface-expressed protein whose mechanism of becoming expressed on the GBS surface has not been known. The surface localization of PGK appears to contribute to bacterial virulence through its ability to bind strongly to host plasminogen, with some binding ability to actin (11). The fact that PGK binds to plasminogen suggests a potential role for PGK in aiding dissemination of the bacteria within the host through the breakdown of host extracellular matrix proteins and endothelium. Modulation of the host plasminogen system by GBS could play a role in the penetration of the blood-brain barrier and the subsequent development of meningitis. The work presented in this thesis focused on exploring the factors associated with GBS virulence, including the surface expression of GBS-PGK. As an invasive pathogen, GBS can also be carried asymptomatically, and disease occurs in the presence of certain triggers. In this thesis, I also studied the effect of polyols in altering GBS phenotypes. Better understanding of the infectious process of GBS may facilitate the development of novel and effective therapy in preventing GBS disease.

7.1 Identification of binding ligands for PGK on the GBS surface:

The main goal of this thesis was to determine the role of surface-expressed GBS-PGK in GBS virulence. Determining the role of surface-expressed GBS-PGK through deletion of the gene for PGK is hampered by PGK's central role in glycolysis. Consequently, it was necessary to first identify the binding ligand of PGK on the GBS

surface, then prevent the expression of PGK on the GBS surface. Our lab has previously made various attempts to investigate the role of surface-expressed GBS-PGK. These include peptide mapping and site-directed mutagenesis of GBS-PGK in order to inhibit binding of the PGK protein to plasminogen, without affecting its glycolytic activity (12). Another example was prevention of GBS-PGK surface expression via transposon Tn*917* mutagenesis of GBS, with the goal of identifying the gene responsible for the translocation of GBS-PGK to the bacterial surface (13). Screening of the Tn*917* mutants led to the identification of one mutant, with a mutation in the *sag1003* gene (later termed *eveB*), with a significant decrease in GBS-PGK expression on the bacterial surface and with increased levels of PGK in the culture supernatant, as compared to the wild type strain (13). This result suggested that the decreased expression of PGK on the surface of GBS *sag1003*::Tn*917* was due to decreased attachment to the bacterial surface, and not due to secretion. Since the *sag1003* gene codes for an ATP-dependent permease transporter (99.4 kDa) that has 8 transmembrane regions, it was suggested that a portion of this protein is potentially exposed to the outer surface of the bacterial membrane and therefore, could theoretically act as a binding ligand for GBS-PGK. While it was shown that the GBS *sag1003*::Tn*917* mutant displays reduced binding of GBS-PGK to the bacterial cell surface, it was not demonstrated if this is directly attributable to the loss of the Sag1003 protein. It was also important to determine whether other proteins in addition to Sag1003 play a role in GBS-PGK binding to the GBS surface. The work presented in chapter two of this thesis discussed the identification of the GBS genes that are involved in GBS virulence, as well as in GBS-PGK surface expression.

My preliminary work in determining the binding ligand of GBS-PGK on the bacterial surface utilized a Far Western Blot assay with recombinant GBS-PGK protein as a probe. The assay identified protein bands, from both the whole cell lysate and the cell wall fraction, to which GBS-PGK was bound (Fig. 2.2). The mass spectroscopy and subsequent BLAST analysis of this peptide band identified the protein as Sag0912 (43 kDa), a hypothetical protein with unknown function. In order to estimate the location of the Sag0912 protein within GBS, the peptide sequence was run on a number of predictors, which indicated that the Sag0912 protein may be located on the cell membrane or cell wall, despite the absence of an N-terminal signal peptide sequence (Fig. 2.4). This localization was later confirmed via a subcellular localization assay of the Sag0912 protein (Fig 4.10). Using rabbit polyclonal antibodies against Sag0912, Sag0912 was found to be present on the GBS cell wall, as well as in the cytoplasm and cell culture media. This suggested that the Sag0912 protein may bind to the GBS surface after secretion through post translational modification of Sag0912. Although it was expected that GBS-PGK would bind to a protein band of 99.4 kDa (the molecular weight of Sag1003) in both the whole cell lysate and the cell wall fraction of GBS, we could not confirm this by Far Western Blot. An explanation for this could be that since Sag1003 is a transmembrane protein, it either did not enter the SDS gel during electrophoresis or it was incompletely extracted or the protein did not transfer onto the nitrocellulose membrane as is seen for some transmembrane proteins.

To be certain of the roles of the Sag1003 and Sag0912 proteins in PGK binding to the GBS surface, knockout mutagenesis of both genes using insertional inactivation with an erythromycin cassette was performed. Insertional mutation of the *sag1003* gene was

completed in order to confirm that the loss of surface GBS-PGK expression in the GBS *sag1003*::Tn*917* mutant was due to the loss of Sag1003 expression and not due to an additional spontaneous mutation elsewhere in the genome. Mutagenesis of the *sag1003* and s*ag0912* genes in GBS resulted in a significant decrease in the surface expression of GBS-PGK (Fig. 2.9). In addition, as compared to the wild type strain, the mutants displayed different GBS phenotypes, such as decrease in ß-hemolysis (Fig. 2.6), CAMP factor (Fig. 2.8) and the production of orange pigmentation (Fig. 2.7). The mutants also showed reduced ability to invade epithelial cells (HeLa cells) (Fig. 2.12) and to resist the phagocytic activity of phagocytic cells within fresh human blood (Fig. 2..11). Based on these interesting findings we have given a name for the *sag0912* and *sag1003* genes as *eveA* and *eveB* (external virulence effector A and external virulence effector B), respectively.

In order to determine whether the *eveA* and *eveB* genes have regulatory effects on the surface attachment of GBS-PGK or the proteins encoded by these genes acting as a binding ligand for GBS-PGK on the bacterial surface, a Far Western Blot was conducted on the purified proteins, EveA and EveB, using the GBS-PGK protein as a probe. Since the EveB protein is a large protein with eight transmembrane domains, it was difficult to express and purify the protein as a whole, and therefore we designed the protein to be expressed as two fragments, EveB (up) and EveB (down). The purified proteins EveA and EveB(up), but not EveB(down) were found to bind the PGK protein on the blot (Fig. 3.8). Thus, the observation that less amount of PGK was expressed on the surface of the mutants was due to the loss of EveA and EveB expression on the mutants' surfaces, since these proteins act as a binding ligand for GBS-PGK. These findings suggest that the *eveA*

and *eveB* genes appear to have a regulatory effect on GBS virulence expression, as well as encode proteins that act as a binding ligand for GBS-PGK on the GBS surface.

While my results demonstrate that GBS-PGK binds to EveA and EveB proteins, the attachment of PGK to the GBS surface does not appear to be entirely dependent on EveA and EveB. The presence of a second ligand physically binding GBS-PGK to the GBS surface is supported by previous results in our lab. Investigators in our laboratory found earlier that recombinant GBS-PGK bound to lipoteichoic acid from Group A Streptococcus (GAS) *in vitro*. It is therefore possible GBS-PGK may become attached to the GBS surface through interactions with lipoteichoic acid (Boone and Tyrrell, unpublished).

Although GBS-PGK seems to bind to various ligands on the bacterial surface, rather than preventing the expression of such ligands, one way to study the role of surface-expressed GBS-PGK would be to knock out the *pgk* gene in GBS.

7.2 Construction of *pgk* mutant in GBS via auxotrophic complementation:

In my attempts to construct the *pgk* mutant, I applied the method called auxotrophic complementation. This method involves constructing a mutation in an essential gene, which renders the organism unable to synthesis the particular organic compound required for its growth, and unless the mutant is complemented auxotrophically with the essential nutrients supplemented in the growth media, the organism is unable to grow. This technique of producing mutations in essential genes has been used successfully in many microorganisms for the purpose of studying the function of the genes products in biological processes (14–18). In our case, the *pgk* gene encodes

for a major enzyme in glycolysis, which catalyzes the reaction of 1,3-bisphoshoglycerate and ADP to produce 3-phosphoglycerate and ATP. After many efforts attempting to complement the *pgk* mutation auxotrophically, I found that the addition of 10% fetal bovine serum (FBS) to the culture media was able to maintain the growth of the mutant. It was expected that FBS contained the essential nutrients for the GBS *pgk* mutant to grow because FBS is well known to contain a large number of nutritional and growth factors that are essential for cell growth; hence, it was used to supplement the media for the *pgk* mutant.

The GBS *pgk* mutant was successfully constructed, as confirmed both phenotypically and genetically. The mutant was partially dependent on exogenous supplementation with FBS to the media for improved growth. In addition, PCR amplification of the *pgk* gene from the mutant revealed inactivation of the *pgk* gene due to insertion of the *ermB* gene, as indicated by a band size larger than the intact *pgk* gene from the wild type strain.

The survival of a viable mutant without any supplementation of the deficient material in the media can be achieved by using a plasmid carrying that essential gene. In this study, I used a previously constructed plasmid in our lab carrying the GBS *pgk* gene. The auxotrophic complementation of the GBS *pgk* mutant with the pUC19/*pgk* plasmid was tested. On media without FBS supplementation, the complemented mutant grew similarly to the wild type. Interestingly, all of the complemented transformants failed to maintain their plasmid and had become prototrophic. The prototrophic phenotype was due to the phenomenon of integration of the intact *pgk* gene via homologous recombination between the *pgk* gene on the plasmid and the knockout *pgk* gene on the

chromosome of the mutant. The phenomenon of homologus recombination that occurred between the *pgk* genes was likely a consequence of how the auxotrophic complementation vector was constructed. The pUC19/*pgk* plasmid was constructed by cloning the *pgk* gene from GBS onto the pUC19, thus creating regions of homologous sequences. One strategy that could be used to eliminate crossing over at the *pgk* gene is to use a *pgk* gene from a different species, with limited homology, for the complementation to prevent homologous recombination and keep the two copies of *pgk* gene. Alternatively, the GBS *pgk* mutant could be constructed using an approach that eliminates the *pgk* sequence from the chromosome entirely.

The involvement of GBS-PGK in bacterial virulence through binding to host plasminogen was also confirmed by an ELISA assay. Plasminogen binding to the surface of the GBS *pgk* mutant was reduced, but not abolished, as compared to the binding to GBS wild type or to the complemented mutant (Fig. 6.5). The observation that some plasminogen still bound to the surface of the mutant may be due to the presence of other plasminogen-binding proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and plasminogen-binding protein (PbsP) on the surface of the GBS mutant (19,20). Although the work presented here is far from complete regarding understanding the role of surface-expressed GBS-PGK in bacterial virulence, my preliminary results lay the foundation for future research identifying the role of PGK in GBS pathogenesis.

7.3 External virulence effectors A and B in GBS pathogenesis:

The results from the *eveA* and *eveB* mutants, while interesting, do not seem likely to provide much insight into the function of surface-expressed GBS-PGK. However,

these results raise some interesting research questions regarding other aspects of GBS virulence. The observation that the mutant GBS strains had reduced ß-hemolysis, orange pigmentation, anti-phagocytic activity and invasion into HeLa cells poses important questions regarding the role of the newly identified proteins, EveA and EveB with regards to GBS virulence.

The findings that *eveA* and *eveB* genes are conserved in all GBS strains regardless of serotype suggest these proteins are important for GBS (Fig. 2.13). The expression of several major virulence genes in GBS is regulated at the transcriptional level by a two-component system (TCS) called CovSR (Control of Virulence Sensor and Regulator), which is the main signaling mechanism used by GBS to respond to their changing environments (21–23). A membrane-bound histidine kinase, CovS is the sensor that detects an environmental stimulus, which induces its autophosphorylation and subsequent phosphorylation of the cytosolic response regulator, CovR (23). Phosphorylation of the CovR protein alters its function as a transcriptional inhibitor, modifying its binding affinity for target promoter regions, thereby changing the expression of specific genes or operons (23). I hypothesize that the EveA and EveB proteins are likely part of a conserved mechanism within the CovSR system, in which the EveA and EveB proteins on the GBS surface may interact with the CovS to modulate the activity of the major regulator of virulence, CovR. The CovSR system controls the expression of most GBS virulence factors, including the ß-hemolysin/cytolysin, orange pigmentation and specific adhesins (24–29). The attenuated virulence of both *eveA* and *eveB* mutants indicates that these genes might have roles in CovSR regulation. This hypothesis is not unrealistic as a previous study by Firon *et al*. reported that another GBS transmembrane protein, Abx1, is

necessary to regulate CovS activity by a protein-protein interaction (30). Notably, the *abx1* mutant demonstrated a significant decrease in hemolytic activity and pigment production, which was mediated via increased CovSR activity (30). More recently, a study by Poupel *et al*. showed that a membrane protein on *Staphylococcus aureus,* SpdC, was observed to interact with the histidine kinase of the WalKR TCS in addition to other nine *S. aureus* histidine kinases, which are involved in controlling the expression of approximately one hundred genes in *S. aureus*, many of which belong to TCS regulons (31). Interestingly, when studying TCS signaling in a cellular context, the regulation of histidine kinase activity by an interacting protein is increasingly being recognized (32–34).

The investigation of immunoreactivity of the purified EveA and EveB proteins with human sera from patients with GBS infection allowed us to determine that EveA and EveB are immunogenic proteins (Fig. 3.7). This suggests that antibodies against EveA and EveB might be protective against GBS infections. However, our results from using polyclonal rabbit antibodies raised against EveA or EveB proteins in *in vitro* studies of invasion (Fig.4.6) or opsonophagocytic killing assays (Fig. 4.7) demonstrated that these antibodies did not protect from GBS infection. Therefore, our investigations indicate that the EveA and EveB proteins are immunogenic, but not protective. It is not a surprise for proteins to be immunogenic but not protective, as this has been demonstrated in other microorganisms (35,36).

While I have stated above that antibodies against EveA or B are not protective, an alternate explanation may be that the antibody-binding affinities are weak due to presence of other molecules impeding the antibodies from binding to the proteins efficiently. Our

observation that PGK binds to the EveA and EveB proteins on the GBS surface could indicate that the antibodies against the EveA and EveB proteins are not binding properly to the corresponding proteins due to the presence of PGK. The PGK protein could be masking the EveA and EveB proteins and inhibiting the antibodies from binding efficiently. Therefore, the antibody-binding affinities should be examined before concluding that the EveA and EveB proteins are not protective.

In consistence with the previous results of decreased bacterial invasion of GBS *eveA::erm* and GBS *eveB::erm* mutants into HeLa cells, the results that the addition of EveA and EveB(down) to epithelial cells prior to infection with GBS decreased GBS invasion, but not adherence, confirms the role of EveA and EveB proteins in GBS pathogenesis (Fig. 4.3) and (Fig. 4.4). For the EveB protein, the downstream region of the EveB protein, EveB (down) with the loop designation from number 5 to 6 (Fig 4.1), was shown to have the most powerful function in GBS invasion as compared to EveB (up); the addition of EveB (up) to HeLa cells did not affect GBS invasion, while EveB (down) does. It is not surprising to see that bacterial proteins which might be involved in controlling the bacterial two-component regulatory system might also act as virulence factors. The SpdC protein that was shown to interact directly with *S. aureus* histidine kinases was also determined to be a virulence factor and involved in bacterial biofilm formation (31). Our investigation of the EveA and EveB proteins, in addition to many identified surface-expressed proteins involved in GBS invasion (37–41), display functional redundancy possibly by different mechanisms. This redundancy may result in a clear advantage for GBS and may represent a kind of backup system in cases where the given biochemical function attributed to a specific protein was rendered non-functional.

Despite the observation that EveA and EveB proteins on the surface of GBS are involved in bacterial invasion into epithelial cells, the mechanism by which EveA and EveB proteins facilitate bacterial invasion of host cells remains to be determined.

Interestingly, the observation that the addition of purified EveA and EveB proteins exogenously to GBS cultures inhibited bacterial growth indicates that EveA and EveB proteins have some antimicrobial activities on GBS (Fig. 4.8). Furthermore, the antimicrobial effects of the EveB protein was observed with the downstream region of the EveB protein, EveB (down), and not with EveB(up). Although the EveA and EveB(down) proteins have antimicrobial effects (Fig. 4.9), their activities in bacterial killing are very low and their minimum inhibitory concentrations (MICs) was measured as 32 and 16 μg/ml, respectively. These MIC values are very high and of little therapeutic value. Typical therapeutic values should be usually 2 μg/ml or less. It is exciting to discover such multifactorial proteins that act as virulent factors when they are expressed on the surface of the bacteria at the appropriate level and can act as antimicrobial agents when they are overexpressed. To be effective as antimicrobial proteins, EveA and EveB should be expressed in very large quantities, as demonstrated by the results of the MICs, which is quite unlikely to happen *in vivo*.

These data show that EveA and EveB at levels expressed sufficient to develop GBS infection are required. The level of expression of specific virulence factors is sometimes critical for virulence, and this was also observed with Abx1: when the Abx1 protein was overexpressed in GBS, the bacteria was avirulent in neonatal rat pups (100% survival), as compared to the wild type stain (30). The production of such low potent antimicrobial proteins by GBS could be a way of self-inducing virulence, in which at

concentrations below those that mediate bacterial damage, these antimicrobial proteins (EveA and EveB) might serve as stimuli to up-regulate the expression of multiple virulence genes by signaling through the two-component regulatory system, thereby enhancing GBS pathogenicity during infection. Consistent with the idea of antimicrobial peptides inducing bacterial virulence, studies have shown that sub-inhibitory concentrations of human antimicrobial peptides (cathelicidin LL-37) were able to stimulate the expression of *Streptococcus pyogenes* virulence genes by binding directly to CovSR TCS. This resulted in increased expression of the CovSR-regulated gene products, including the capsule biosynthetic enzymes, the IL-8 protease PrtS/ScpC and the anti-phagocytic protein/IgG protease Mac/IdeS, thereby contributing to bacterial virulence (42,43).

Although the EveA and EveB(down) proteins are actually large proteins with molecular weights of 43 and 20 kDa, respectively, it is not surprising that they can act as antibacterial proteins since previous studies have shown that various large molecular weight antibacterial proteins, such as bacteriocins (class III), with a size range between 25-80 kDa, that are produced by bacteria have antibacterial properties (44–51). Bacteriocins are heat-stable antimicrobial peptides that are produced by bacteria that can exhibit both broad and narrow range inhibition spectra. Some of these large bacteriocins, such as colicins from *E. coli*, have a domain-type structure, in which each domain has different functions. These domains include an amino-terminal translocation (T) domain, which is implicated in the transfer across the outer membrane via the translocator protein; a central receptor-binding (R) domain, which is bound with a bacterial outer membrane receptor; and a carboxy-terminal cytotoxic (C) domain, which has antibacterial activity

(52,53). Antibacterial proteins production in GBS and other bacteria may be involved in bacterial competition and virulence determination, and their production by bacteria could be induced under stress conditions. The release of antibacterial proteins from the cells could be mediated by the action of the lysis protein that is lethal for both the producing cell and any bacteria that are sensitive to that antibacterial protein.

My results allow me to propose a model for the role of EveA and EveB proteins in GBS virulence (Fig 7.1). My results indicate that EveA and EveB proteins act as binding ligands for another virulence factor, such as the surface-expressed PGK; regulate some virulence gene expression, such as the genes for ß-hemolysis and pigmentation; are involved in bacterial invasion into HeLa cells, and can resist the phagocytic activity of phagocytic cells in human blood. The results also suggest that appropriate levels of EveA and EveB protein expression are required for GBS virulence and high volumes of EveA and EveB proteins were observed to have an antimicrobial effect on GBS.

7.4 Triggering GBS virulence by polyols:

Although GBS is a commensal bacterium of the adult gastro-intestinal tract and is present asymptomatically in the vaginal flora of 30% of healthy women (1), its ability to cause infections is multifactorial (24). The signals dictating the GBS switch from commensalism to virulence are mainly unknown. One factor that could be associated with GBS virulence is the presence of polyols. Polyols, which are also called sugar alcohols, are sugar-free sweeteners that are commonly created by hydrogenation of sugars. Polyols have also been found in the placentas of ruminants and in the coelomic and amniotic fluids of humans (54,55). The most common polyols are sorbitol, mannitol,

xylitol and erythritol. Because of their lower caloric content compared to sugars, polyols are added to food and especially, chewing gum. In addition to their advantages as non-caloric sweeteners, they are not broken down by bacteria in the mouth or metabolized to acids, and thus do not contribute to tooth decay (56). Polyols have been suggested to have preventive properties against caries (57). Several studies showed that polyols can decrease polysaccharide-producing oral streptococci glass surface adhesion and biofilm formation that contributes to plaque accumulation, and erythritol was found to be more effective at this (58–61). In contrast to its effect on oral streptococci, erythritol was shown to have a reverse effect on *Brucella* and *Chlamydia*, such as triggering the expression of virulent traits and enhancing bacterial growth (62–66). Based on these findings, I hypothesized that GBS has similarities with brucellosis and chlamydiosis in that the cause is bacterial infection that could affect the fetus by penetrating the placentas and the amniotic membranes. I conducted a study to investigate the effect of the polyols, erythritol, sorbitol and mannitol, on triggering GBS virulence. Our findings that 1% of erythritol, but not sorbitol or mannitol, was able to stimulate the surface expression of GBS-PGK (Fig. 5.2), increase GBS resistance to phagocytic cells (Fig. 5.3) and increase bacterial invasion into HeLa cells (Fig. 5.4) suggest that erythritol plays a role in altering GBS phenotypes. These findings show that the presence of GBS in environments enriched with erythritol can affect the nature of the bacteria and transform it from a non-harmful commensal bacterium into a pathogenic one.

The concentration of 1% of erythritol is equal to 80 µM/ml, and this is considered to be a high concentration compared to the normal concentration of erythritol that is present in the umbilical vein (11.19 µM/ml) and the coelomic fluids (12 µM/ml). It could

be that the concentration of erythritol is increased in some cases of pregnancy due to the presence of an erythritol producer, such as yeast in the area where the GBS resides (54,55,60). Previous studies determined that some yeasts, such as Candida can produce erythritol in high concentrations (60,67–69). It is unknown whether yeasts that affect humans, and especially the commensal yeasts residing in the human vaginal tract would also produce the erythritol locally at that specific area. If commensal vaginal yeasts could also produce the erythritol at a concentration optimal for triggering the GBS virulence, it would provide an explanation for our observation that high concentrations of erythritol stimulates GBS virulence, thereby allowing the commensal GBS to be converted to its pathogenic version when present in such a sharing niche as this erythritol producer.

7.5 Future Directions:

My research focused on identifying and understanding virulence factors in GBS. While the development of a complete understanding of how surface-expressed GBS-PGK contributes to GBS virulence is not complete, my results from constructing a GBS *pgk* mutant by an auxotrophic complementation method have laid the foundation for future research in this area. In addition, my results on determining the role of the newly identified proteins, EveA and EveB in GBS invasion and regulation of the gene expression of some other virulence determinants have provided potential virulence functions for the EveA and EveB proteins. Lastly, my results relating to polyols showed that polyols may act as triggers that alter GBS phenotypes, particularly, they might act as signals controlling the bacterial switch from commensalism to virulent. These exciting findings provide interesting opportunities for further research on GBS virulence.

7.5.1 Virulence of GBS Δ*pgk* mutant in an animal model of invasive GBS infection:

Plasminogen binding to the bacterial surface has been shown to be an important virulence factor in most bacterial pathogens. The biological significance of plasminogen binding to GBS-PGK located on the surface of GBS is unknown. I successfully created a GBS *pgk::erm* mutant displaying less plasminogen binding to the surface of the mutant. The creation of the GBS *pgk* mutant provided a tool to explore the significance of GBS-PGK on the GBS surface as it relates to plasminogen. To understand the biological significance of the created GBS *pgk* mutant, a virulence assay using an animal model should be used. It is expected that mice injected with the mutant strain will survive longer than those mice injected with the wild type strain. It is also possible that there will be no difference between the wild type strain and the mutant strain assayed, since there are many virulence factors associated with GBS. However, this seems unlikely as it has been previously demonstrated that Brucella PGK is critical for the full bacterial virulence and that a *Brucella pgk* mutant was highly attenuated in immunocompromised mice (70). This assay will determine whether or not the surface-expressed GBS-PGK is as important as *Brucella* PGK in the invasive GBS disease process.

7.5.2 EveA or EveB might affect CovSR signaling:

The observation that a mutation of the *eveA* and *eveB* genes in GBS reduced the mutants' hemolysis and pigmentation raises interesting research questions for future study. Particularly, the question of whether the EveA or EveB proteins in GBS have inhibitory effects on the CovS activity or whether these proteins protect or sequester the CovR protein, thereby impeding its phosphorylation by CovS either directly or indirectly.

In order to interpret the relationship between the EveA or EveB proteins and the CovSR two-component system, inactivation of the *covS* and *covR* genes in the wild type strain and in both mutants, NCS13 *eveA::erm* and NCS13 *eveB::erm,* needs to be achieved. Deletion of the *covS* and *covR* genes from the *eveA* and *eveB* mutant strains followed by assaying the β-hemolysis and pigmentation should determine if the EveA and EveB proteins are involved in regulating the activity of either CovS or CovR proteins. Since transcription of the *cyl* operon, which encodes the ß-hemolycin/cytolysin and orange pigmentation, is controlled negatively by the CovSR system in GBS (21,71), inactivation of the *covS* or *covR* genes in the wild type strain will result in hyperhemolytic and hyperpigmented mutant strains. If my hypothesis that the EveA or EveB proteins regulate the CovSR system is correct, then inactivation of the *covS* and *covR* genes in both Δ*eveA* and Δ*eveB* mutants will restore the pigmentation and hemolysis to levels similar to those observed in the Δ*covS* and Δ*covR* mutants of the wild type mutant strains. An alternative approach would be to overexpress the EveA or EveB proteins from plasmids in GBS wild type strain. Based on my hypothesis, overexpression of the EveA or EveB proteins should result in increased hemolysis and pigment production.

In addition to controlling the expression of GBS hemolysis and pigmentation, the CovSR system also regulates the expression of various virulence factors, such as BibA, ScpB and FbsA (27,71,72). In order to confirm the role of the EveA and EveB proteins as inhibitors of the CovSR signaling pathway, a Western blot analysis of the BibA, ScpB and FbsA proteins can be conducted. This would examine the expression of the BibA, ScpB and FbsA proteins in the *covS* and *covR* mutants of both *eveA* and *eveB* mutant strains, in the wild type strain, in the Δ*eveA* and Δ*eveB* mutant strains, as well as the

overexpressed wild type strain with *eveA* or *eveB* from plasmids. Again if my hypothesis is correct, it is expected that inactivation of the *covS* or *covR* genes in both *eveA* and *eveB* mutant strains or the overexpression of *eveA* and *eveB* will dramatically increase the BibA, ScpB and FbsA protein production. Using a microarray to characterize the changes in gene expression of these GBS mutant strains as compared to the wild type NCS13 strain should also provide insight into which genes are regulated in response to the EveA or EveB protein interaction with the CovSR system. If there was a similarity between the *eveA* or *eveB* overexpression mutants and the *covSR* deletion mutants, it would suggest that the EveA and EveB proteins have a specific effect on the CovSR two-component system.

In order to determine whether the EveA or EveB proteins have an inhibitory function on the CovSR activity through direct interaction with the CovS or CovR proteins, a bacterial adenylate cyclase two-hybrid (BACTH) system can be used to test the physical interactions of the EveA and EveB proteins with different putative partners. The full length, membrane-bound CovS histidine kinase or the CovR cytoplasmic response regulator could be fused to the C-terminal domain of the T25 subunit of the *Bordetella pertussis* adenylate cyclase, while the full-length EveA or EveB proteins could be fused to the C-terminal domain of the T18 subunit, using plasmids pKT25 and pUT18C, respectively. The physical interactions can be assayed in *E. coli* DHT1 cells co-transformed with combinations of the pKT25 and pUT18C derivatives carrying the translational fusions. Upon protein-protein interactions, the close proximity between the T18 and T25 subunits would restore the adenylate cyclase activity, leading to cAMP synthesis and activation of the lactose operon. Interaction efficiencies between the hybrid

proteins can be tested both by spotting the resulting strains on LB plates containing X-Gal and by measuring the β-galactosidase activity in 96-well plate assays. The results would determine if these proteins are able to form homodimers and interact with the CovS or CovR proteins.

7.5.3. Identifying pathogenicity of *eveA* and *eveB* GBS mutants *in vivo*:

The *in vitro* assays of GBS *eveA::erm* and GBS *eveB::erm* mutant bacteria showing that these mutant strains are attenuated. The contribution of EveA and EveB proteins to GBS infection *in vivo* can be investigated using a mouse model of GBS infection. A mouse model of invasive GBS infection has previously been developed to measure the pathogenicity of specific GBS mutant strain in the mouse model (73). Groups of CD1 mice will be intravenously injected with wild type GBS NCS13 strain, GBS *eveA::erm* and GBS *eveB::erm* bacteria. Mice will be monitored and euthanized after 48 h and bacteria will be counted in blood, lungs and brain homogenates. It is expected that the number of bacteria in the blood and tissues of GBS *eveA::erm* and GBS *eveB::erm* infected mice groups will be significantly lower compared to the wild type bacteria detected in the blood, lungs and brain of infected mice

7.5.4. Identifying host protein binding to EveA and EveB:

This thesis demonstrated that the addition of the EveA or EveB(down) proteins to epithelial cells prior to GBS infection inhibits GBS invasion. What remains unknown is which host protein(s) the EveA and EveB proteins interact with to initiate GBS invasion. Future studies using a Far Western Blot on which HeLa cell membranes will be extracted

and subjected to a SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with purified EveA or EveB(down) proteins, should serve as a valuable tool to address this question. The proteins will be detected using polyclonal rabbit antibodies against the EveA or EveB(down) proteins, and anti-rabbit IgG antibodies conjugated with alkaline phosphatase as a secondary antibody. Following the development with (NBT/CIBC) substrate, any identified bands to which EveA or EveB(Down) proteins belong will be identified using mass spectroscopy. This identification will help us to understand the involvement of the EveA and EveB proteins in the host-pathogen interaction of GBS pathogenesis and its related diseases.

7.5.5. Protection provided by EveA and EveB against GBS disease *in vivo*:

My results from testing the immunoreactivity of the EveA and EveB proteins, both EveB(up) and EveB(down), with human sera from patients with GBS infections that was detected by Western blotting suggest that the EveA and EveB proteins on GBS are immunogenic. Subsequently, using invasion and opsonophagocytic killing assays, I tested the protection provided by polyclonal rabbit antibodies against EveA, EveB(up) and EveB(down) in GBS infection *in vitro*. My results showed that these polyclonal antibodies were not protective against GBS infection *in vitro*. It is unclear whether the EveA and EveB proteins are not protective or if this observation was due to manufacturing problems in raising the antibodies. Thus, it is important to confirm whether EveA and EveB proteins can elicit protective immunity against GBS infection using an *in vivo* study. In order to determine the level of protection provided by the EveA and EveB proteins, a mouse maternal immunization model can be used. A maternal

immunization/neonatal pup challenge model of invasive GBS infection has previously been developed to measure the protective efficacy of antigens in the mouse model (74,75). It is unknown whether the proteins will provide protection against invasive GBS infection or not. Complete (100%) or some protection provided by the proteins would suggest that the EveA and EveB proteins could be used not only as potential components for a multi-valent GBS vaccine, but also as carriers or adjuvants in polysaccride conjugate vaccines.

7.5.6. Polyols and GBS virulence:

My results provide some interesting insight into how polyols (erythritol, sorbitol and mannitol) can alter GBS phenotypes. My results suggest that growing GBS in the presence of 1% of erythritol was able to increase the GBS-PGK surface expression, GBS antiphagocytic activity and GBS invasion into HeLa cells. Regarding the effect of polyols, erythritol was previously found to have an effect on *Brucella* and *Chlamydia* in triggering the expression of virulence factors and increasing bacterial multiplications (63,64,66). It would be interesting to determine if virulence genes in GBS are also regulated in response to 1% erythritol. Using a microarray to characterize the changes in gene expression of GBS grown in the presence and absence of 1% erythritol should provide insight into which genes are regulated in response to erythritol, as well as the relative contribution of the various identified genes to GBS virulence.

Furthermore, my results showing that growing GBS in the presence of 2% and 4% of polyols (erythritol, sorbitol and mannitol) prior to GBS infection of HeLa cells significantly decreased the invasion into the cells poses an interesting question as to

whether high concentrations of polyols have a negative effect on GBS. It was not confirmed whether this decreased bacterial invasion into HeLa cells was due to decreased bacterial adherence to the epithelial cells or simply decreased invasion. If the decreased bacterial invasion into the HeLa cells was due to decreased bacterial adherence, it would not be surprising since previous studies on oral streptococci determined that higher concentrations (4% or 10%) of polyols (erythritol, sorbitol or xylitol) were able to significantly decrease bacterial adherence to glass surfaces, and subsequently decrease biofilm formation (58,76–78). In addition, the effect of high concentration of polyols on the growth of oral streptococci and *Streptococcus pneumoniae* has been extensively studied both *in vitro* and *in vivo* (59,78–82). Thus, it is not unexpected to see high concentrations of polyols affecting the growth and adhesion/invasion of GBS. It would be interesting to investigate if higher concentrations of polyols (higher than 4%) will have a negative effect on GBS growth, GBS adherence and biofilm formation. It would also be important to compare the results of the three polyols, erythritol, sorbitol and mannitol, used for the experiments to identify the most effective polyol in reducing GBS virulence. If my hypothesis is correct regarding the bactericidal effects of higher concentrations of polyols on GBS, it may be beneficial to use polyols to reduce the GBS colonization in GBS-infected pregnant mothers so as to prevent GBS transmission from mother to infant and to prevent the occurrence of GBS early onset disease (EOD). Thus, it may be possible to use polyols for universal prophylaxis in all pregnant women.

7.6 Conclusion:

While much remains to be discovered regarding GBS pathogenesis, the data presented in this thesis explored the function of several GBS virulence factors and their role in GBS disease. Although my work did not definitively demonstrate that surface-expressed GBS-PGK is involved in GBS virulence, the constructed GBS *pgk* mutant will assist in characterizing the function of surface-expressed GBS-PGK in GBS virulence in future research studies. The novel functions of the EveA and EveB proteins on the GBS surface have been also identified. The EveA and EveB proteins have been recognized as acting as binding ligand for the surface-expressed GBS-PGK, in addition to being involved in regulating the expression of other GBS virulence factors, such as ß-hemolysin and orange pigmentation. Furthermore, the EveA and EveB proteins were observed to be involved in GBS resistance to phagocytic killing and in the bacterial invasion into epithelial cells. Also, the addition of the EveA or EveB proteins to epithelial cells prior to GBS infection reduced GBS invasion into these cells. Furthermore, I have identified that polyols, specifically 1% erythritol, can alter GBS phenotypes, such as increasing the bacterial PGK surface expression, resistance to phagocytic killing, and invasion into epithelial cells. The data presented in this thesis advances our understanding of the factors in GBS, as well as in the host that are involved in GBS pathogenesis.

7.7 Limitations of statistical method that was used in this thesis and some alternative statistical analysis:

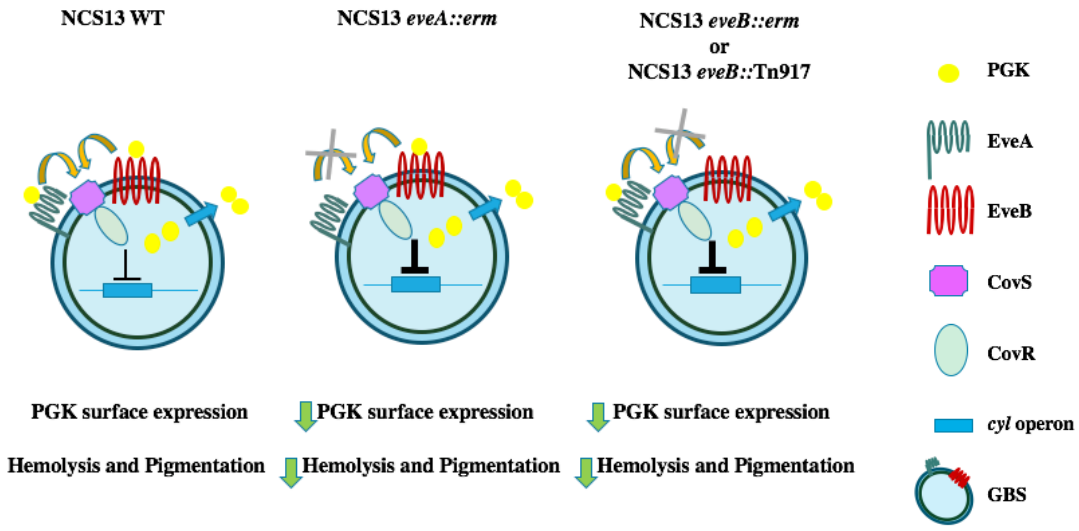
In my thesis, I used the statistical analysis Student t test to analyze my research data. The t-test can be used to determine if the means of two sets of data are significantly

different from each other. Student t test is appropriate to use if the data of two groups are being compared are parametric (would follow a normal distribution) with assumed equal variances. If the two samples that are being compared have unequal variances or unequal sample sizes, the Welch's unequal variances t-test can be used. If the data are substantially nonparametric and the sample size is small, the t-test can give misleading results.

If parametric data of more than two groups are being compared, the t-test is not optimal and the best statistical analysis that could be use in this case is one-way analysis of variance ANOVA. One-way ANOVA is used to test for differences among at least three independent groups (means) since the two group case can be covered by a t-test. ANOVA provides a statistical test of whether two or more population means are equal, and therefore generalizes the t-test beyond two means.

In the presence of an outlier or when the data distribution of two independent samples are asymmetric or have large tails, the t-test is not appropriate to use. In this case and for nonparametric data (log transformed or scored data), the Mann-Whitney U test is more appropriate than the t-test. The Mann–Whitney U test is a nonparametric test of the null hypothesis that it is equally likely that a randomly selected value from one sample will be less than or greater than a randomly selected value from a second sample.

A



B

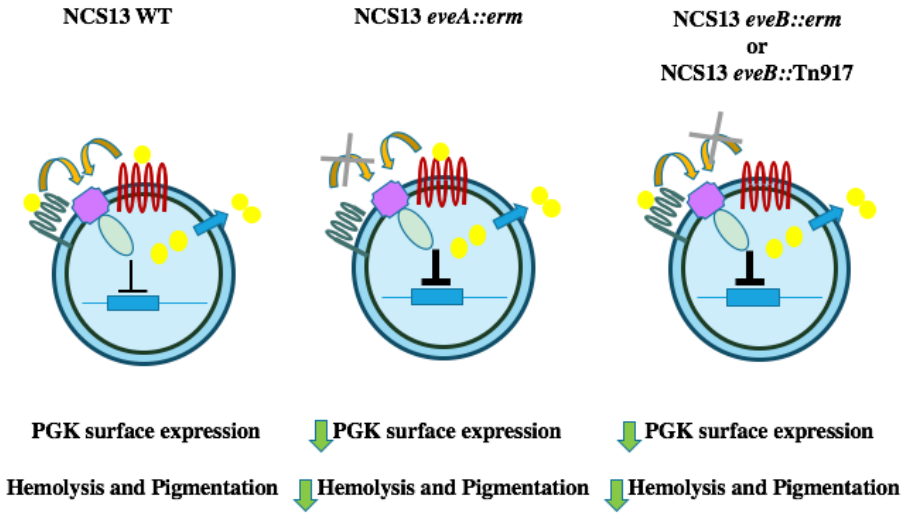


Figure 7.1. Proposed model for the role of EveA and EveB in GBS virulence. (A) Diagram of the regulatory network through CovSR TCS controlling GBS hemolysin and pigment production. The EveA and EveB in a wild type context, might interact with CovS to maintain the equilibrium between kinase and the phosphatase activity of the CovS. Activation of the CovR by CovS increase its inhibitory activity on *cyl* operon transcription. In this condition, the *cyl* operon is expressed at a level defining the wild

type ß- hemolytic and pigmentation phenotype. In addition, EveA and EveB act as binding ligands for surface expressed GBS-PGK. In the absence of EveA or EveB, the CovS is locked in its kinase competent form that activates CovR, thus inhibiting the expression of the *cyl* operon. In this condition, the mutants are non-hemolytic and non-pigmented. In addition, less GBS-PGK was expressed on the surface of the mutants. (B) Schematic representation of the role of EveA and EveB(down) proteins in GBS entry into human epithelial cells. Treatment of HeLa cells with EveA and EveB proteins prior GBS infection reduced bacterial invasion which confirm the role of these proteins in GBS invasion.

7.8 References:

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