University of Alberta

Characterization of Phosphoglycerate Kinase Expressed on the Surface of Group B Streptococcus

by

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Abstract:

Group B *streptococcus* (GBS) is a major cause of invasive disease in the neonatal and adult populations. To mediate invasive disease, GBS encodes a variety of surface expressed and secreted components. One surface expressed protein that may contribute to GBS virulence is phosphoglycerate kinase (PGK; a glycolytic enzyme). Glycolytic enzymes have been identified on the surface of many Gram positive bacteria, despite the absence of any known secretion or surface attachment signal. My results demonstrate that the SecA2 locus, in particular the Srr1 fimbrial protein, is required for transport of PGK across the GBS membrane. Once across the bacterial membrane, PGK becomes attached to the bacterial surface through interaction with lipoteichoic acid as well as a second ligand that also requires Srr1 for expression. While the surface localization of PGK suggests it may play a role in GBS virulence, confirming their role in virulence has been hampered by its role in glycolysis. Due to the essential cytoplasmic role of PGK in metabolism, traditional knock-out mutagenesis is not possible to determine its function on the bacterial surface. My results demonstrate that GBS-PGK may contribute to GBS virulence through its ability to bind to actin, fibrin, fibrinogen, fibronectin and plasminogen. Site directed mutagenesis, preventing interaction with host proteins without affecting the glycolytic activity, has previously been used to demonstrate a role for the glycolytic enzymes α-enolase and glyceraldehyde-3-phosphate dehydrogenase in virulence. Using truncation followed by peptide mapping experiments the actin and plasminogen

binding sites of GBS-PGK were located to the amino acids 126-134 and amino acids 302-306. Using site directed mutagenesis, targeting these two locations within the GBS-PGK molecule, I have reduced the actin and plasminogen binding by GBS-PGK without affecting the glycolytic activity.

In conclusion, the research presented in this thesis identifies the pathway utilized by GBS-PGK to become surface expressed, identifies potential virulence functions of surface expressed GBS-PGK and identifies mutations within the *pgk* gene that could prevent these virulence functions without affecting glycolysis. This work contributes to our understanding of surface expressed glycolytic enzymes and should facilitate future research determining the function of surface expressed GBS-PGK.

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List of Abbreviations

2D	Two dimensional
α	Alpha
β	Beta
γ	Gamma
ε	Epsilon
6-ACA	6-aminocaproic acid
µg	microgram
µl	microliter
µm	micrometer
°C	degrees Celsius
A	Absorbance
ADP	Adenosine 5'-diphosphate
AMP	Antimicrobial peptide
ATP	Adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
C-	Carboxy-
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleic acid
DPPC	Dipalmotyl phophatidylcholine
ELISA	Enzyme linked immunosorbent assay
EOD	Early onset disease
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Group A streptococcus
GBS	Group B streptococcus
h	Hours
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
LB	Luria-Bertani
LDH	Lactate dehydrogenase

LOD	Late onset disease
M	Molar
mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger Ribonucleic acid
N	Normal
N-	Amino-
NaCl	Sodium chloride
NaH2PO4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NBT	Nitro-blue tetrazolium chloride
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometers
PAGE	Polyacrylamide gel electrophoresis
PBS	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*
RNA	Ribonucleic acid
sc-PGK	Phosphoglycerate kinase form *Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline
TBST	0.1% tween 20 in Tris buffered saline
TH	Todd Hewitt
tPA	Tissue-type plasminogen activator
tRNA	Transfer Ribonucleic acid
uPA	Urokinase plasminogen activator

Table of Amino Acids

Amino Acid	Three Letter Code	Single letter Code	Side Chain pK
Nonpolar			
Glycine	Gly	G	
Alanine	Ala	A	
Valine	Val	V	
Leucine	Leu	L	
Isoleucine	Ile	I	
Methionine	Met	M	
Phenylalanine	Phe	F	
Tryptophan	Trp	W	
Proline	Pro	P	
Polar			
Serine	Ser	S	
Threonine	Thr	T	
Cysteine	Cys	C	8.33
Tyrosine	Tyr	Y	10.13
Asparagine	Asn	N	
Glutamine	Gln	Q	
Negatively Charged			
Aspartic Acid	Asp	D	3.9
Glutamic Acid	Glu	E	4.07
Positively Charged			
Lysine	Lys	K	10.79
Arginine	Arg	R	12.48
Histidine	His	H	6.04

Chapter 1:

Introduction

Group B streptococcus (GBS), also known as *Streptococcus agalactiae*, is a major cause of invasive disease in the neonatal and adult population (1-6). Early research studying GBS virulence demonstrated that during the course of infection, GBS becomes internalized within host cells (7). While the exact mechanism of internalization has not been fully elucidated, the requirement for actin cytoskeleton rearrangements has been well established (7-9). Previous work in our laboratory, attempting to characterize these actin rearrangements, identified phosphoglycerate kinase (PGK; a glycolytic enzyme) on the GBS surface (10). Initially, the identification of PGK on the GBS surface seemed to be an odd discovery as glycolytic activity outside the bacterial membrane does not seem to have any apparent purpose. However, the presence of glycolytic enzymes on the surface of Gram positive bacteria appears to be a general phenomenon (11, 12). Two, well-studied, examples are α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition to their role in glycolysis, these two enzymes are believed to contribute to bacterial virulence through their ability to bind to host proteins (12). In contrast to these two surface expressed enzymes, the function of surface expressed GBS-PGK has not been established.

The ultimate goal of this work was to determine the function of PGK expressed on the GBS surface. Similar to work with α-enolase and GAPDH, determining the function of PGK on the GBS surface is hampered by its role in glycolysis. Due to this essential cytoplasmic function, traditional knock-out mutagenesis is not possible to determine the function of surface expressed GBS-

PGK. One method that has been used to determine the role of surface expressed glycolytic enzymes was to prevent transport of the enzyme to the bacterial surface (13). A second method that has been utilized to determine the function of surface expressed glycolytic enzymes was to use site directed mutagenesis to abolish binding to host proteins without affecting their glycolytic activity (14-16). The work described in this thesis sought to determine how GBS-PGK becomes surface expressed as well as determine potential virulence functions of GBS-PGK in order to facilitate future experiments determining the function of surface expressed GBS-PGK.

To understand the potential virulence functions of surface expressed GBS-PGK, it is necessary to consider what is currently known regarding GBS virulence. The intent of this introductory chapter is to orient the reader to GBS disease and discuss potential roles for surface expressed GBS-PGK. This chapter is also intended to introduce the reader to the current knowledge regarding surface expressed glycolytic enzymes on gram positive bacteria.

1.1 Group B streptococcal disease:

1.1.1.1 Neonatal GBS disease.

It has been estimated that between 10-30% of women are asymptomatically colonized by group B streptococcus (3). While colonized women generally do not have symptoms, bacterial colonization during pregnancy

can result in severe disease of the infant. From the 1970's to the early 1990's, GBS was heralded as the leading cause of invasive neonatal disease by bacteria with incidence of neonatal sepsis or meningitis of 1.7 cases per 1000 live births (Fig. 1.1) (3). While one study suggested as high as 70% of the cases of meningitis in infants under the age of 1 month are caused by GBS (17), this study did not include *Escherichia coli* and therefore likely overestimated the prevalence of GBS infections. An estimation that GBS was responsible for 40-50% of invasive bacterial disease within the first week of life (5) is likely more accurate. Since then, improved prevention techniques have reduced the prevalence of invasive GBS disease in the neonate (Fig 1.1) (3).

Neonatal GBS disease can be separated into early onset disease (EOD) and late onset disease (LOD). EOD occurs when GBS is transferred from the mother to infant in utero or during passage through the birth canal. This form of GBS disease presents within the first week of life and often presents as pneumoniae that quickly progresses to bacteremia and septic shock (18). In contrast LOD can occur up to several months in age and has a high rate of progression to meningitis (19). Neurological sequelaes including seizures, hearing loss and cognitive impairment follow GBS meningitis in up to 50% of cases (3, 19, 20). A third, less characterized manifestation of GBS infection, is midgestation stillbirths (21). It is difficult to definitively determine the prevalence of GBS in midgestation stillbirths due to a lack of studies; however, the studies that have been performed suggest that GBS may be responsible for nearly a quarter of all spontaneous abortions (22) and occur at a frequency of 2.8 cases/1000 pregnancies (23).

1.1.1.2 Adult GBS disease.

In addition to being a major cause of invasive bacterial disease in the neonatal population, invasive GBS disease in non-pregnant adults is also rising. In the early 1990's invasive GBS disease in non-pregnant adults had reached 2.4-4.4 cases per 100 000 (2). In a more recent population based study, the rate of GBS infections was found to be 7.3 cases per 100 000 persons (24). The majority (75%) of GBS infections in the adult population occur in people with predisposing conditions that compromise the immune system (2). These predisposing conditions include older age (> 65 years), diabetes mellitus, obesity, cancer, COPD, cardiovascular disease, liver disease, kidney disease and immunosuppression as well as alcohol and substance abuse (2, 6, 24). In the elderly population invasive GBS disease is approximately 10 × more prevalent than in the general adult population (2, 6), particularly for those living in nursing homes (6). In addition to predisposing conditions resulting in a compromised immune system, medical conditions that affect the integrity of the skin and mucosal membranes also increase the risk of developing GBS disease (2, 6).

Typically, GBS disease in non-pregnant adults often presents as bacteremia or skin and soft tissue infections (2, 6, 24). GBS disease can also present as respiratory infections, genitourinary infections, joint and bone infections, abdominal infections, endocarditis or infections of the central nervous system. More severe presentations such as necrotizing fasciitis and streptococcal toxic shock syndrome are rare, but are becoming increasingly recognized (2).

1.1.2 Development of GBS disease.

While GBS is also known to cause invasive disease in the adult population, the majority of research focuses on the development of invasive neonatal disease. As a result, this review will focus on the development of invasive GBS disease in the neonate. It is expected that many of the same virulence characteristics that are necessary for the development of invasive neonatal disease also contribute to the development of invasive disease in the adult population. To cause invasive neonatal disease, GBS colonizes a number of niches within both the mother and infant (Fig 1.2). For the purposes of this chapter, the development of invasive GBS disease will be broadly divided into three categories: adhesion, dissemination and survival. To facilitate these processes, GBS expresses a number of surface expressed and secreted virulence factors.

1.1.2.1 Adhesion.

The first step in the development of invasive GBS disease is adhesion to host cells. During the development of invasive neonatal disease, GBS is known to adhere to a number of host cells including: the vaginal mucosa, the placental membranes, the lung epithelium, macrophages and the brain endothelium.

Initial interaction with host cells is thought to occur via lipoteichoic acid expressed on the GBS surface (18, 25). Deletion of the *iag* gene encoding the anchor for lipoteichoic acid resulted in a mutant GBS strain that shed lipoteichoic

acid into the culture supernatant and had reduced ability to adhere and invade blood brain barrier cells (25). GBS also express pili (26, 27). Deletion of the *pilA* gene resulted in reduced adherence to A549 cells (pulmonary epithelial cells) and hBMEC (brain endothelial cells) (28, 29). This decreased adhesion by GBS strains lacking the *pilA* gene has since been found to be due to the ability of PilA to bind to collagen (30). GBS has also been demonstrated to express a serine rich repeat protein (Srr-1) on its surface that mediates adhesion to host cells (31, 32). The Srr-1 protein of GBS is believed to mediate adhesion to host cells through its ability to bind keratin 4 (31). In a more recent publication, deletion of the *srr-1* gene was found to decrease adherence to A549 cells as well as reduce virulence in a neonatal rat sepsis model (32). Interestingly, deletion of the *srr-1* gene reduced adhesion to HEp-2 cells (human epithelial cells) without affecting internalization (31), suggesting that Srr-1 mediates an adhesion event that does not lead to internalization. (31). Finally, the GBS surface protein FbsA is believed to mediate adhesion to host cells through its ability to bind fibrinogen (33). Deletion of the *fbsA* gene reduced adhesion to both A549 and hBMEC cells (33, 34).

1.1.2.2 Dissemination.

GBS colonization of the vaginal mucosa can progress to an ascending infection of the placental membrane allowing the bacterium to gain access to the amniotic fluid (35). Aspiration of contaminated amniotic fluid is a major source of GBS infection of the infant's lung (36). In addition GBS crosses the lung

epithelium and pulmonary endothelium to gain access to the infant blood stream. Finally, GBS crosses the blood brain barrier resulting in meningitis. GBS crosses host barrier tissues through three main mechanisms: internalization within host cells, tissue destruction and paracellular invasion.

It was first discovered that GBS could become internalized within epithelial and endothelial cells in the early 1990's (Fig 1.3) (7, 8, 37). Becoming internalized within and trancytosing eukaryotic cells is one mechanism used by GBS to cross barrier tissues (7, 35, 37). The importance of becoming internalized is underscored by the observation that GBS strains isolated from invasive disease are more efficiently internalized than carriage isolates (38). Internalization within epithelial cells by GBS has been demonstrated to rely on changes in the actin cytoskeleton (7, 9) and in some cases microtubules appear to play a role in GBS internalization (35). GBS surface proteins mediate internalization through interaction with host cell surface components. Deletion of the *fbsA* gene resulted in decreased internalization within A549 and hBMEC cells (33, 34), however, this decrease in internalization likely reflects the decreased adhesion discussed above. In contrast, the fibrinogen binding GBS surface protein FbsB has been found to specifically promote internalization into lung epithelial cells (39). Deletion of *fbsB* reduced internalization within A549 cells by 70% without affecting adhesion (39). The surface protein ScpB has been demonstrated to mediate GBS internalization through binding fibronectin (40, 41). Deletion of the *scpB* gene resulted in a 50% reduction in fibronectin binding (41) and reduced internalization into HEp-2 and A549 cells by 82% and 67% respectively, without reducing

adhesion (42). Encoded close to ScpB is the gene encoding the laminin binding protein Lmb. Disruption of the *lmb* gene resulted in an 80% reduction in binding to laminin (43) and a 50% reduction in internalization within hBMEC cells with no reduction in adhesion (44). The alpha-C protein, encoded by the *bca* gene, has also been demonstrated to be involved in GBS internalization. Deletion of *bca* reduced internalization within the human epithelial cell line ME180 by 67% without affecting adhesion (45). The alpha-C protein mediates internalization through binding of a cell surface glycosaminoglycan (46), or the $\alpha 1\beta 1$-integrin (47). While Srr-1 does not play a role in invasion of HEp-2 cells (31), it has been demonstrated to play a role in invasion of hBMEC cells (48); the differences in the results from these two papers may highlight different strategies GBS uses to invade epithelial and endothelial cells. The GBS pilus has also been implicated in GBS internalization (29). Deletion of the *pilB* gene, encoding the major structural subunit of the pilus, was found to reduce internalization within hBMEC cells by 60% without affecting adherence (29). Finally, the β-hemolysin has also been previously demonstrated to contribute to GBS internalization (49). One hypothesis to explain these results is that the β-hemolysin may have adhesive properties, or cell damage by the toxin may induce engulfment (49). Another potential explanation would be that the pores formed by β-hemolysin allow secreted GBS factors to enter the eukaryotic cell cytoplasm to mediate internalization (50).

GBS has also been demonstrated to cross barrier tissues without becoming internalized through destruction of the host barrier tissue (49, 51). In addition to

mediating bacterial dissemination, cellular damage caused by GBS increases the severity of many of the symptoms of GBS disease (49). A major contributor to cellular damage is the β-hemolysin (49, 52). GBS strains containing deletions in the *cylE* gene, which encodes the β-hemolysin, do not cause destruction of lung epithelial cells and are less able to cause invasive disease in rabbit models (49, 51, 52). The β-hemolysin has been shown to be neutralized by the free extracellular phospholipid, dipalmotyl phosphatidylcholine (DPPC) (49, 52), which is a major component of lung surfactant (18). The effect of DPPC on the β-hemolysin, and the fact that preterm infants lack DPPC, is believed to be one of the reasons that preterm infants are particularly susceptible to GBS infection (51).

In addition to internalization and destruction of host cells, GBS has been demonstrated to cross host barrier tissues through a paracellular route (Fig 1.4) (53). While this form of invasion has not been well studied in GBS, it has also been documented for other streptococcal species. Paracellular invasion by *Streptococcus pneumoniae* and group A streptococcus (GAS) has been demonstrated to rely on recruitment of plasmin activity to the bacterial surface (54, 55). The recruited plasmin activity is believed to contribute to paracellular invasion via breakdown of extracellular matrix proteins such as fibronectin and laminin (55) as well as cadherins located in the adherens junctions (54). GBS has also been demonstrated to recruit plasminogen to the bacterial surface, which is activated to plasmin by host plasminogen activating factors (56). The importance of recruiting plasmin activity to the GBS surface is highlighted by the observation that pre-incubation of GBS cells with plasminogen and tissue type plasminogen

activator (tPA) significantly increased virulence in a mouse model (56). It seems likely that GBS, similar to GAS and *S. pneumoniae*, utilizes the recruited plasmin activity to cross host barrier tissues through a paracellular route. One GBS surface protein known to recruit plasminogen to the GBS surface is Skizzle (57). While Skizzle has been demonstrated to be a surface expressed plasminogen binding protein that contributes to plasminogen activation in vitro (57), no Skizzle knockout strain of GBS has been made so it is not clear what contribution Skizzle on the GBS surface has on recruitment and activation of plasminogen on the GBS surface. In addition to Skizzle, plasminogen has been demonstrated to bind to the GBS surface through a fibrinogen intermediate (56).

1.1.2.3 Survival within the host.

To cause disease, GBS must be able to avoid clearance by the human immune system. GBS typically infects individuals that lack antibodies targeting GBS (2, 3). As a result, GBS has only the innate immune system to contend with during the course of infection. To overcome the innate immune system, GBS express virulence factors that provide protection against antimicrobial peptides (AMPs), prevent opsonization by the complement system and promote survival within neutrophils and macrophages.

One major obstacle faced by GBS is the presence of antimicrobial peptides (AMPs). These peptides are small, typically positively charged and generate pores in the bacterial membrane to disrupt membrane integrity (58). AMPs are an

integral part of the innate immune system and are produced by epithelial cells as well as neutrophils (58). GBS resistance to AMPs is mediated by at least three mechanisms. The first identified mechanism used by GBS to resist killing by AMPs is the incorporation of D-alanine into the lipoteichoic acid (59). Incorporation of D-alanine into the lipoteichoic acid, mediated by the *dlt* operon, increases the surface charge on the bacteria repelling the positively charged AMP (59). Deletion of *dltA* resulted in loss of D-alanine incorporation into the lipoteichoic acid, increased sensitivity to cationic antimicrobial peptides and increased killing by human neutrophils and macrophages (59). A second protein involved in resistance to AMPs is PbP1a, encoded by the *ponA* gene (58). It is not yet known how PbP1a provides protection against AMP killing as deletion of the *ponA* gene did not result in any major changes to the bacterial surface. Finally the pilus appears to provide some protection against AMP killing by trapping the AMPs to prevent them from reaching the bacterial membrane (60). GBS resistance to AMPs has been found to be regulated by the CiaR/H two component system, as deletion of *ciaR* resulted in increased sensitivity to AMPs (61). How the CiaR/H two component system regulates resistance to AMPs is not entirely clear as it does not appear to affect expression of the known AMP resistance genes.

Central to the innate immune response are the phagocytic cells, neutrophils and macrophages. In order for neutrophils and macrophages to effectively clear GBS, opsonization of the GBS cells is necessary. Preventing deposition of the complement component C3, is one mechanism GBS utilizes to

prevent opsonization. GBS prevents deposition of C3 through the polysaccharide capsule, BibA, the β protein, the C protein and CspA. Regardless of serotype, the GBS capsule contains a terminal sialic acid, which interferes with C3 deposition (62). The BibA protein is believed to provide anti-phagocytic activity through its ability to bind the complement regulator protein C4bp (63). The β-protein binds factor H, a regulator of the alternate pathway of complement (64), preventing C3 activation. The CspA cleaves host fibrinogen, coating the bacteria with fibrin interfering with complement mediated opsonophagocytic clearance (65). In addition to cleaving fibrinogen, CspA has been demonstrated to cleave the chemokines GRO-α, GRO-β, GRO-γ, NAP-2 and GCP-2 preventing recruitment of neutrophils (66). The ScpB protein has also been demonstrated to prevent recruitment of neutrophils through its ability to cleave C5a (67-70).

In the absence of opsonization, macrophages do not effectively kill internalized GBS (71). This is likely due to lack of activation of the macrophages as activation with LPS and interferon-γ decreased intracellular survival of GBS (71). To survive within non-activated macrophages, GBS encodes proteins involved in AMP resistance as well as glutathione, a superoxide dismutase (SodA), the orange carotenoid pigment and the β-hemolysin. Glutathione, SodA and the orange carotenoid pigment provide protection for GBS against oxidative killing (72, 73). The β-hemolysin provides protection against killing by macrophages as it induces apoptosis in the phagocyte (73). As a pore forming toxin, the β-hemolysin may also allow effector molecules to gain access to the cytoplasm of the phagocyte (50).

1.1.3 Treatment and prevention strategies.

In the 1970's GBS was heralded as the leading bacterial cause of invasive neonatal disease. At this time approximately 80% of GBS disease was EOD (1), occurring within the first week of life. The rate of EOD was estimated at 1.7 cases/1000 live births and at this time neonatal GBS disease had a fatality rate over 50%. Currently, pregnant women are screened for GBS colonization at 35-37 weeks gestation and antibiotic prophylaxis is given to those found to be colonized with GBS (74). With the introduction of antibiotic prophylaxis to prevent transfer of GBS from mother to infant, the rate of EOD GBS infections has been reduced to 0.4 cases/1000 live births (Fig 1.1) (3). Recognition of GBS disease in the neonate has also improved, decreasing the fatality rates from 55% in the 1970's to about 4-6% currently (1, 3). Further reduction in the rate of EOD GBS infections will either require better screening techniques or universal prophylaxis, since the majority of EOD now occurs in infants whose mother tested negative for GBS colonization (3). One interesting option for universal prophylaxis is the use of bacteriophage lytic proteins (75, 76). The main advantage to using a bacteriophage lytic protein such as PlyGBS90-1 (76) is that, because of the narrow spectrum of activity, it is unlikely that universal use of PlyGBS90-1 would have any effect on bacteria other than GBS. This narrow spectrum of activity also makes it less likely that GBS will become resistant.

While intrapartum prophylaxis has been effective at reducing the prevalence of GBS-EOD, preventing GBS in the neonatal and prenatal population is far from complete. The incidence of GBS-LOD has remained stable despite

intrapartum prophylaxis (3). In addition, the current screening and prevention strategies fail to prevent the occurrence of GBS-induced midgestation abortions (21). This manifestation of GBS infection, in particular, needs to be addressed as the few studies that have been performed suggest GBS may be one of the major causes of spontaneous abortion (21). In a study out of Alabama, pregnant women were tested for bacteria in their urine (bacteriuria). Of the women tested, 2% had bacteriuria caused by GBS; of this 2%, 14% spontaneously aborted and were found to have GBS colonization of the amniotic fluid and infant (23). In a later study out of Denmark, the presence of GBS in the urine was again strongly linked to abortion. In women who aborted, 24% had GBS in the urine compared to 1% of women with an uncomplicated pregnancy (22). While the lack of studies makes it difficult to form any definitive conclusions about the prevalence of GBS in spontaneous abortions, these studies suggest that GBS is responsible for approximately one quarter of all spontaneous abortions and that GBS may cause abortions at a frequency of 2.8/1000 pregnancies. The continued presence of GBS in the neonatal population despite antibiotic prophylaxis, along with the concern of antibiotic resistance highlight the need to develop a more complete strategy to control GBS infections. GBS is known to cause invasive disease in the elderly population, another population that has largely been ignored for the development of prevention strategies (6, 24).

The need for a more complete prevention strategy to control GBS infections has led to investigation into vaccine targets. It is believed that maternal vaccination against group B streptococcus would be more effective at preventing

neonatal GBS disease than the current antibiotic prophylaxis (77). Initially, vaccines targeting the GBS capsule were developed and assayed for their ability to provide protection against neonatal GBS disease. These vaccines were found to elicit a protective immune response in 63% of vaccinated mothers (78). Conjugation of the capsular polysaccharides to tetanus toxin improved the response to 80-93% (79). One major limitation to targeting the capsule is the presence of multiple serotypes (80). Targeting conserved surface proteins may be a more effective approach to provide protection against all GBS serotypes. Two GBS surface proteins that have been found to be present on virtually all GBS strains isolated from humans are ScpB and Lmb (81, 82). Similarly, pilus proteins and Sip have also been identified as immunogens that would provide protection against nearly all GBS isolates (83). Vaccines targeting the GBS surface proteins ScpB, Lmb, Sip and the pilus have all been demonstrated to provide protection against GBS infection using mouse models (19, 80, 83, 84). While all of these proteins are well conserved across the GBS serotypes, and antibodies directed against these proteins provide protection against GBS disease, introduction of vaccines into the pregnant population is difficult. Also, due to the current use of antibiotic prophylaxis to prevent GBS-EOD, it is not feasible to design a clinical trial to assess the efficacy of GBS vaccines to prevent GBS-EOD (4, 19). One strategy to assess efficacy of the vaccine in the absence of invasive neonatal disease is to assay antibody production and transfer to the neonate to predict vaccine efficacy (19, 85). A second option is to focus on a different manifestation of invasive GBS disease, such as in the elderly population (86).

Once the vaccine has been shown to be protective and safe in the elderly population it may be easier to introduce into the pregnant population to prevent GBS disease in the neonate.

1.2 Surface expressed glycolytic enzymes:

For many years, glycolytic enzymes were believed to only be involved in energy generation (12). The first indication that these enzymes may play a role other than metabolism was the discovery that they make up a large portion of the structural component of the eye lens (87, 88). Since then, the eukaryotic glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described to have many functions unrelated to glycolysis. Both GAPDH and enolase have been demonstrated to have nuclear functions regulating gene transcription (89, 90), telomere length, DNA mismatch repair, export of tRNA's and mRNA stability (89, 91). GAPDH has also been demonstrated to play a role in endocytosis, nuclear membrane assembly and cytoskeleton dynamics (91). Glycolytic enzymes have also been identified as surface expressed and secreted proteins of gram positive bacteria (Fig 1.5), including GBS (12, 92-94). How these enzymes become surface expressed is not known as they contain no known signal sequence for secretion or surface attachment. However, their surface localization suggests that these enzymes may play a role in bacterial virulence.

1.2.1 Potential role in virulence.

While the localization of glycolytic enzymes on the surface of gram positive bacteria suggest they may play a role in virulence, assigning a function to these proteins has been hampered by their essential role in metabolism. In some cases the presence of two genes, one encoding the cytoplasmic enzyme and the other encoding the secreted protein, has allowed gene knock-outs to be used to determine their function (95). In most cases however, including GBS-PGK, only one gene is present in the bacterial genome and this approach is not feasible. As a result, the majority of research focusing on these enzymes has been limited to functional studies using purified proteins. Two well studied surface expressed glycolytic enzymes are α-enolase and GAPDH. Functional studies demonstrate that these proteins have the capacity to bind multiple host proteins (14-16, 96-106), suggesting that surface expressed α-enolase and GAPDH may contribute to adhesion, dissemination and survival within the host. While α-enolase and GAPDH are believed to play a role in bacterial virulence, little research has been performed to determine the function of surface expressed GBS-PGK.

1.2.1.1 Adhesion.

Adhesion to eukaryotic cells is a prerequisite for the development of invasive disease. GAPDH has been identified as a potential adhesin for *Streptococcus suis* (107, 108). Tn*916* mutagenesis resulting in loss of a 39 kDa

protein, eventually identified as GAPDH, from the bacterial surface significantly reduced adherence to bovine tracheal cells and porcine tracheal rings (107). In a follow up study porcine tracheal rings were pre-incubated with recombinant GAPDH resulting in decreased adherence by *S. suis* (108). Similarly prevention of GAPDH expression on the surface of GAS through the addition of a hydrophobic tail resulted in decreased adherence to human pharyngeal cells (13). While these results seem to demonstrate that GAPDH contributes to adhesion, in neither case was it possible to definitively demonstrate that the loss of adhesion was due to the loss of GAPDH on the bacterial surface. In the case of GAPDH on the surface of *S. suis*, loss of GAPDH expression was due to Tn*916* insertion into genes that may have affected expression of proteins other than GAPDH. In the case of GAPDH on the surface of GAS, sequestering GAPDH to the GAS cytoplasm resulted in altered expression of a number of virulence genes (109). It does seem likely however that loss of GAPDH from the bacterial surface would reduce adhesion as it has been demonstrated to bind host proteins that could contribute to bacterial adhesion and colonization. Similarly, α-enolase is capable of binding many of the same proteins as GAPDH and may contribute to adhesion as well.

Both α-enolase and GAPDH have been identified as mucin binding proteins (98, 100-103). α-enolase from both *Streptococcus mutans* and *Streptococcus gordonii* has been demonstrated to bind salivary mucin. GAPDH isolated from *Mycoplasma genitalium* preferentially bound to human vaginal/cervical mucin but also bound bovine submaxillary type I mucin to high

levels, while binding to porcine gastrointestinal mucin was not seen (98). These results are in contrast to GAPDH isolated from *Lactobacillus plantarum* which was found to bind human colonic mucin (102, 103). Studies, characterizing the interaction between GAPDH and mucin, identified five sugars that inhibit binding. Binding by *M. genitalium* GAPDH was inhibited by fucose, galactose, sialic acid, N-acetylglucosamine and N-acetylgalactosamine, indicating that GAPDH binds mucin through the mucin associated sugars (98). This is in agreement with GAPDH isolated from *L. planatarum* which was shown to bind mucin through interaction with blood group antigens attached to the mucin (103).

The eukaryotic glycolytic enzymes GAPDH, aldolase, lactate dehydrogenase and phosphoglycerate kinase have been demonstrated to bind to actin (99). It has also been demonstrated that GAPDH isolated from GAS is also capable of binding actin (110). Although actin is traditionally thought of as a cytoplasmic protein, it has been identified on the surface of lymphocytes, monocytes, endothelial cells and L cells (111-115). This surface localization indicates that the ability for surface expressed glycolytic enzymes to bind actin may facilitate adhesion to these eukaryotic cells. However, with the exception of GAPDH, it is not clear if bacterial glycolytic enzymes retain the capacity to interact with actin. As a result, it is not clear if surface expressed glycolytic enzyme would contribute to adhesion through actin binding.

Additionally, α-enolase, GAPDH and PGK have all been demonstrated to bind plasminogen (14, 105, 106, 116, 117). Interaction with plasminogen has been demonstrated as a major virulence characteristic of gram positive bacteria,

contributing to bacterial dissemination (54-57, 118). In addition to contributing to bacterial dissemination, plasminogen binding by *Streptococcus pneumoniae* and group A streptococcus has been shown to lead to increased attachment to eukaryotic cells and may be involved in initial colonization (54, 55). In contrast to plasminogen mediated degradation of the extracellular matrix, plasminogen binding to facilitate adhesion requires that the plasminogen remain un-activated (55). In a 2007 paper by Magalhaes *et al*. GBS was found to express five plasminogen binding proteins (56). In this paper, GAPDH was identified as one of the plasminogen binding proteins (56). In a more recent publication, Skizzle was identified as a second plasminogen binding protein (57). Skizzle was found to not only bind plasminogen, but this binding was found to enhance activation of plasminogen (57).

1.2.1.2 Dissemination.

Fibronectin is a 450 kDa glycoprotein found both as a soluble protein in the blood plasma and as an insoluble protein in the extracellular matrix (119). Nearly all invasive gram positive bacteria encode surface expressed fibronectin binding proteins (42, 120-127). In *Staphylococcus aureus* and GAS, the recruited fibronectin binds to the $\alpha 5\beta 1$ integrin and CD46 to facilitate internalization (119, 122, 126-129). As previously discussed, the ScpB protein of GBS is one of the fibronectin binding proteins on the surface of GBS (40, 41). Deletion of the *scpB* gene resulted in a loss of approximately 50% of the fibronectin binding capacity

of GBS, suggesting the presence of a second fibronectin binding protein on the GBS surface (41). α-enolase, PGK and the E1 β subunit of pyruvate dehydrogenase have all been previously demonstrated to bind fibronectin (41, 96, 97, 130), indicating that one or more of these glycolytic enzymes could contribute to GBS fibronectin binding.

Laminin is another extracellular matrix protein that is through to be involved in internalization of bacteria (127). Similar to fibronectin binding, GAS internalization via laminin has been demonstrated to be dependent on the β1 integrin (127). However, unlike fibronectin mediated internalization, laminin mediated internalization does not appear to be dependent on the α5 integrin (127). While GBS has been demonstrated to express the laminin binding protein, Lmb (43, 44), residual laminin binding is still seen in GBS strains containing deletions in the *lmb* gene (43), suggesting the presence of other laminin binding proteins. In *Staphylococcus aureus*, an additional laminin binding protein was identified as α-enolase (131). Conceivably, α-enolase may act as a laminin binding protein in other gram positive bacteria, such as GBS.

As previously discussed, a number of glycolytic enzymes have been previously demonstrated to bind actin (99, 110). While this binding activity may be involved in adherence to eukaryotic cells expressing actin on their surface, it is also possible that secreted glycolytic enzymes may gain access to the host cell cytoplasm through pore forming toxins (50) to directly interact with the host cytoskeleton. Since GAPDH has previously been demonstrated to play a role in endocytosis and cytoskeleton dynamics (91), it seems possible that it could also

facilitate internalization of GBS. Also, because the cytoskeleton is involved in phagosomal maturation (132), interaction with the actin cytoskeleton may also contribute to intracellular survival through preventing phagosomal maturation.

Another major mechanism utilized by gram positive bacteria to cross epithelial and endothelial barrier tissues is through the acquisition and subsequent activation of plasminogen on the bacterial surface resulting in degradation of the extracellular matrix and basement membrane (106). In the case of GBS, plasminogen recruited to the bacterial surface is activated by the host activators urokinase (uPA) plasmin activator and tPA (56). Plasminogen has been demonstrated to enhance GBS virulence in a mouse model (56), presumably through providing GBS with proteolytic activity. Two GBS surface proteins that have been demonstrated to bind plasminogen are GAPDH (56) and Skizzle (57). α-enolase and PGK from other sources have also been demonstrated to bind the eukaryotic proteins plasmin and plasminogen (13, 14, 97, 104-106, 133). Binding to plasminogen by both GAPDH and α-enolase has been shown to be at least partially due to two C-terminal lysine residues (105, 130). An additional plasminogen binding region (FYDKERKVY) has been identified in α-enolase of *Streptococcus pneumoniae* spanning amino acids 248-256 (14, 134). Interestingly, the GBS *α-enolase* gene contains a point mutation within this identified plasminogen binding region that appears to improve binding to plasminogen (135). To date the contribution each of these surface expressed proteins has on recruitment of plasminogen to the GBS surface is not known.

Functional studies characterizing plasminogen binding by α-enolase of *S. pneumoniae* led to the generation of an α-enolase protein that is not capable of binding plasminogen (14, 134). Incorporation of this mutant α-enolase gene in place of the genomic copy resulted in a 55% reduction in binding of plasminogen to the bacterial surface (14). This loss of plasminogen recruitment correlated to reduced degradation of basement membrane and extracellular matrix proteins (15) as well as reduced degradation of fibrin (15). While in this case the functional studies eventually allowed the authors to demonstrate a role for α-enolase in *S. pneumoniae* virulence, in other cases the functional studies have not been as successful. The C-terminal lysine residues of GAPDH have been demonstrated to be involved in binding plasmin (35). Removal of these lysine residues from the GAPDH protein results in loss of binding between GAPDH and plasmin, however replacement of the genomic *gapdh* gene with this mutant *gapdh* gene did not result in reduced plasmin recruitment to the GAS surface (136). These results highlight the need to confirm the in vitro experiments, with in vivo models to definitively demonstrate the role of glycolytic enzymes on the bacterial surface.

1.2.1.3 Survival within the host.

Iron is an essential nutrient for almost all organisms (137). As a result, acquisition of iron is a necessary step for the survival and multiplication of invading pathogens within the host. In the serum of mammals, iron is extremely limiting due to the presence of the iron binding glycoprotein transferrin.

Staphylococcus aureus expresses a cell wall anchored protein, which has been identified as GAPDH, with transferrin binding capability to facilitate the uptake of iron (137, 138). Expression of GAPDH has been previously shown to be increased in the absence of iron. The role GAPDH plays in iron acquisition has more recently come into question as purified GAPDH has been shown to not bind transferrin (139).

α-enolase from *Streptococcus sobrinus* and GAPDH of GBS have been implicated in providing protection against the host immune response in mouse models (140, 141). These two proteins were both found to inhibit the adaptive immune response by inducing expression of the anti-inflammatory cytokine interleukin-10 (140, 141). The IL-10 response for both α-enolase and GAPDH was found to occur rapidly after inoculation (2 and 6 hours respectively) (140, 141). In addition, GAPDH from GAS has been shown to bind the neutrophils and macrophage chemoattractant C5a, both on the bacterial surface and in the culture supernatant (142). Binding of C5a by GAPDH on the GAS surface is necessary for cleavage of C5a by ScpA (142). Also, C5a binding by secreted GAPDH is thought to contribute to GAS virulence by sequestering C5a and inhibiting its chemotactic function (142). GBS encodes a similar C5a peptidase, ScpB (69, 70), suggesting that GAPDH may play a similar role in GBS.

In addition to providing protection against the initiation of an inflammatory response and preventing influx of neutrophils, glycolytic enzymes may also contribute to survival within phagocytes. As has been previously discussed, actin binding by bacterial glycolytic enzymes (99, 110) that have

gained access to the host cell cytoplasm may alter intracellular trafficking. Since maturation of phagosomes relies on cytoskeleton dependent membrane trafficking events (132), it stands to reason that glycolytic enzymes may delay or halt the maturation of phagosomes. In support of this statement, GAPDH from *L. monocytogenes* has been demonstrated to prevent phagosome maturation through inhibition of Rab5a (143). This inhibition of Rab5a is linked to GAPDH ADP-ribosylating ability (143). GAPDH from GAS has been demonstrated to have similar ADP-ribosylating properties (144), suggesting that GAPDH from GAS may also inhibit phagosome maturation to contribute to GAS virulence.

1.2.3 Transport to the bacterial surface.

Since glycolytic enzymes do not contain any known secretion or surface attachment signals, these proteins are known as anchorless surface proteins (11). The presence of these enzymes on the surface of numerous gram positive and fungal pathogens despite the absence of any known signal sequences has led to the hypothesis that they are expressed on the surface through a specialized transport system (12). To date, this specialized transport system has not been identified. While the mechanism by which these enzymes become surface expressed remains unknown, a number of observations have been made that may aide in determining how glycolytic enzymes become surface expressed. In addition to being expressed on the bacterial surface, glycolytic enzymes have been identified in the bacterial supernatant (131, 145, 146). Coupled with the

observation that exogenously added glycolytic enzymes will attach to the bacterial surface (105), these results suggest that glycolytic enzymes become surface expressed by being secreted into the bacterial supernatant followed by passive re-association with the bacterial surface (12).

The first step in expressing glycolytic enzymes on the bacterial surface is secretion of the protein across the bacterial membrane. In *Listeria monocytogenes*, deletion of the *secA2* gene resulted in a loss of surface expressed α-enolase (147). While this result suggests that SecA2 is involved in secretion of glycolytic enzymes, since these experiments did not assess secreted proteins (147), it is also possible that SecA2 is necessary for expression of the ligand responsible for attaching α-enolase to the bacterial surface. It also has not been determined how α-enolase was targeted for SecA2 dependent secretion, as it does not contain the known SecA2 secretion signal. As a result, α-enolase was been deemed "moonlighting" protein (147). A 2004 paper by Boel *et al*. demonstrated that lysine residue at amino acid 341 of α-enolase is modified by covalent linkage to 2-phosphoglycerate (148). This modification occurred only in 1-2% of the total α-enolase, but 10% of the extracellular α-enolase was found to be modified (148). This data, along with the observation that the modification is reversible, led to the conclusion that modification by 2-phosphoglycerate is required for export of α-enolase (148). While modification of α-enolase by covalent attachment of 2-phosphoglycerate was found to be necessary for secretion, the experiments were performed using *E. coli* and therefore may not be applicable to secretion in a gram positive bacteria. However, post-translational modification of glycolytic enzymes

to facilitate transport to the bacterial surface is a possibility as 2D-PAGE analysis has identified more than one spot corresponding to these enzymes (94). Finally, it has been demonstrated that addition of a hydrophobic tail to the C-terminus of GAPDH sequestered the protein in the bacterial cytoplasm (13), suggesting the signal sequence for secretion of GAPDH is localized to the C-terminus of the protein.

The second step in surface expression of glycolytic enzymes is the, possibly passive, re-association with the bacterial surface (11, 12). Interestingly, in *S. pneumoniae* strains that express a capsule, α-enolase attaches to the outer surface of the capsule (Fig 1.6) (105); while in strains that do not express a capsule, α-enolase attaches to the outer surface of the bacterial cell wall (Fig 1.6) (105). These results suggest either that α-enolase may attach to the *S. pneumoniae* surface through interaction with more than one surface component or that it attaches through interaction with a single surface component stretching through the cell wall and capsule. GAPDH has been found attached to the surface of *Streptococcus gordonii* only at pH 6.5 and below, at pH 7.5 90% of the GAPDH is found in secreted form (149). These results suggest that GAPDH binding to the surface of *S. gordonii* is mediated by a pH dependent charge interaction. Similar results were obtained for α-enolase and GAPDH association with the surface of *Lactobacillus crispatus* was found only to occur at low pH (pH 4.5) and incubation of the bacteria at pH 8 resulted in loss of both glycolytic proteins from the bacterial surface (150). In both *S. gordonii* and *L. crispatus* , interaction of glycolytic enzymes with the bacterial surface was thought to be

mediated through interaction with lipoteichoic acid. Based on the location of α-enolase on encapsulated and non-encapsulated strains of *S. pneumoniae* (Fig 1.6), it seems likely that α-enolase also attaches to the surface of *S. pneumoniae* via interaction with lipoteichoic acid. In contrast, GAPDH has been demonstrated to be firmly associated with the surface of GAS (110) through a mechanism that is not pH dependent. In the case of GAS, association of glycolytic enzymes with the bacterial surface has been proposed to occur through interaction with the M-protein (151).

1.2.4 Potential vaccine development.

As proteins present on the surface of gram positive bacteria, glycolytic enzymes may be candidates for vaccine development. Antibodies against these surface expressed proteins have been demonstrated to provide protection against lethal infection from a number of pathogenic bacteria in mouse models. For example, inoculation of mice with antibodies targeting PGK has been demonstrated to provide protection against lethal GBS infections (94). Similarly, immunization against both GAPDH and fructose-bisphosphate aldolase has been demonstrated to provide protection against *S. pneumoniae* infection (92). These observations suggest that glycolytic enzymes, particularly PGK, could potentially be used as a target for development of a vaccine to prevent GBS disease. Two features of glycolytic enzymes that make them particularly tempting for development of vaccines is their presence on the surface of a number of different

gram positive bacteria (152) and the amino acid similarities between glycolytic enzymes from different bacterial species. These features raise the possibility of developing a vaccine that could provide protection against more than one species of bacteria (152). While the amino acid similarities between glycolytic enzymes from different bacterial species raise the possibility for a vaccine providing protection against more than one bacterial species, it also raises the possibility of unwanted cross-reactivity. Since glycolytic enzymes are expressed by human cells as well as non-pathogenic bacteria (153), the development of an auto-immune response and an immune response targeting the normal flora is a possibility.

Autoimmune disorders following bacterial infection have been reported (154-156); in many cases, these infections result in antibodies that cross react with human glycolytic enzymes (154-156). Given the similarities between bacterial and human glycolytic enzymes it is not surprising that generation of antibodies to bacterial glycolytic enzymes could result in antibodies that cross-react with their human counterpart (154, 156). Antibodies to enolase have been implicated in acute rheumatic fever (154), rheumatoid arthritis (155, 156) asthma (157) and primary biliary cirrhosis (158). Antibodies to GAPDH have been identified in patients suffering from lupus (159), dilated cardiomyopathy (160) multiple sclerosis (161) and rheumatic fever (145). Given the array of autoimmune disorders resulting from antibodies targeting glycolytic enzymes, to develop a vaccine against PGK, it will be necessary to target a region that is unique to GBS-PGK. A second issue that may be more difficult to reconcile is the effect vaccines

targeting glycolytic enzymes will have on the normal flora. Glycolytic enzymes have been demonstrated to be present on the surface of a number of non-pathogenic gram positive bacteria (153), suggesting that vaccines targeting these enzymes may eliminate non-pathogenic organisms as well. Since GBS-PGK is likely to be more similar to PGK from other bacterial species than human PGK it may be more difficult to identify unique amino acid sequences from GBS-PGK.

1.3. Objectives and hypotheses:

Objective 1: To determine how GBS-PGK is transported to and attached to the GBS surface.

Hypothesis: GBS-PGK is likely secreted across the GBS membrane via the SecA2 secretion system. Once Secreted, GBS-PGK is likely attached to the bacterial surface through interaction with lipoteichoic acid on the GBS surface.

Objective 2: Identify potential virulence functions of surface expressed GBS-PGK.

Hypothesis: PGK, from GBS and other sources, has previously been demonstrated to bind actin, fibronectin and plasminogen. If GBS-PGK retains these binding functions, they likely will contribute to GBS virulence.

Objective 3: Prevent the virulence functions of GBS-PGK without affecting glycolysis.

Hypothesis: If the binding sites within GBS-PGK responsible for interaction with host proteins can be identified, site directed mutagenesis of these sites should result in a GBS-PGK molecule that is glycolytically active but lacks the ability to bind host proteins.

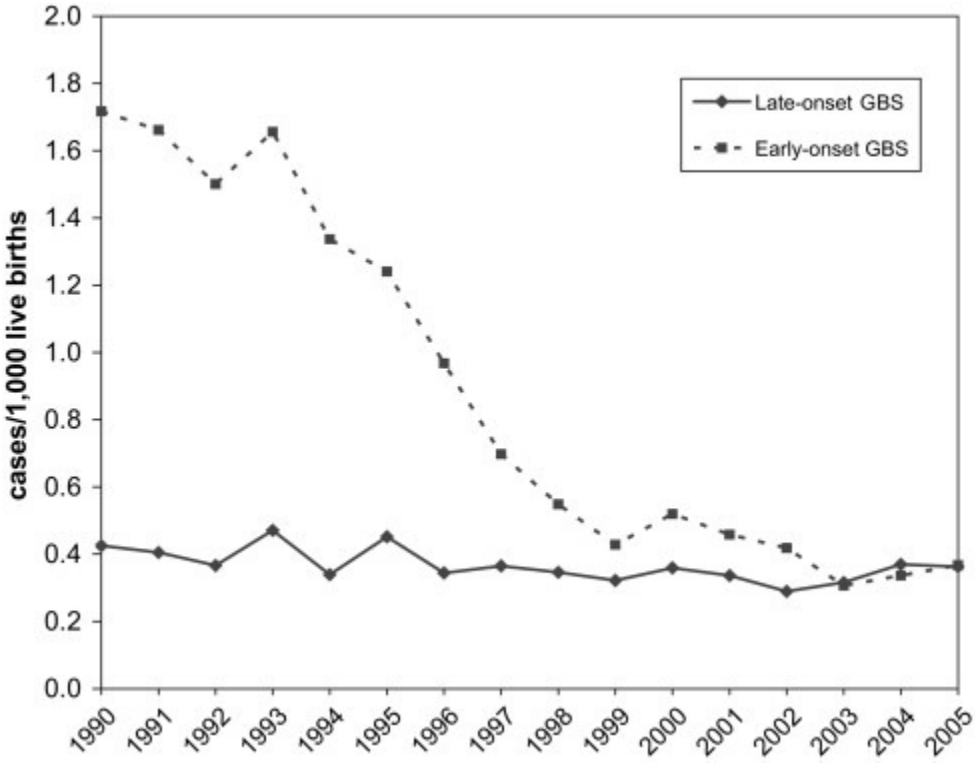

Fig 1.1. Incidence of group B streptococcal infections in North America 1990-2005. In the early 1990's the incidence of early onset GBS infections were approximately 1.7 cases/1000 live births. With the introduction of prevention strategies the incidence of early onset GBS disease dropped to approximately 0.4 cases/1000 live births. Over this time period the incidence of late onset GBS disease remained relatively stable at 0.4 cases/1000 live births. (Adapted from Verani *et al.* 2010; (3)).

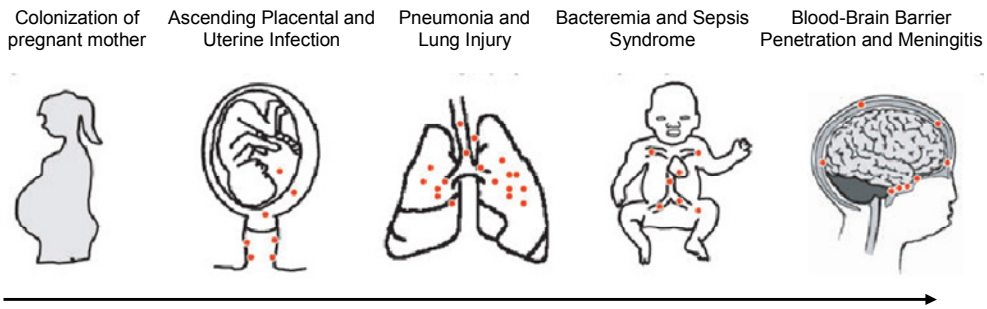

Figure 1.2. Progression of GBS infection in the neonate. GBS infection of the neonate begins with asymptomatic colonization of the mother. This colonization leads to an ascending infection resulting in contamination of the amniotic fluid. Inhalation of contaminated amniotic fluid by the infant results in colonization of the infant's lung resulting in pneumonia and lung injury. GBS gains access to the bloodstream through invasion of the lung epithelium resulting in bacteremia and sepsis syndrome. GBS circulating in the bloodstream eventually gain access to the blood-brain barrier. Invasion of the blood brain barrier results in the development of meningitis. (Adapted from Doran *et al*. 2004;(36)).

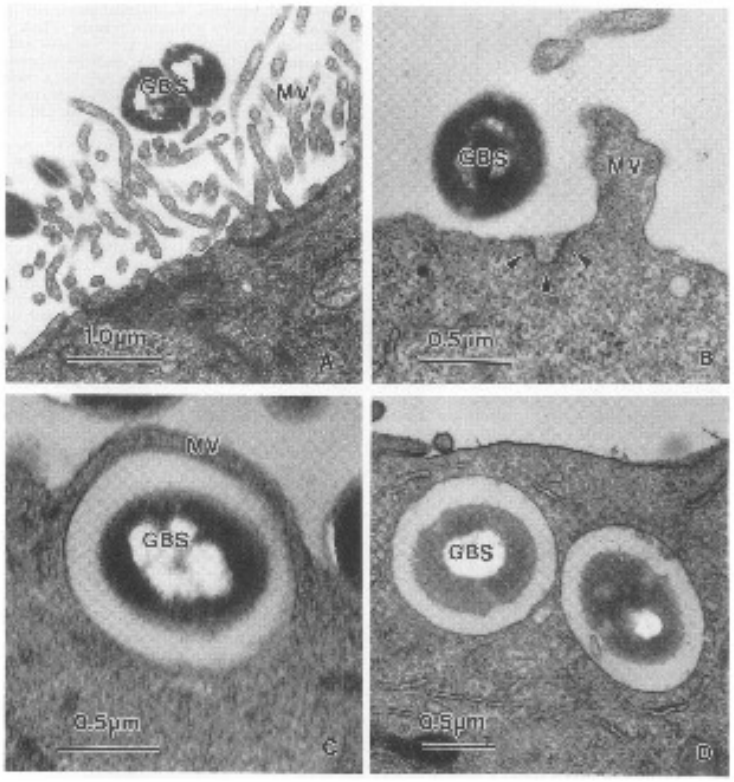

Figure 1.3. Electron micrographs of GBS strain COH-1 entering cultured A549 respiratory epithelial cells. One hour post inoculation (A and B), GBS cells were seen associated with microvilli (MV). Two hours post inoculation (C and D), GBS cells can be observed enclosed within membrane-bound vacuoles. (Adapted from Rubens *et al*. 1992; (7)).

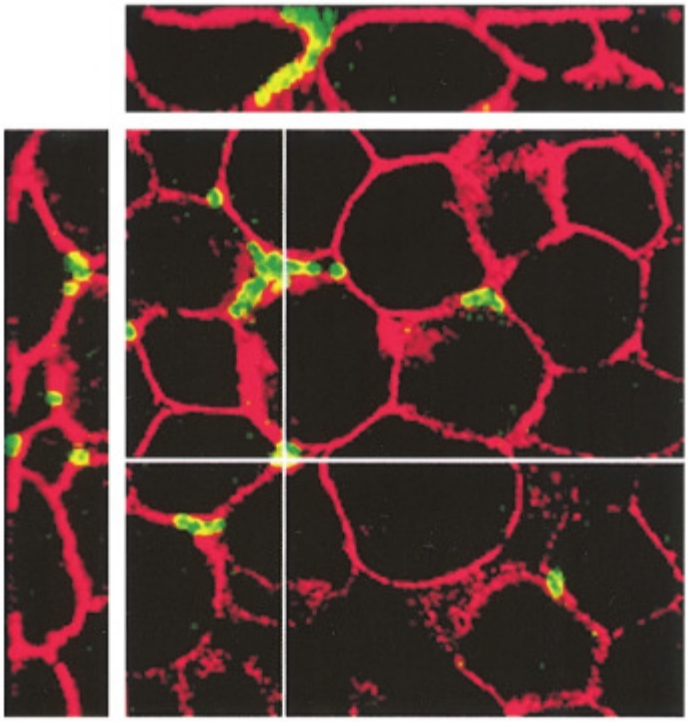

Figure 1.4. Confocal immunofluorescence microscopy image of GBS associated with the perimeter of Caco2 epithelial cells. Caco2 cells were incubated for 4 h with GBS strain 2603 V/R. Cells were stained with rabbit anti-GBS serotype V capsular polysaccharide and labeled with Alexa Fluor 488-conjugate antibodies (green). The F actin within the eukaryotic cells was labeled using Texas red-conjugate phalloidin (red). Top and side strips are relative to Z sections through the monolayer at the white lines drawn on the main panel. GBS cells can be seen in close proximity with actin and located between the polarized Caco2 cells. (Adapted from Soriani et al. 2006; (53)).

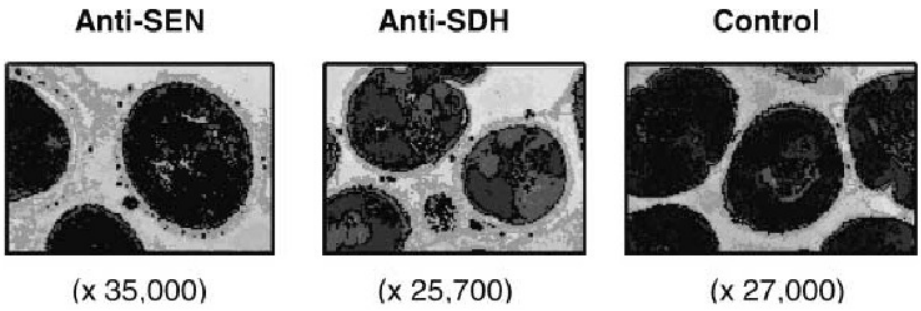

Figure 1.5. detection of α-enolase and GAPDH on the surface of group A streptococcus. α-enolase was detected on the surface of group A streptococcus using anti-SEN (1A10) monoclonal antibodies linked to 5 nm gold particles. GAPDH was detected on the surface of group A streptococcus using anti-SDH (4F12) monoclonal antibodies linked to 10 nm gold particles. Association of the gold particles with the group A streptococcal surface can be seen using both the anti-SEN and anti-SDH antibodies, while no localization can be seen when probing with the anti-mouse IgG control antibodies. (Adapted from Pancholi and Chhatwal 2003; (12)).

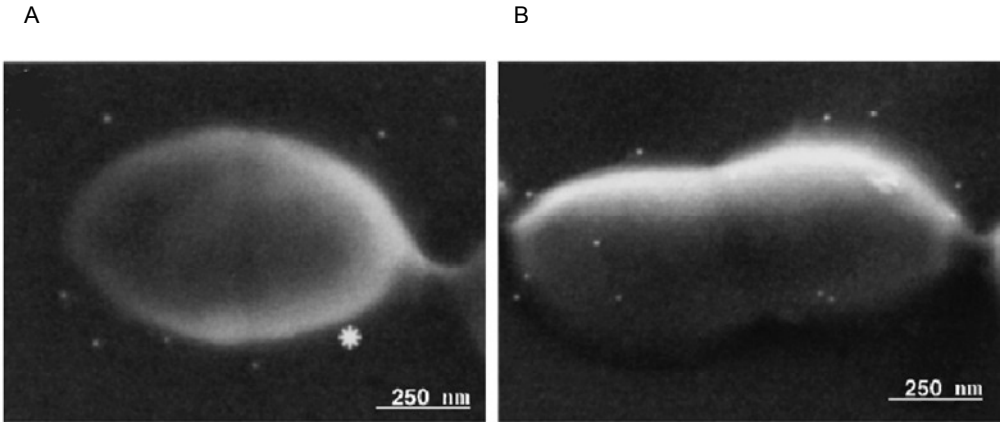

Fig 1.6. Electron microscopic localization of α-enolase on the surface of *Streptococcus pneumoniae*. α-enolase was detected on the surface of the encapsulated *S. pneumoniae* strain ATCC 11733 (A) and the non-encapsulated *S. pneumoniae* strain R6x (B) using anti-Eno antibodies and 15 nm protein A-gold particles. The white dots represent gold particles, while the white star indicates the location of the capsule. α-enolase was detected at the surface of the cell wall on the R6x strain, while α-enolase was detected at the outer surface of the capsule on the ATCC 11733 strain. (Adapted from Bergmann *et al.* 2001; (105)).

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Chapter 2:

Phosphoglycerate Kinase is a Surface Expressed Group B Streptococcal Protein with the Ability to Bind a Variety of Host Proteins

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2.1. Introduction:

Group B streptococcus (GBS) has long been recognized as a major bacterial pathogen involved in invasive neonatal disease as well as invasive disease in the adult population (1-3). To cause invasive disease, GBS must cross host barrier tissues to gain access to sterile body sites such as the circulatory system, meninges, deep tissues etc. (1, 4-6). One mechanism GBS uses to cross these barrier tissues is to become internalized within the host cells through bacterial induced actin cytoskeleton rearrangements (5, 7-12). The mechanism(s) of cell invasion by GBS seems to be multifactorial involving a number of GBS surface binding proteins including fibrinogen binding proteins A and B, alpha C protein, laminin binding protein, ScpB and pili to name a few (13-16). In addition to becoming internalized, GBS has been demonstrated to cross host barrier tissues through β-hemolysin induced tissue destruction (17) and paracellular invasion (18).

While many GBS surface proteins have well characterized roles, the glycolytic enzyme phosphoglycerate kinase (PGK) is one GBS surface protein without a clearly defined function outside of the cytoplasm (19, 20). Other glycolytic enzymes, including α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have also been identified on the surface of GBS suggesting they may have roles beyond glycolysis (20, 21). Surface presentation of glycolytic enzymes is not restricted to GBS as other gram positive bacteria such as group A Streptococcus (GAS), *Streptococcus pneumoniae*, *Staphylococcus aureus, Bacillus anthracis* and the fungal pathogen, *Candida*

albicans, express these glycolytic proteins on their surface (19, 20, 22-24). Due to their essential role in glycolysis, it has not been possible to prevent expression of these enzymes in order to determine their function on the cell surface (22, 25-28). However, functional studies using purified proteins suggest these enzymes may have functions unrelated to glycolysis (21, 23, 25-34). Using PGK as an example, PGK from GBS and other sources has been demonstrated to bind actin, fibronectin and plasminogen (13, 32, 35). Binding to actin, fibronectin and plasminogen by PGK suggests a potential role for surface expressed group B streptococcal phosphoglycerate kinase (GBS-PGK) in attachment to and internalization within host cells by GBS as well as breakdown of extracellular matrix proteins to facilitate bacterial dissemination.

The objective of this chapter was to determine if PGK could contribute to GBS virulence by assaying its ability to bind eukaryotic proteins and characterizing its expression outside the GBS cell membrane.

2.2. Materials and methods:

2.2.1. Production, purification and generation of antibodies to GBS-PGK.

The *pgk* gene was PCR amplified from chromosomal DNA isolated from a previously described serotype V GBS strain, NCS13 (7, 19), using the primers PGK-pstF (TTCCTGCAGTTATTTTCAGTCAATGC) and PGK-hinR (TTCAAGCTTTTTTCAGTCAATGCTGCCAAACC) (Integrated DNA technologies; CA, USA). The resulting amplicon was ligated into the expression plasmid pQE30, and transformed into chemically competent *Escherichia coli* M15. Recombinant GBS-PGK (rGBS-PGK) was expressed and purified as an N-terminal hexahistidyl tagged protein under native conditions using the Qiaexpressionist kit (Qiagen; Mississauga, ON, CA). Ten milliliter overnight cultures were used to seed 100 ml Luria-Bertani broth supplemented with 60 μg/ml carbenicillin and 25 μg/ml kanamycin. After 1 h incubation at 35°C, expression of rGBS-PGK was induced by the addition of 2mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 h incubation at 35°C bacterial cultures were harvested by centrifugation at 5000 ×g and the cellular pellet was frozen overnight at -70°C. The bacterial pellet was resuspended in 1 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH 8.0) and treated with lysozyme (1 mg/ml) for 30 min on ice. Bacterial cells were then sonicated in an ice water bath for 15 min and centrifuged 10 000×g for 20 min. The resulting

supernatant was collected and incubated with 1 ml 50% Ni-NTA slurry (Qiagen) for 1 h at 4°C with shaking. The slurry was loaded into a 1 ml polypropylene column (Qiagen) and washed 8× with 1 ml aliquots of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). Purified protein was eluted 6× with 500 µl aliquots of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) (Fig 2.1).

The enzymatic activity of the purified rGBS-PGK was determined as previously described (36) by adding 2.4 µg to a 3 ml reaction mix containing 50 mM potassium phosphate, 0.83 mM glyceraldehyde-3-phosphate, 0.3 mM β-nicotinamide adenine dinucleotide, 0.2 mM adenosine 5`-diphosphate, 4.2 mM magnesium sulfate, 133 mM glycine and 1 unit glyceraldehyde-3-phosphate dehydrogenase. The absorbance at 340nm (A_{340}) was measured for five min and the change in absorbance was used to calculate the enzymatic activity. Commercial PGK (0.05 units; Sigma-Aldrich, St. Louis, MO, USA) isolated from *Saccharomyces cerevisiae* (sc-PGK) was used as a positive control and potassium phosphate (100mM) was used as a negative control. Purified, enzymatically active (Fig 2.2), rGBS-PGK was used to generate anti-rGBS-PGK antibodies in New Zealand white rabbits and the antibodies were purified using protein A affinity chromatography (Genscript; Piscataway, NJ, USA).

2.2.2. Quantification of interactions with host proteins.

The interaction between rGBS-PGK and host proteins actin, fibronectin, laminin, plasmin and plasminogen was assayed using ELISA. Host proteins: bovine actin (1 μg/well), human fibronectin (1 μg/well) , human plasmin (1 μg/well), human laminin (1 μg/well) and human plasminogen (0.1 μg/well) (Sigma-Aldrich) were immobilized to flat bottom 96 well polystyrene plates (Maxi-sorp; NUNC, Thermo Fischer Scientific, Nepean, Ontario, Canada) by diluting in 0.1 M sodium carbonate solution (pH 9.5) and incubating 8 h at room temperature. Plates were washed 1× with tris buffered saline (TBS), blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich), 0.1% tween 20 in TBS for 16 h at room temperature and incubated with increasing concentrations of rGBS-PGK (100 μl; 0-57.8 μg/ml) diluted in blocking buffer for 2 h. Plates were washed 3× with TBS and probed with anti-rGBS-PGK antibodies (50 μl; 1:300 in blocking buffer) for 1 h at room temperature. Plates were then washed 3× with TBS and incubated with goat anti-rabbit-IgG alkaline phosphatase conjugate (50 μl; 1:200 in blocking buffer) for 1 h at room temperature. Plates were washed 3× with TBS and developed with 100 μl 4-nitrophenol phosphate (Sigma-Aldrich) for 30 min at room temperature before stopping the reaction with 25 μl NaOH (3 N). Absorbance at 405nm (A_{405}) was measured using an Athos LP400 microplate reader (Bio-Rad Laboratories; Mississauga, ON, CA).

The role of rGBS-PGK lysine residues in binding to actin and plasminogen was determined using ELISA in the presence of the lysine analogue

6-aminocaproic acid (6-ACA) similar to previously described (27). Actin (1 μg/well) and plasminogen (0.1 μg/well) were fixed to wells of a 96 well polystyrene plate (Maxi-sorp). Plates were washed 1× with TBS, blocked with 5% BSA, 0.1% tween 20 in TBS for 16 h at room temperature. Plates were washed 1× with TBS and incubated with rGBS-PGK (25 μg/ml) diluted in blocking buffer containing increasing concentrations of 6-ACA (0-50 mM) for 2 h. Plates were washed 3× with TBS and incubated with anti-rGBS-PGK antibodies (50 μl; 1:300 in blocking buffer) for 1 h at room temperature. Plates were then washed 3× with TBS and incubated with goat anti-rabbit-IgG alkaline phosphatase conjugate (50 μl; 1:200 in blocking buffer) for 1 h at room temperature. Plates were washed 3× with TBS and developed with 100 μl 4-nitrophenol phosphate (Sigma-Aldrich) for 30 min at room temperature before stopping the reaction with 25 μl NaOH (3 N). Absorbance at 405nm (A_{405}) was measured using an Athos LP400 microplate reader.

Interaction of rGBS-PGK with immobilized fibrin and fibrinogen was also assayed using ELISA. Fibrin and Fibrinogen (0.1μg/well) were immobilized to flat bottom 96 well polystyrene plates (Maxi-sorp) by diluting in 0.1 M sodium carbonate solution (pH 9.5) and incubating 8 h at room temperature. Plates were washed 1× with TBS and incubated 16 h with blocking buffer. Wells were washed 1× with TBS and incubated with increasing concentrations of rGBS-PGK (0-60 μg/ml in blocking buffer) for 2 h. Plates were washed 3× with TBS and probed with anti-rGBS-PGK antibodies (1:300 in blocking buffer) for 1 h at room temperature. Plates were washed 3× with TBS and incubated with goat anti-

rabbit-IgG alkaline phosphatase conjugate (1:200 in blocking buffer) for 1 h at room temperature. Plates were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate for 30 min at room temperature before stopping the reaction with 25 µl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader.

ELISA was also used to demonstrate binding of actin and plasminogen to immobilized rGBS-PGK. Increasing amounts of rGBS-PGK (0.125-1 µg/well) was immobilized to flat bottom 96 well polystyrene plates (Maxi-sorp) by diluting in 0.1M sodium carbonate solution (pH 9.5) and incubating 8 h at room temperature. Plates were washed 1× with TBS and blocked incubated for 16 h with blocking buffer. Plates were washed 1× with TBS and incubated with either actin or plasminogen (0 or 20 µg/ml in blocking buffer) for 1 h. Wells were washed 3× with TBS and with either anti-actin (Millipore; clone C4) or anti-plasminogen (Sigma-Aldrich; clone 3E6) antibodies (50 µl; 1:300 in blocking buffer) for 1 h at room temperature. Wells were washed 3× with TBS and incubated with anti-mouse antibodies (50 µl; 1:200 in blocking buffer; Sigma-Aldrich) for 1 h at room temperature. Wells were washed 3× with TBS and developed for 30 min at room temperature with 100 µl 4-nitrophenol phosphate before stopping the reaction with 25 µl NaOH (3N) and measuring the A_{405} using an Athos LP400 microplate reader.

A_{405} measurements from plates incubated with 0 µg/ml actin or plasminogen were subtracted from the A_{405} measurements obtained from plates

incubated with 20 μg/ml actin or plasminogen to control for non-specific binding of the antibodies to rGBS-PGK.

2.2.3. Interaction between rGBS-PGK and host proteins visualized using a far western blot.

The eukaryotic proteins plasminogen, plasmin, actin, fibronectin and laminin (0.5μg each) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad laboratories). BSA and rGBS-PGK (0.5 μg each) were also separated and Western transferred to be used as negative and positive controls respectively. The membrane was washed 1× 15min with TBS, blocked with 5% BSA, 0.1% tween 20 in TBS for 1 hour at room temperature, washed again 1× 15min before being incubated for 16 hours at 4°C with rGBS-PGK (25 μg/ml) diluted in blocking buffer. A duplicate membrane incubated in blocking buffer without the addition of rGBS-PGK was used as a negative control. The membrane was washed 1× 15 min with TBS and probed with anti-GBS-PGK antibodies (1:1 500 in blocking buffer) for 3h at 4°C. The membrane was washed 3× 15 min with 0.1% tween 20 in TBS (TBST), 2× 15 min with TBS and incubated with goat anti-rabbit-IgG alkaline phosphatase conjugate (1:10 000 in blocking buffer) for 1 hour at room temperature. The membrane was washed 3× 15 min with TBST and 2× 15min with TBS before developing with SigmaFast BCIP/NBT (Sigma-Aldrich) for approximately 5 minutes. Development was stopped using 3 changes of distilled water.

2.2.4. Subcellular localization of phosphoglycerate kinase.

A single colony of NCS13 was used to inoculate 100 ml Todd Hewitt (TH) broth and was incubated for 16 h at 35°C. The overnight culture was centrifuged at 5000 ×g for 20 min at 4°C. The supernatant was collected as the extracellular fraction and filtered through a 0.22 μm filter. The resulting pellet was washed, re-suspended in 10 ml spheroplast forming buffer (20% sucrose, 1mM $MgCl_2$, 33mM Tris, pH 8.0), treated with 1mg/ml lysozyme (Sigma-Aldrich) at 35°C for 2 h and centrifuged at 15 000×g for 15 min. 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 min and collected as the cytoplasmic fraction. One milliliter of the overnight culture was centrifuged and resuspended in 100 μl lysozyme (50 mg/ml in distilled water) and incubated for 30 min at 35°C to generate the whole cell lysate. The supernatant fraction and sterile TH broth (negative control) were precipitated using ammonium sulfate and resuspended in 1/10 volume TBS. The whole cell lysate and cytoplasmic fractions were diluted 1:10 using TBS. The GBS fractions were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was washed 1× 15 min with TBS and incubated 30 min with blocking buffer (0.1% tween 20, 5% BSA in TBS) at room temperature. The membrane was washed 1× 15 min with TBS and probed with anti-rGBS-PGK antibodies (1:1500 in blocking buffer) for 16 h at 4°C. The membrane was then washed 3×

15 min with TBST, 2× 15 min with TBS and incubated for 1 h with goat anti-rabbit-IgG alkaline phosphatase conjugate (1:10 000 in blocking buffer). The membrane was then washed 3× 15 min with TBST and 2× 15 min with TBS before being developed with BCIP/NBT for 5 min. Development was stopped using 3 changes of distilled water.

2.2.5. Fluorescence microscopy of GBS probed with anti-rGBS-PGK antibodies.

The GBS strain NCS13 was grown 16 h in 1 ml TH broth at 35°C and centrifuged 20 800 ×g for 1 min. The supernatant was removed and the bacterial pellet was re-suspended in 1 ml PBS. Bacterial cells were diluted 1:10 in PBS, a 10 µl bacterial suspension was added to a glass slide and was air dried. Bacteria were fixed to the slide using a 5 min methanol treatment and washed once with PBS and once with water. The slides were treated with anti-rGBS-PGK antibodies diluted 1:50 in PBS for 1 h at room temperature. Slides were washed once with PBS, once with water and incubated with fluorescein isothiocyanate (FITC) labeled anti-rabbit-IgG antibodies (1:50; Sigma-Aldrich) for 1 h at room temperature in the dark. Slides were washed twice with PBS and once with water, allowed to air dry and visualized using a fluorescence microscope (Olympus Microscopes, Carsen Group Inc.) under 1000× magnification. Image was captured using a 1.5 second exposure.

2.2.6. Statistical Analysis.

Initial experiments demonstrating rGBS-PGK binding to immobilized eukaryotic proteins were performed in triplicate. All other ELISA experiments characterizing rGBS-PGK interaction with actin and plasminogen were performed triplicate in triplicate. Data points correspond to the average value of all replicates and error bars represent one standard deviation. Data was analyzed using the Students t test and a p-value <0.05 was considered statistically significant.

2.3. Results:

2.3.1. Binding of rGBS-PGK to host proteins.

PGK has previously been shown to bind actin, fibronectin and plasminogen (13, 32, 35). In addition to these three host proteins, other glycolytic enzymes have been shown to bind plasmin and laminin (26, 31, 37-39). Based on these previous reports, rGBS-PGK was assayed for the ability to bind to actin, fibronectin, plasmin, plasminogen and laminin. rGBS-PGK bound to immobilized plasminogen, plasmin, fibronectin and actin (Fig. 2.3A). Of these four proteins, immobilized plasminogen bound the greatest amount of rGBS-PGK (Fig. 2.3A). Due to the enhanced ability of plasminogen to bind rGBS-PGK, it was necessary to use 10 fold less plasminogen in the binding assay to obtain quantifiable results. Immobilized actin bound the second greatest amount of rGBS-PGK (Fig. 2.3A). Immobilized fibronectin bound the third greatest amount of rGBS-PGK, while rGBS-PGK binding to immobilized plasmin was barely detectable (Fig. 2.2A). No binding was observed between immobilized laminin and rGBS-PGK (Fig 2.3A).

Interaction with plasminogen by the glycolytic enzyme α-enolase has been shown to be dependent on lysine residues and interactions can be inhibited by the lysine analogue 6-aminocaproic acid (6-ACA) (27). To determine the role lysine residues play in rGBS-PGK binding to actin and plasminogen, ELISA in the presence of increasing concentrations of 6-ACA (0-50 mM) was performed.

Addition of 6-ACA resulted in significant ($p<0.01$) reduced binding of rGBS-PGK to immobilized plasminogen at all concentrations assayed, while no effect was seen for the interaction between rGBS-PGK and actin (Fig. 2.2B). These results suggest that rGBS-PGK binding to plasminogen but not actin is dependent on lysine residues within the rGBS-PGK molecule.

To confirm the results obtained from the ELISA assay, binding to plasminogen, plasmin, actin, fibronectin and laminin was also assayed using a far Western blotting technique. A 100 kDa band corresponding to rGBS-PGK binding to plasminogen was clearly visible (Fig. 2.4), while only a faint 42 kDa band corresponding to rGBS-PGK binding to actin could be seen (Fig. 2.4). No bands corresponding to rGBS-PGK binding to BSA, fibronectin, laminin and plasmin were observed (Fig. 2.4). These results support my ELISA results demonstrating a higher degree of rGBS-PGK binding to actin and plasminogen, compared to binding to fibronectin, plasmin and laminin.

The interaction of rGBS-PGK with actin and plasminogen was further confirmed by assaying actin and plasminogen binding to immobilized rGBS-PGK using ELISA (Fig. 2.5). As the amount of rGBS-PGK immobilized to the wells increased, a corresponding increase in the A_{405} measurements was observed for both actin (Fig. 2.5A) and plasminogen (Fig. 2.5B). In all cases the increased A_{405} measurements were found to be statistically significant ($p<0.01$) compared to wells containing the lowest amount of rGBS-PGK (0.125 μg/well). These results, along with the results from the far Western blot assay, confirm the interaction of rGBS-PGK with actin and plasminogen.

Activation of plasminogen recruited to the GBS surface by host plasminogen activators provides GBS with plasmin activity, which may contribute to breakdown of fibrin clots (40). To gain evidence to support that surface expressed GBS-PGK could facilitate breakdown of fibrin clots by bringing the plasmin activity in close contact with the fibrin, binding of rGBS-PGK to immobilized fibrin and fibrinogen was assayed using ELISA (Fig. 2.6). Similar to plasminogen, it was necessary to immobilize 10 fold less fibrin and fibrinogen to the 96 well plates to obtain quantifiable data. Immobilized fibrin was found to bind more rGBS-PGK than immobilized fibrinogen but less than immobilized plasminogen (Fig 2.6 and 2.3).

2.3.2. Subcellular localization of PGK in GBS.

To determine the location of GBS-PGK within NCS13, anti-rGBS-PGK antibodies were used to probe the extracellular, cell wall and cytoplasmic fractions of an overnight culture of NCS13 using Western blot. A 42 kDa protein band, the molecular weight of GBS-PGK, was visible in all three fractions (Fig. 2.7). The protein band was visibly strongest in the cytoplasmic fraction and the visibly weakest in the extracellular fraction (Fig. 2.7). This 42 kDa band was also visible in the whole cell lysate (positive control) but not in the lane corresponding to sterile TH broth (negative control). These results demonstrate that GBS-PGK is present in the GBS cytoplasm, cell wall as well as secreted into the bacterial supernatant. Since no PGK band was seen in the lane corresponding to sterile TH

broth, the PGK present in the cell wall and culture supernatant must have a GBS origin. Additional protein bands of lower molecular weight than 42 kDa reacted with the anti-rGBS-PGK antibody in the cytoplasmic and cell wall fractions, but were not visible in the whole cell lysate (cell wall and cytoplasmic) or extracellular fraction. Since these bands did not occur in the whole cell lysate of GBS, it is likely the extra bands seen below PGK in the cell wall and cytoplasmic fractions of GBS represent PGK that has been cleaved during the lysozyme and sonication steps.

Fluorescence microscopy was used to confirm the presence of PGK on the GBS cell surface (Fig. 2.8). When overnight cultures of NCS13 were probed with anti-rGBS-PGK antibodies, the fluorescence appeared to stain the outer surface of GBS (Fig. 2.8). This observation provides further evidence that GBS-PGK is present on the bacterial cell surface. The antibodies did not appear to stain the cytoplasm of any of GBS cells. The presence of PGK on the GBS surface during fluorescence microscopy suggests that the PGK in the cell wall fraction of the Western blot was not due to lysis of the GBS cells during fractionation.

2.4. Discussion:

Glycolytic enzymes are typically considered cytoplasmic proteins necessary to fulfill a defined role of energy generation. However, over the last twenty years, investigators have identified glycolytic enzymes expressed on the surface of a variety of gram positive bacteria. In addition, many of these proteins have been found to possess host protein binding abilities, suggesting that they may play a role in bacterial virulence. The two best studied examples are α-enolase and GAPDH. I have demonstrated that GBS-PGK, like α-enolase and GAPDH, is expressed on the GBS surface as well as secreted into the culture supernatant (Fig 2.7 and 2.8). My results also demonstrate that rGBS-PGK binds the host proteins actin, fibrin, fibrinogen, fibronectin, plasmin and plasminogen (Fig 2.3-2.6). Based on the extracellular location of GBS-PGK and its host protein binding ability, it seems likely that GBS-PGK contributes to GBS virulence.

Recruitment of plasminogen to the bacterial surface is a well known virulence characteristic of gram positive bacteria. Interaction with plasminogen has been demonstrated to contribute to adhesion to host cells by GAS and *S. pneumoniae* (41, 42). In addition to contributing to adhesion, activation of the recruited plasminogen to plasmin via plasminogen activators contributes to dissemination within the host (22, 43). Two proteins that are thought to be involved in recruiting plasminogen to the GBS surface are GAPDH (43) and Skizzle (44). My results demonstrate that PGK expressed on the surface of GBS

(Fig 2.6 and 2.7) may also recruit plasminogen to the bacterial surface (Fig 2.3-2.5). The relative role that each of these proteins play in recruiting plasminogen to the GBS surface is not clear, as no knock-out mutants have been made for any of these three proteins. The binding of rGBS-PGK to plasminogen was retained after electrophoresis under denaturing conditions suggesting that the GBS-PGK binding epitope of plasminogen is not dependent on overall folding of the plasminogen protein. While some refolding of the plasminogen protein may occur after transfer to the nitrocellulose membrane, complete refolding of the plasminogen molecule is unlikely. A common feature of plasminogen binding proteins is the critical importance of lysine residues interacting with the Kringle domains within the plasminogen molecule (27). My finding that the lysine analogue, 6-ACA, blocks rGBS-PGK binding to plasminogen (Fig. 2.2B) suggests GBS-PGK also binds plasminogen through GBS-PGK lysine residues interacting with the plasminogen Kringle domains.

In addition to plasminogen, rGBS-PGK was also found to bind fibrin and fibrinogen (Fig 2.6). These results suggest that surface expressed GBS-PGK may contribute to GBS virulence by mediating dissolution of fibrin clots, evasion of the host immune system or indirect recruitment of plasminogen. Plasmin is a key enzyme in the fibrinolytic cycle, mediating degradation of fibrin and fibrinogen. As a secreted protein of GBS, the fibrin binding ability of GBS-PGK suggests that GBS-PGK may accumulate at the site of fibrin clots. Recruitment of plasminogen by fibrin bound GBS-PGK, and the subsequent activation by host plasminogen activators, could result in dissolution of the fibrin clot. GBS is known to cleave

fibrinogen to a form that is similar to fibrin, which accumulates on the GBS surface and prevents opsonization of GBS (45). Fibrinogen cleavage on the GBS surface has been previously demonstrated to be mediated by CspA (45). As a fibrinogen binding protein on the GBS surface, GBS-PGK may contribute to this process by recruiting fibrinogen to the GBS surface, allowing cleavage by CspA. Following cleavage of fibrinogen by CspA, GBS-PGK may be involved in binding the resulting fibrin like products to the GBS surface. Finally, fibrinogen binding to the surface of GBS has previously been demonstrated to be involved in indirect recruitment of plasminogen (43). As a fibrinogen binding protein on the GBS surface, GBS-PGK may be indirectly involved in recruiting plasminogen. While my results demonstrate that GBS-PGK is expressed on the GBS surface and can bind fibrin and fibrinogen, previous publications demonstrate that FbsA and FbsB are the major fibrinogen binding proteins on the GBS surface (15, 46). As a result it is not clear if surface expressed GBS-PGK binds fibrinogen to the surface of NCS13.

My results also demonstrate that rGBS-PGK binds fibronectin (Fig. 2.3A), similar to previously described (13). Fibronectin binding by GBS surface proteins has been demonstrated to play a role in internalization of GBS into host cells (13, 47, 48). Fibronectin binding by GBS has previously been demonstrated to occur through ScpB (13). Deletion of the scpB gene resulted in a 50% loss of binding to fibronectin (13). My results indicate that surface expressed GBS-PGK may be a second fibronectin binding protein on the GBS surface. These results agree with

previous publications that identified GBS-PGK as a potential fibronectin binding protein (13).

Finally, my results demonstrate that rGBS-PGK binds actin (Fig 2.3-2.5). While actin is typically considered a cytoplasmic protein, it has been identified as a surface protein of lymphocytes, monocytes, endothelial cells and L cells (49-53). This suggests that, as an actin binding protein on the surface of GBS, GBS-PGK may mediate adhesion to these host cells. Also, I have demonstrated that GBS-PGK is secreted as well as surface expressed (Fig 2.7). This, along with the observation that GBS expresses a pore forming toxin (54, 55), suggest that GBS-PGK may gain access to the host cell cytoplasm to interact directly with the actin cytoskeleton (56). It has previously been demonstrated that expression of GBS-PGK in the cytoplasm of eukaryotic cells results in disruption of the actin cytoskeleton (57). This disruption of the actin cytoskeleton may contribute to GBS internalization. Perhaps this is one of the mechanisms by which the β-hemolysin contributes to GBS internalization (55). It is also possible that disruption of the actin cytoskeleton by GBS-PGK gaining access to the host cell cytoplasm may disrupt intracellular trafficking resulting in delayed maturation of the phagosome, contributing to intracellular survival.

In conclusion, I have demonstrated that GBS-PGK can bind actin, fibrin, fibrinogen, fibronectin and plasminogen. I have also demonstrated that, in addition to being present on the bacterial cell surface GBS-PGK is also secreted into the extracellular environment. These results confirm that GBS-PGK is expressed in a location that could contribute to bacterial virulence and displays

binding properties that would increase GBS virulence.

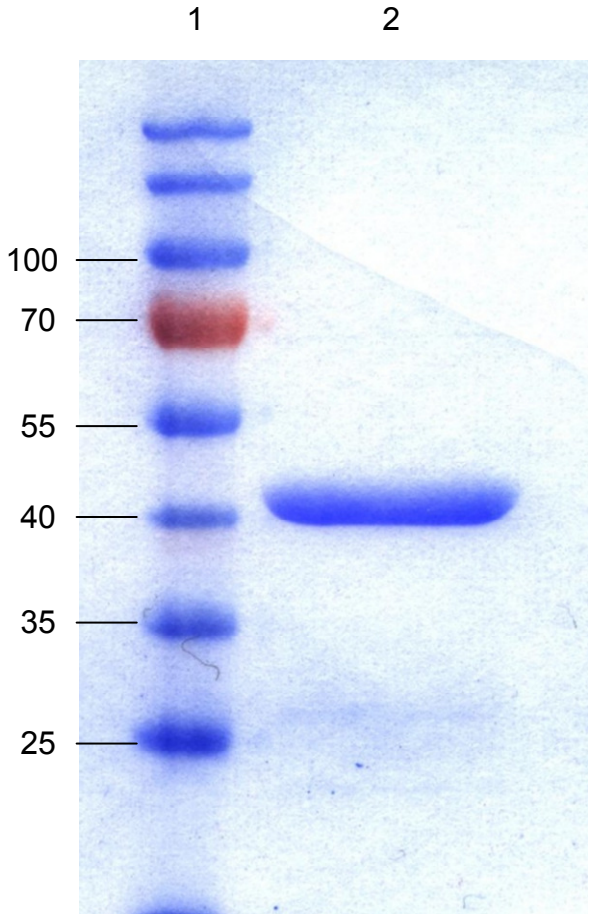

Figure 2.1. SDS-PAGE analysis of purified rGBS-PGK. The pgk gene from NCS13 was PCR amplified, ligated into the plasmid pQE30 and transformed into *Escherichia coli* M15. Expression and purification of rGBS-PGK was performed under native conditions using the Qiaexpressionist kit. The PageRuler prestained protein ladder (1) along with the resulting purified rGBS-PGK (2) was separated by 10% SDS-PAGE and stained using coomassie brilliant blue.

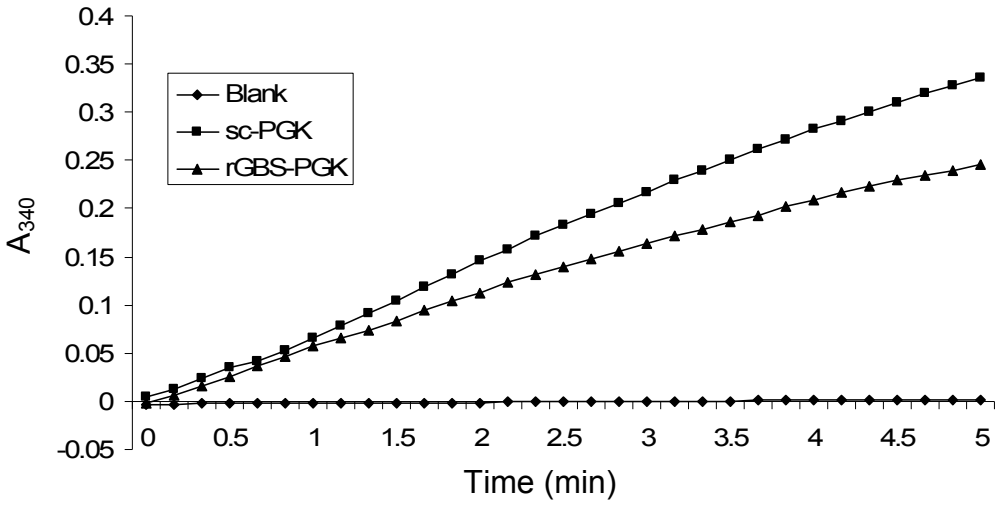

Figure 2.2. Enzymatic activity of purified rGBS-PGK. The enzymatic activity of rGBS-PGK (▲) was determined by adding 2.4 µg to a 3 ml reaction mix containing 50 mM potassium phosphate, 0.83 mM glyceraldehyde-3-phosphate, 0.3 mM β-nicotinamide adenine dinucleotide, 0.2 mM adenosine 5`-diphosphate, 4.2 mM magnesium sulfate, 133 mM glycine and 1 unit glyceraldehyde-3-phosphate dehydrogenase. The absorbance at 340 nm (A_{340}) was measured for five min and the change in absorbance was used to calculate the enzymatic activity. Commercial PGK (0.05 units; Sigma-Aldrich) isolated from *Saccharomyces cerevisiae* was used as a positive control (■) and potassium phosphate (100mM) was used as a negative control (♦).

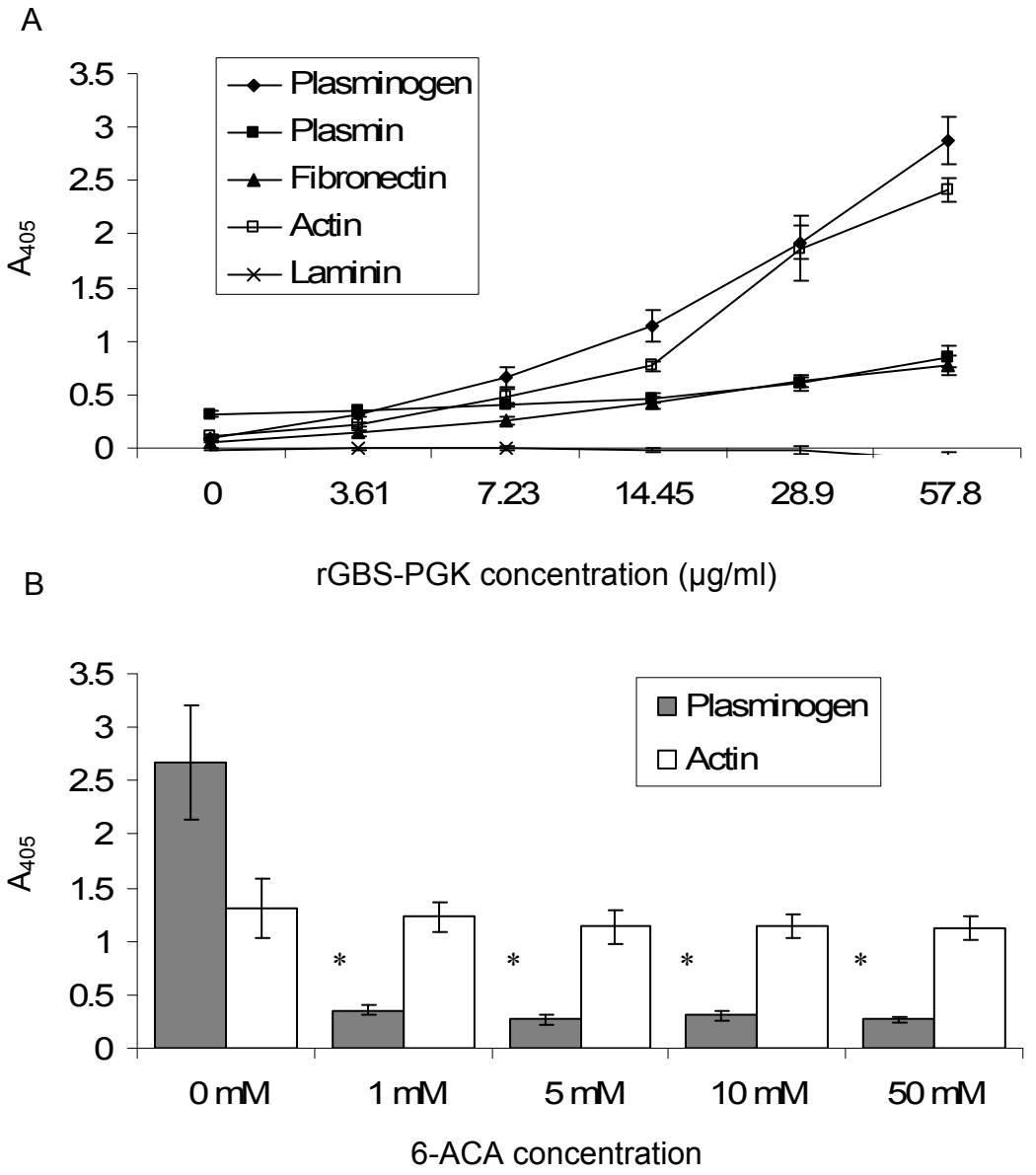

Figure 2.3. Characterization of rGBS-PGK binding to various eukaryotic proteins. (A) Proteins were immobilized onto 96 well plates (using 1 µg each for plasmin, fibronectin, actin and laminin and 0.1 µg for plasminogen). Plates were then incubated with increasing concentrations of rGBS-PGK (0-57.8 µg/ml). (B) 0.1µg plasminogen and 1 µg actin were immobilized onto 96 well plates and incubated with 25 µg/ml rGBS-PGK mixed with increasing concentrations of the lysine analogue 6-aminocaproic acid (6-ACA; 0-50 mM). The level of rGBS-PGK bound to each well was then assayed using rabbit anti-rGBS-PGK, followed by anti-rabbit-IgG alkaline phosphatase conjugate antibodies, developed with 4-nitrophenol phosphate and the absorbance at 405 nm (A_{405}) was measured. Data points represent the average value of experiments run in triplicate; error bars represent 1 standard deviation. * indicates p value <0.05.

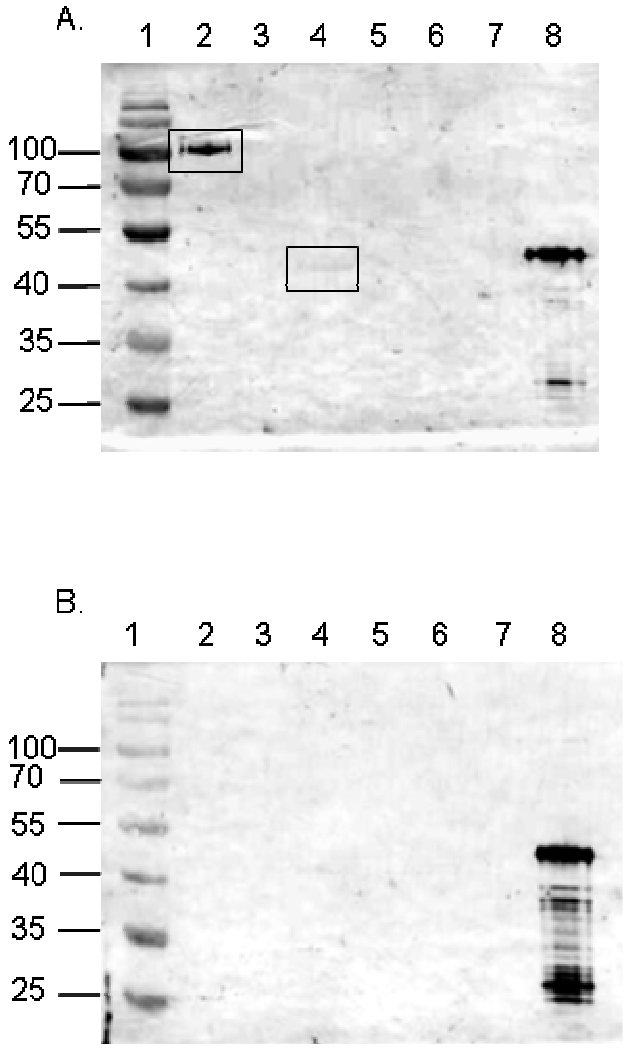

Figure 2.4. Interaction of rGBS-PGK with actin and plasminogen visualized using far-Western blot. Host protein binding by rGBS-PGK was confirmed through a Far Western assay. Plasminogen (lane 2.), plasmin (lane 3.), actin (lane 4.), fibronectin (lane 5.) and laminin (lane 6.) (0.5 µg each) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated for 16 h at 4°C with (A) or without (B) rGBS-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 conjugated anti-rabbit-IgG (1:10 000 in blocking buffer) for 1 h at room temperature. The membrane was developed using SigmaFast BCIP/NBT for approximately 5 min before stopping with 3 changes of distilled water. Lane 1 is a SDS-PAGE standards ladder (PageRuler prestained protein ladder; Fermentas). Lane 7 is BSA (negative control) and lane 8 is rGBS-PGK (positive control).

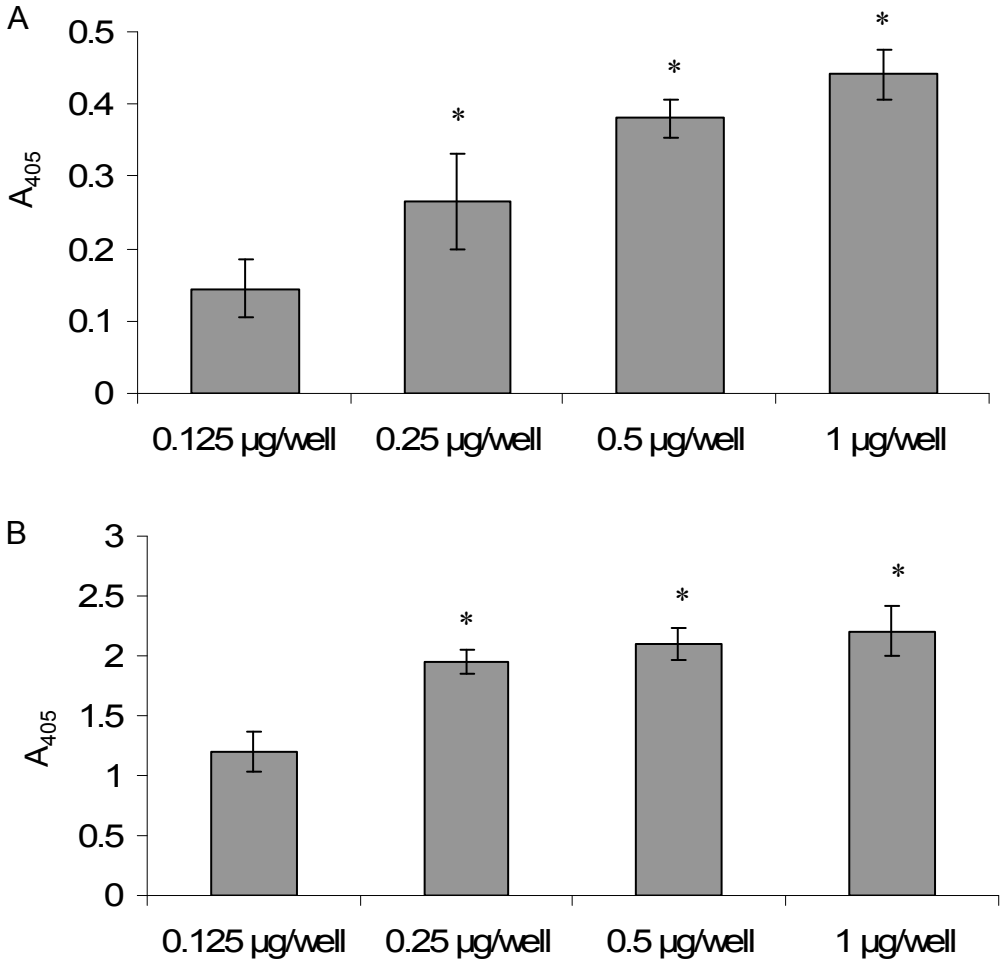

Figure 2.5. Binding of actin and plasminogen to immobilized rGBS-PGK using ELISA. Increasing amounts of rGBS-PGK (0.125-1 µg/well) was immobilized onto 96 well plates. Following overnight incubation with blocking buffer, plates were incubated with either (A) actin or (B) plasminogen. The amount of actin and plasminogen remaining in the wells was assayed using either anti-actin followed by anti-rabbit IgG alkaline phosphatase conjugate or anti-plasminogen followed by anti-mouse IgG alkaline phosphatase conjugate antibodies. Plates were developed using 4-nitrophenol phosphate and the A_{405} was measured. Data points represent the average value of experiments run triplicate in triplicate; error bars represent 1 standard deviation. * indicates significantly ($p < 0.01$) higher A_{405} value compared to wells containing 0.125 µg rGBS-PGK.

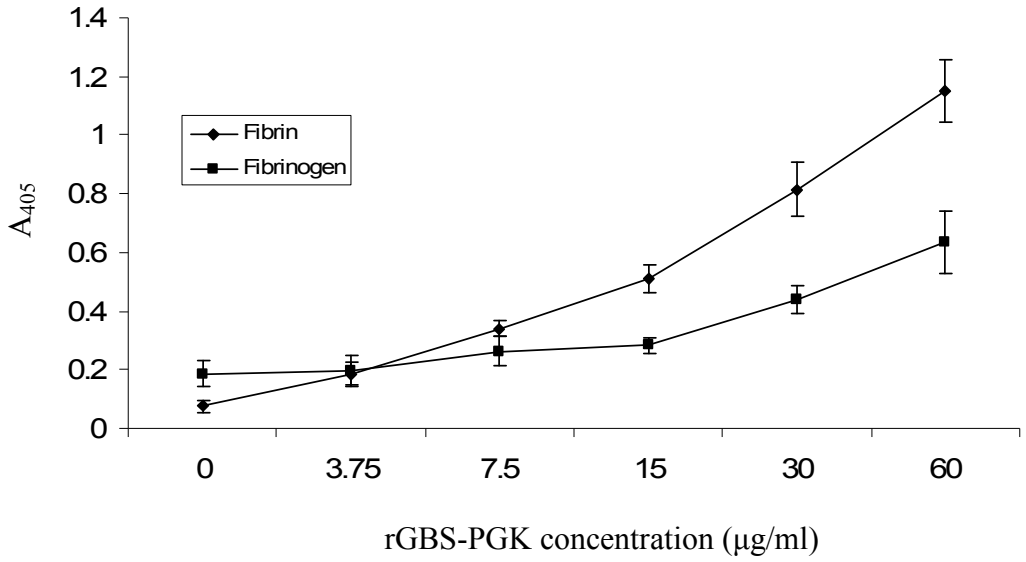

Figure 2.6. Binding of rGBS-PGK to fibrin and fibrinogen. Fibrin and fibrinogen (0.1 μg/well) were fixed to wells of a 96 well polystyrene plate. Following overnight blocking, with 5% BSA, 0.1% tween 20 in TBS, plates were incubated with increasing concentrations of rGBS-PGK (0-60 μg/ml in blocking buffer). The amount of rGBS-PGK remaining in the wells was determined using anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed with 4-nitrophenol phosphate and the absorbance at A_{405} was measured. Data points represent the average A_{405} values obtained from experiments performed triplicate in triplicate.

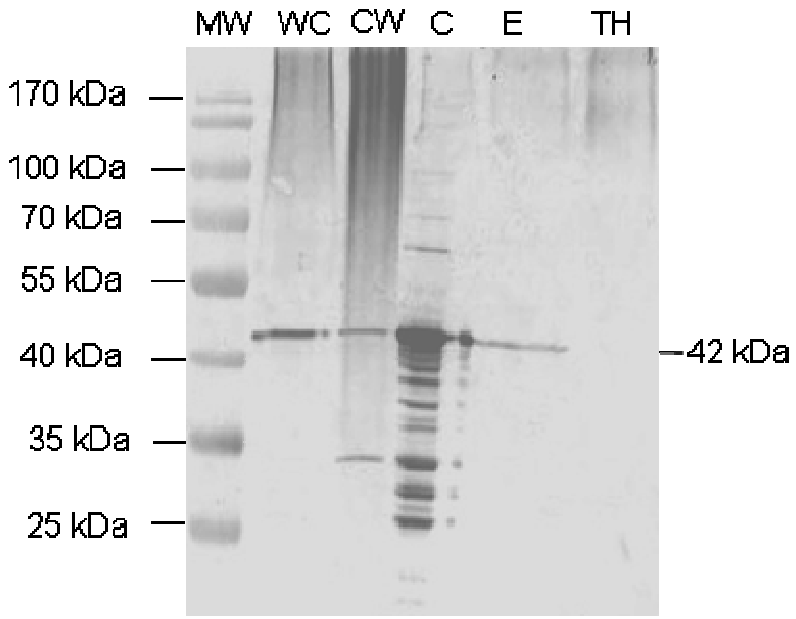

Figure 2.7. Subcellular localization of GBS-PGK. An overnight culture of GBS strain NCS13 was used to identify the locations of GBS-PGK. Bacterial cell fractions were subjected to a 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with rabbit anti-GBS-PGK antibodies overnight. The western blot was then incubated with anti-rabbit-IgG alkaline phosphatase conjugate antibodies and developed using SIGMAFAST BCIP/NBT. Lane designations correspond to whole cell lysate (WC), cell wall (CW), cytoplasm (C), extracellular fraction (E) and sterile Todd Hewitt broth (TH) which was used as a negative control.

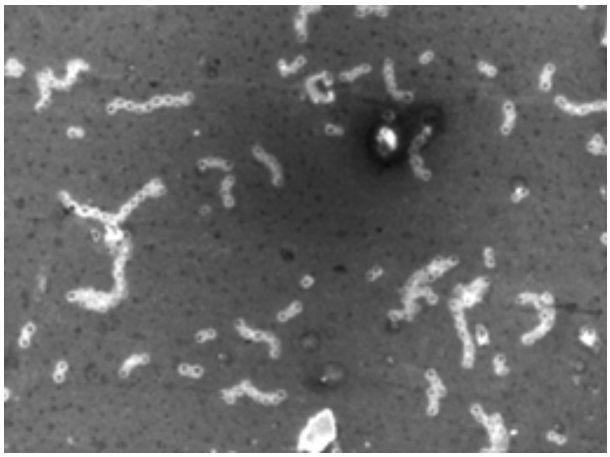

Figure 2.8. Fluorescence microscopy of an overnight GBS culture probed with anti-rGBS-PGK antibodies. A 1 ml overnight culture of the GBS strain NCS13 was diluted 1:10 in PBS and methanol fixed to a glass slide. The slides were treated with anti-rGBS-PGK followed by FITC labeled anti-rabbit-IgG antibodies diluted 1:50 in PBS for 1 h at room temperature. Slides were washed twice with PBS and once with water, allowed to air dry and visualized using a fluorescence microscope (Olympus Microscopes, Carsen Group Inc.) under 1000× magnification. Image was captured using a 1.5 second exposure.

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Chapter 3:

Identification of Genes Affecting Expression of Phosphoglycerate Kinase on the Surface of Group B Streptococcus

A version of this chapter has been accepted for publication in the Canadian Journal of Microbiology as:

Boone, Tyler J. and Gregory J. Tyrrell. Identification of Genes Affecting Expression of Phosphoglycerate Kinase on the Surface of Group B Streptococcus.

3.1 Introduction:

Streptococcus agalactiae (group B streptococcus; GBS) is an opportunistic bacterial pathogen that can cause invasive disease in both the neonatal and adult population (1-3). GBS virulence factors include a variety of surface expressed and secreted proteins (4-7). While many GBS surface proteins have been demonstrated to be involved in causing disease, the functions of other surface expressed proteins are not as clear. One protein expressed on the GBS surface without a clear function is group B streptococcal phosphoglycerate kinase (GBS-PGK) (8, 9). We, and others, have previously demonstrated that PGK binds actin, fibronectin and plasminogen (10-13). The surface localization of GBS-PGK and its ability to bind these host proteins suggest that GBS-PGK may play a role in GBS virulence. However, determining the function of surface expressed GBS-PGK has been hampered by its role in glycolysis. Due to its essential metabolic function, traditional knock-out mutagenesis of the *pgk* gene is lethal in GBS. As a result it may be necessary to prevent transport of PGK to the GBS surface, without affecting the enzymatic activity to determine the function of PGK on the GBS surface.

PGK and other glycolytic enzymes have been identified on the surface of GBS (8, 9) and other streptococcal species (14-19). How these enzymes become surface expressed is unknown, since they do not contain any known secretion or surface attachment signals (20). The expression of glycolytic enzymes on the surface of numerous gram positive bacteria in the absence of known secretion

signals has led to the hypothesis that glycolytic enzymes may be transported to the bacterial surface through a specialized transport system (21). Identifying the transport mechanism used by glycolytic enzymes to become surface expressed may provide some insight into methods that could be used to prevent expression of PGK on the GBS surface. The objective of this chapter was to identify the GBS genes involved in surface expression of GBS-PGK.

3.2 Materials and Methods:

3.2.1 Development of a high throughput assay to quantify surface expression of GBS-PGK.

Expression of PGK on the surface of GBS strains NCS13 (8, 22) and COH1 (23) was first assayed using fluorescence microscopy. NCS13 is a serotype V GBS strain isolated from a leg wound in a elderly patient, which was found to be highly invasive in vitro (22), while COH1 is a serotype III strain of GBS isolated from a neonatal sepsis case that is commonly used to study GBS virulence (23-25) Single colonies of NCS13 and COH1 were inoculated into 1 ml Todd Hewitt (TH) broth and incubated for 20 h at 35°C. Bacterial cultures were washed 1× and resuspended in 1 ml phosphate buffered saline (PBS). Bacterial cells were diluted 1:10 in PBS; a 10 µl bacterial suspension was added to a glass slide and was air dried. Bacteria were fixed to the slide using a 5 min methanol treatment and washed once in PBS and once in water. The slides were treated with anti-rGBS-PGK antibodies (1:50 in PBS) (13) for 1 h at room temperature. Slides were washed once with PBS, once with water and incubated with FITC labeled anti-rabbit-IgG antibodies (1:50 in PBS; Sigma-Aldrich) for 1 h at room temperature. Slides were washed twice with PBS and once with water, allowed to air dry and visualized using a fluorescence microscope (Olympus Microscopes, Carsen Group Inc.) under 1000× magnification. Image was captured using a 1.5 second exposure.

Surface expression of GBS-PGK was next assayed using ELISA similar to previously used to quantify lipoteichoic acid on the GBS surface (23). Single colonies of NCS13 and COH1 were inoculated into 1 ml TH broth and incubated for 20 h at 35°C. Bacterial cultures were washed 1× and resuspended in 1 ml tris buffered saline (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, CA) by overnight incubation at 4°C. Wells were washed 1× with TBS and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in TBS for 1 h. After blocking, the wells were washed 3× with TBS and incubated 1 h with anti-rGBS-PGK antibodies (1:300 in blocking buffer;). Wells were washed 3× with TBS and incubated 1 h with anti-rabbit IgG-alkaline phosphatase conjugate antibodies (1:200 in blocking buffer; Sigma-Aldrich). Wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate (Sigma-Aldrich) for 30 min at room temperature before stopping the reaction with 25 µl NaOH (3N). The absorbance at 405 nm (A_{405}) was measured using an Athos LP400 microplate reader (Bio-Rad Laboratories Ltd. Mississauga, ON, CA).

3.2.2 Effect of growth phase on surface expression of GBS-PGK.

To determine the effect of growth phase on surface expression of GBS-PGK, single colonies of NCS13 were inoculated into 1 ml Todd Hewitt (TH) broth and incubated for 2, 3, 4, 7 and 20 h at 35°C. The absorbance at 600 nm

(A_{600}) was measured and used to determine the bacterial concentration. Bacterial cultures were washed 1× with 1 ml tris buffered saline (TBS) and resuspended in TBS to the same A_{600} as cultures grown 20 h. One hundred microliters of washed bacterial cultures were fixed to the wells of a 96 well polystyrene plate and assayed using the above described ELISA assay. The A_{405} values were compared to the average A_{405} value from wells containing NCS13 grown 20 h to determine the $\%A_{405}$.

3.2.3 Effect of cell density on ELISA quantification of surface expressed GBS-PGK.

To determine the effect of minor differences in the number of bacteria fixed to wells of the 96 well plates, NCS13 cultures grown for 20 h were washed 1× and resuspended in 1 culture volume of TBS. Washed bacterial cultures were either diluted or concentrated to bacterial cell concentrations of 40%, 50%, 60%, 70%, 80%, 90%, 100% 140%, 160% or 180%. One hundred microliters of these bacterial suspensions were fixed to wells of a 96 well polystyrene plate and assayed using the above described ELISA. The A_{405} values obtained were compared to the average A_{405} value from wells containing a 100% suspension of NCS13 to determine the $\%A_{405}$.

3.2.4 Tn917 mutagenesis.

Generation of a Tn*917* mutagenesis library was performed similar to previously described for *Streptococcus mutans* (26). Briefly, NCS13 was transformed with the temperature sensitive plasmid pTV1-OK carrying the transposon Tn*917* and grown on Todd Hewitt (TH) agar containing 500 μg/ml kanamycin (Gibco, Invitrogen, Carlsbad, CA, USA) at 30°C. Single colonies were inoculated in TH broth containing 500 μg/ml kanamycin and 0.02 μg/ml erythromycin (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 30°C. One milliliter of this overnight culture was used to inoculate 10 ml TH broth containing 500 μg/ml kanamycin and 0.02 μg/ml erythromycin. After overnight incubation 20 μl and 200 μl aliquots were plated onto TH agar containing 10 μg/ml erythromycin. Plates were incubated at 42°C for 16 h followed by 24 h incubation at 35°C.

Colonies arising from Tn*917* mutagenesis were assayed for GBS-PGK expression on their surface using an ELISA similar to previously used to quantify lipoteichoic acid on the GBS surface (23). Colonies of NCS13 containing Tn*917* transposon insertions were inoculated into 1 ml TH broth containing 10 μg/ml erythromycin and incubated 20 h at 35°C. These overnight cultures were washed 1× and resuspended in 1 ml tris buffered saline (TBS). The overnight bacterial cultures exhibiting visibly reduced growth were resuspended in 100 μl TBS to maintain a similar bacterial density. One hundred microliters of washed bacterial

cultures were fixed to the wells of a 96 well polystyrene plate and assayed using the above described ELISA.

NCS13 strains containing Tn*917* insertions that had altered expression of surface expressed GBS-PGK were sub-cultured and eight individual colonies were selected. These eight colonies were further assayed for surface expression of GBS-PGK in duplicate. The A_{405} measurements from NCS13 strains containing Tn917 insertions were compared to those from the parent NCS13 strain using the student's t test to determine statistical significance.

GBS-PGK secreted by NCS13 and mutant NCS13 isolates with significantly reduced surface expression of GBS-PGK was assayed using a similar ELISA protocol. Colonies of NCS13 were inoculated into TH broth containing 10 μg/ml erythromycin and incubated 20 h at 35°C. These overnight cultures were centrifuged 20 800×g for 10 min and the supernatant was collected. Culture supernatants were sterilized by passage through a 0.22 μm syringe filter. One hundred microliters of the culture supernatants were fixed to wells of a 96 well polystyrene plate and assayed using the above described ELISA.

3.2.5 Identification of Tn917 insertion site.

The Tn*917* insertion sites for mutant NCS13 strains showing altered surface expression of GBS-PGK was determined using single primer PCR, similar to previously described (26, 27). Genomic DNA was isolated from NCS13 and NCS13 strains containing the Tn*917* insertions. The primer rtp-1 (Table 3.1),

reading outward from Tn*917*, was used to PCR amplify DNA from these genomic DNA preparations. The PCR amplification was performed with the following cycling conditions: 5 min at 94°C; 20 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec; 30 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec; 30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec; and 72°C for 7 min. Amplicons unique to NCS13 strains containing Tn*917* insertions were gel purified using the QiaQuick gel extraction kit (Qiagen, Mississauga, ON, CA). Gel purified amplicons were re-amplified using the following conditions 94°C for 5 min; 35 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec; and 72°C for 7 min and sequenced using the nested outward reading primer rtp-2 (The Institute for Biomolecular Design, IBD; University of Alberta, Edmonton, AB, CA. table 3.1). Sequences generated were compared to the serotype V GBS 2603V/R genome (28) to determine the location of the Tn*917* insertion.

3.2.6 Sensitivity of Tn917 mutants to nisin and bacitracin.

Overnight cultures of NCS13, NCS13*sag0979*::Tn*917*, NCS13*sag0980*::Tn*917* and NCS13*sag1003*::Tn*917* were diluted in TH broth or TH broth supplemented with 10 µg/ml erythromycin to a final concentration of 1×10^6 cfu/ml. Fifty microliters of bacterial suspension was used to inoculate 50 µl TH or TH supplemented with 10 µg/ml erythromycin containing increasing concentrations of either nisin or bacitracin (0-1000 µg/ml; Sigma-Aldrich) on a 96 well plate. Plates were incubated for 24 h at 35°C and the absorbance at 620 nm

(A_{620}) was measured using an Athos LP400 microplate reader. The minimum inhibitory concentration (MIC) was defined as the concentration of nisin or bacitracin that resulted in an A_{620} reading below 0.1.

3.2.7 Southern blot.

To confirm single insertion of the Tn*917* transposon, Southern blot analysis was used. Generation of a DIG labeled probe was performed using the PCR DIG Probe Synthesis Kit (Roche; Laval, QC, CA). The primers ermBF and ermBR were used to PCR amplify a probe from the plasmid pTV1-OK in the presence of DIG-dUTP. Amplification was carried out using 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec. This cycling was followed by incubation at 72°C for 10 min. Genomic DNA was isolated from GBS isolates containing Tn*917* insertions using the DNEasy DNA isolation kit (Qiagen) and subjected to southern blot similar to previously described (27). Genomic DNA (1 μg) was digested with *Hin*d III and resolved on a 0.7% agarose gel at 100V for 1 h. DNA was depurinated by incubating for 5 min in 250 mM HCl. After rinsing several times with distilled water the gel was incubated 15 min with denaturing solution (0.5M NaOH, 1.5M NaCl). The gel was rinsed 1× with distilled water and incubated for 30 min in neutralization solution (1M tris, 1.5M NaCl pH8). The gel was rinsed 1× with water and the DNA was transferred onto positively charged nylon membrane overnight. DNA was linked to the nylon membrane using UV irradiation exposing the gel to 150 mJ. Membrane was

incubated at 42°C in hybridization buffer (6×SSC, 0.004% SDS, 0.235× Denhardt's reagent) containing 1.5% blocking reagent. Following the 1 h incubation in pre-hybridization buffer the membrane was incubated overnight at 42°C in 50mL hybridization solution containing 8 µl dig labeled probe. Membrane was washed for 1 min with 6× SSC and incubated for 1 ½ h in 6× SSC containing 0.1% SDS at 65°C. Membrane was washed for 1 min in maleate solution (0.1M malic acid, 0.15M NaCl pH 7.5) before incubating for 2 h at room temperature with maleate solution containing 1.5% blocking solution. Membrane was then incubated for 1h with anti-dig alkaline phosphatase conjugate antibody (1:500 diluted in maleate buffer containing 1% blocking reagent). The membrane was washed 4× 15 min in maleate solution containing 0.3% tween 20 and equilibrated for 5 min with Buffer C (100mM Tris, 100mM NaCl pH 9.5) before developing with SigmaFast BCIP/NBT.

3.2.8 Binding of rGBS-PGK to lipoteichoic acid.

The interaction of rGBS-PGK with lipoteichoic acid isolated from group A streptococcus (Sigma-Aldrich) was assayed, using an ELISA similar to previously described (29). Lipoteichoic acid from group A streptococcus was used because it is commercially available and similar to GBS lipoteichoic acid (30). Lipoteichoic acid was resuspended in 0.1 M sodium carbonate solution (pH 9.5) and immobilized to wells of a 96 well polystyrene plate (Maxi-sorp; NUNC) for 8 h at 4°C. As a negative control, BSA (0.1 µg/ml) was also immobilized to

wells of the same 96 well plate. Wells were washed 1× 10 min with TBS and incubated overnight in blocking buffer (5% BSA, 0.1% tween 20 in TBS) at room temperature. Wells were then washed 3× 10 min with TBS and incubated with decreasing concentrations of rGBS-PGK (60-0 μg/ml in blocking buffer) for 1 h at room temperature. Wells were then washed 3× 10 min with TBS and incubated with anti-rGBS-PGK (1:300 in blocking buffer) antibodies for 1 h at room temperature. Wells were washed 3× 10 min with TBS and incubated with anti-rabbit IgG alkaline phosphatase conjugate antibodies (1:200 in blocking buffer) for 1 h at room temperature. Wells were washed 3× 10 min with TBS and developed with 100 μl 4-nitrophenol phosphate for 30 min at room temperature before stopping the reaction with 25 μl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader.

Experiments were performed triplicate in duplicate. The A_{405} values from wells incubated with 0 μg/ml rGBS-PGK were subtracted from the A_{405} values from wells incubated with all other rGBS-PGK concentrations to control for non-specific interaction of the antibodies with BSA and lipoteichoic acid. The A_{405} readings from wells containing lipoteichoic acid were compared to wells containing BSA using the student's t-test to determine statistical significance.

3.2.9 Effect of pH on surface expression of GBS-PGK.

The effect of pH on surface expression of GBS-PGK was assayed using ELISA. Single colonies of NCS13 were used to inoculate TH broth adjusted to

pH 5.3, 6.1, 7.6, 8.2 and 9.1 and incubated 20 h at 35°C. Bacterial cultures were washed 3× and resuspended in 1 culture volume of TBS. Washed bacterial cultures were fixed to wells of a 96 well polystyrene plate (Maxi-sorp) by incubation overnight at 4°C. Wells were washed 1× with TBS and blocked with 5% BSA in TBS for 1 h at room temperature. After blocking the wells were washed 3× with TBS and incubated 1 h with anti-rGBS-PGK antibodies (1:300 in blocking buffer). Wells were washed 3× with TBS and incubated 1 h with anti-rabbit IgG-alkaline phosphatase conjugate antibodies (1:200 in blocking buffer). Wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate for 30 min before stopping the reaction with 25 µl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader

Experiment was performed triplicate in triplicate. The A_{405} values readings from NCS13 grown at different pH was compared to the average A_{405} readings from NCS13 grown in non-modified TH broth (pH 8.2) to determine the $\%A_{405}$. Data points obtained from growth at different pH were compared to growth at pH 8.2 using the student's t-test to determine statistical significance.

3.2.10 Effect of pH on secretion of GBS-PGK.

The effect of pH on secretion of GBS-PGK was assayed using Western blot. Overnight NCS13 cultures (10 ml), grown in TH broth at pH 5.3, 6.1, 7.6, 8.2 or 9.1, were centrifuged 3000 ×g for 10 min. The supernatants were collected and passed through a 0.22 µm filter. The final pH was measured using an

Accumet pH meter 915 (Fisher Scientific; Ottawa, ON, CA) and the supernatant proteins were concentrated 10× using a centrifugal filtration unit with a 10 kDa cutoff. The concentrated supernatant proteins were separated by 10% SDS-PAGE and transferred, electrophoretically, to a 0.45 μm nitrocellulose membrane. Nitrocellulose membranes were washed 1× 15 min with TBS and blocked for 30 min with 5% BSA, 0.1% tween 20 in TBS. After blocking, membranes were washed 1× 15 min with TBS and incubated overnight at 4°C with anti-rGBS-PGK antibodies (1:1250 in blocking buffer). Membranes were then washed 3× 15 min with TBST, 2× 15min with TBS and incubated 1 h with anti-rabbit IgG alkaline phosphatase conjugate antibodies (1:10 000 in blocking buffer) at room temperature. Membranes were washed 3× 15 min with TBST, 2× 15 min with TBS and developed with SigmaFast BCIP/NBT for 5 min before stopping the reaction with 3 changes of distilled water. Membranes were analyzed using Image J to determine the band intensities corresponding to secreted GBS-PGK.

3.2.11. Effect of pH on attachment of rGBS-PGK to the GBS surface.

The effect of pH on the attachment of exogenously added rGBS-PGK was assayed using ELISA. Overnight cultures of NCS13 were resuspended in one culture volume of either TBS or phosphate buffered saline (PBS) adjusted to pH 4 or pH 8 and supplemented with rGBS-PGK (10 μg/ml). Resuspended bacterial cultures were incubated for 1 h at 35°C, washed 5× with and resuspended in TBS. One hundred microliters of washed bacterial cultures were fixed to wells of a 96

well polystyrene plate (Maxi-sorp) by overnight incubation at 4°C. Wells were then washed 1× with TBS and incubated 1 h with blocking buffer (5% BSA in TBS). After blocking the wells were washed 3× with TBS and incubated 1 h with anti-poly histidine antibodies (1:300 in blocking buffer). Wells were washed 3× with TBS and incubated 1 h with anti-mouse-alkaline phosphatase conjugate antibodies (1:200 in blocking buffer). Wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate for 30 min at room temperature before stopping the reaction with 25 µl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader. Experiment was performed triplicate in triplicate. Data points obtained at pH 8 were compared to those obtained at pH 4 using the student's t-test to determine statistical significance.

3.2.12 Effect of pH on release of GBS-PGK from the GBS surface.

Release of PGK from the NCS13 surface by incubation at high pH and high osmolarity was assayed using ELISA. Overnight cultures of NCS13 (5 ml) were split into 1 ml aliquots and resuspended in 50 mM tris adjusted to pH 4, pH 8 or pH 8 supplemented with 2 M NaCl and incubated at room temperature for 1 h with constant agitation. Cells were then washed 3× and resuspended in 1 ml of TBS. As a control, a 1 ml aliquot of the overnight culture was washed 3× and resuspended in 1 ml TBS. One hundred microliters of washed bacterial cultures were fixed to wells of a 96 well polystyrene plate (Maxi-sorp) by overnight incubation at 4°C. Wells were washed 1× with TBS and blocked with 5% BSA in

TBS for 1 h at room temperature. After blocking the wells were washed 3× with TBS and incubated 1 h with anti-rGBS-PGK antibodies (1:300 in blocking buffer). Wells were washed 3× with TBS and incubated 1 h with anti-rabbit IgG-alkaline phosphatase conjugate antibodies (1:200 in blocking buffer). Wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate for 30 min before stopping the reaction with 25 µl NaOH (3N). The absorbance at A_{405} was measured using an Athos LP400 microplate reader.

Experiment was performed triplicate in triplicate. The A_{405} values obtained were compared to the average A_{405} value from overnight cultures of NCS13 to determine the $\%A_{405}$. Data points obtained from incubation at pH 8 and pH 8 in the presence of 2M NaCl were compared to data points obtained from incubation at pH 4 using the student's t-test to determine statistical significance.

3.3 Results:

3.3.1 Development of a high throughput assay to quantify surface expression of GBS-PGK.

To facilitate a transposon mutagenesis screen, it was necessary to develop a high throughput method to assay surface expression of GBS-PGK. To this end an ELISA assay was developed and compared to fluorescence microscopy to quantify the level of PGK expressed on the bacterial surface. Fluorescence microscopy demonstrated visibly higher binding of anti-rGBS-PGK to the surface of NCS13 compared to COH1 (Fig. 3.1A and B). Similar results were obtained using ELISA as significantly higher A_{405} values were obtained from NCS13 cultures compared to COH1 (Fig. 3.1C). Based on these results, I have concluded that ELISA is capable of providing a relative quantification of surface expressed GBS-PGK. This suggests that ELISA may be a feasibly high throughput assay that could be used to identify mutant NCS13 cultures with reduced surface expression of GBS-PGK.

3.3.2 Effect of growth phase on surface expression of GBS-PGK.

Expression of GBS surface proteins has previously been demonstrated to be affected by growth phase (31). To determine if growth phase affects surface expression of GBS-PGK specifically, the amount of surface expressed GBS-PGK

was assayed using NCS13 cultures grown for 2, 3, 4, 7 and 20 h. The average A_{600} values were 0.257, 0.825, 1.100, 1.151 and 1.224 respectively (Fig. 3.2). The amount of GBS-PGK expressed on the surface of these bacterial cultures was assayed using ELISA. Experiments were performed duplicate in triplicate. With the exception of cultures grown for 4 h, which was slightly lower, there was no significant difference in the A_{405} values obtained from all five time points (Fig. 3.2). These results suggest that expression of PGK on the surface of the GBS strain NCS13 is not dependent on growth phase. Based on these results, I have concluded that expression of GBS-PGK on the surface of NCS13 after 20 h should be representative of surface expressed GBS-PGK at all other time points.

3.3.3 Effect of cell density on ELISA quantification of surface expressed GBS-PGK.

The quantification of surface expressed GBS-PGK could also be affected by the bacterial cell density used to fix to wells of the 96 well plates. Since it was expected that at least some of the mutant NCS13 strains generated during the Tn*917* transposon mutagenesis screen would have altered levels of growth, determining the effect of different cell densities was necessary to determine if the changes in the A_{405} values were due to altered cell growth or were in fact due to altered levels of surface expressed GBS-PGK. To determine the effect of cell density differences, overnight cultures of NCS13 were either concentrated or diluted and fixed to wells of a 96 well plate. Experiments were performed

duplicate in triplicate. Assaying these cultures for surface expression of GBS-PGK using ELISA demonstrated that changes in the bacterial cell density did have a slight effect on the A_{405} measurements (Fig 3.3). However, if the bacterial density was between 70-180% the differences in the A_{405} measurements were found to be not significant ($p > 0.05$). In addition, the differences in the A_{405} measurements at any of the bacterial concentrations assayed were less than 0.1. Based on these results, I have concluded that minor differences in the bacterial cell density were not likely to affect the results of the Tn*917* screen.

3.3.4 Tn917 transposon mutagenesis.

To identify GBS genes that are involved in GBS-PGK surface expression, Tn*917* transposon mutagenesis was performed. A total of 4557 Tn*917* mutants of GBS NCS13 were assayed, using anti-rGBS-PGK antibodies, to quantify the level of GBS-PGK expressed on the bacterial surface. Mutant isolates with A_{405} readings below 100% or above 150% compared to the parent strain were subcultured and eight colonies per mutant strain were re-plated after subculture. The levels of GBS-PGK expressed on the surface of these colonies were re-assayed in duplicate (Fig. 3.4). A total of five mutant strains were subcultured and re-assayed. During these experiments the average A_{405} value obtained from the parent NCS13 strain was 0.853 and had a standard deviation of 0.05. For each plate the A_{405} reading from the parent NCS13 strain was defined as 100%. Of the five subcultured mutant strains, two mutant strains had significantly ($p<0.01$)

increased levels of GBS-PGK on their surface and one had significantly ($p<0.01$) reduced levels of GBS-PGK on its surface. The remaining two mutant strains had reduced levels of GBS-PGK on their surface but the difference was not significant.

To determine the transposon insertion site, DNA sequences reading outward from the transposon were compared to the GBS 2603V/R genome (28). DNA sequences in the NCS13 mutants with homologies to genes in the 2603V/R genome were given the same identifiers as those identified in the 2603V/R genome. The Tn*917* mutagenesis screen identified five insertion sites, within four genes, affecting surface expression of GBS-PGK (Fig. 3.4). Tn*917* insertions in *sag1003* significantly ($p<0.01$) reduced expression of GBS-PGK on the bacterial surface resulting in an A_{405} measurement of 56% compared to the parent NCS13 strain. Tn*917* insertion in the *sag0966* or *sag0979*(1) (base 1650 of the 1661 base long *sag0979*) gene significantly ($p<0.01$) increased surface expression of GBS-PGK resulting in A_{405} readings of 167% and 230% respectively compared to the parent NCS13 strain. Finally, Tn*917* insertion in the *sag0979*(2) (base 1215 of the 1661 base long *sag0979*) or *sag0980* gene resulted in A_{405} readings of 85% and 88% respectively compared to the parent NCS13 strain but these reductions were not significant (p-values of 0.08 and 0.11 respectively; Fig. 3.4).

The identified genes were then analyzed using BLAST to identify homologies to other genes encoding proteins with known functions. The *sag0966* was predicted to encode IS*1381* transposase OrfB. The *sag0979* gene was predicted to encode OppA2, a substrate binding protein for ATP dependent import

of peptides (32). The *sag0980* gene was predicted to encode a protein with homology to both SatE from *Streptococcus mutans*, a protein with unknown function, as well as MutG from *Streptococcus sanguinis,* a protein predicted to be involved in ATP dependent efflux of antimicrobial peptides. Finally the *sag1003* gene was predicted to encode a permease protein predicted to be involved in ATP dependent efflux of antimicrobial peptides.

Tn*917* insertion into *sag1003* resulted in decreased amounts of surface expressed GBS-PGK. To differentiate between decreased transport across the GBS membrane and decreased attachment to the bacterial surface, the proteins secreted by overnight cultures of NCS13, and NCS13*sag1003*::Tn*917* were assayed using ELISA. Significantly higher ($p<0.01$) A_{405} measurements were obtained from wells containing secreted proteins of NCS13*sag1003*::Tn*917* than from the parent NCS13 strain. The average A_{405} reading from the overnight culture supernatant of the parent NCS13 strain was found to be 0.0735 ± 0.006, while the average A_{405} reading from NCS13*sag1003*::Tn*917* was 0.115 ± 0.05. This suggests that the decreased expression of PGK on the surface of NCS13*sag1003*::Tn*917* was a result of decreased attachment to the bacterial surface.

3.3.5 Sensitivity of Tn917 mutants to nisin and bacitracin.

With the exception of *sag1003*, all of the genes identified in the Tn*917* screen were located within a 15 gene span (*sag0966-sag0980*) of the 2124 gene

GBS genome (28). Based on this clustering, I hypothesized that this region of the GBS genome may be responsible for attachment of GBS-PGK to the GBS surface. BLAST analysis of the genes within this 15 gene region identified that the genes *sag0973-sag0977* are homologous to genes involved in bacitracin or nisin (antimicrobial peptides) resistance (Fig 3.5). In addition *sag1003* was predicted to encode a protein involved in efflux of antimicrobial peptides. These results led me to assay the Tn*917* mutants for increased sensitivity to nisin and bacitracin (Fig. 3.6). Only NCS13*sag1003*::Tn*917* demonstrated increased sensitivity to bacitracin and nisin (bacitracin: NCS13-125 μg/ml vs. NCS13*sag1003*::Tn*917*-15.62 to 31.25 μg/ml) (nisin: NCS13-125 to 250 μg/ml vs. NCS13*sag1003*::Tn*917*- 31.25 μg/ml). NCS13*sag0979*(2)::Tn*917* and NCS13*sag0980*::Tn*917* showed no change in MIC's compared to NCS13 for either nisin or bacitracin. Due to a dramatically reduced level of growth, it was not possible to assay NCS13*sag0979*(1)::Tn*917* for sensitivity to nisin and bacitracin. These results demonstrate that at least *sag1003* encodes a protein involved in resistance to antimicrobial peptides.

While performing the sensitivity assays it was observed that all of the Tn*917* mutants did not grow to the same bacterial concentration as the parent strain. Single colonies of NCS13, NCS13*sag0979*(2)::Tn*917*, NCS13*sag0980*::Tn*917* and NCS13*sag1003*::Tn*917* inoculated into 1 ml of TH broth and incubated 20 h at 35°C were found to have bacterial concentrations of 8.4×10^8 cfu/ml, 1.2×10^8 cfu/ml, 1.8×10^8 and 9.5×10^7 cfu/ml respectively. It was also noted that all three GBS mutants exhibited reduced hemolysis

surrounding the colonies after 24 h growth on sheep blood agar plates at 35°C (Fig 3.7). This reduced hemolysis was most notable in the strains containing Tn*917* insertion in the *sag0980* and *sag1003* genes. These observations suggest that in addition to affecting surface expression of GBS-PGK, the genes identified in the Tn*917* screen play a role in general fitness of NCS13 and regulate expression of the β-hemolysin. These results are surprising in light of previous work demonstrating that deletion of *oppA2* in the GBS strain O90R had no effect on bacterial growth (32). Similarly, deletion of *mutE* has been shown to have no effect on growth of *Streptococcus mutans* (33).

3.3.6 Southern Blot Analysis.

Because Tn*917* insertion into the identified genes resulted in more than one alteration in the bacterial phenotype, Southern blot was used to confirm that the mutant NCS13 isolates contained a single insertion of the Tn*917* transposon. Southern blot analysis was performed on genomic DNA from the mutant strains using a probe directed against the transposon. Single bands were seen in each lane of the southern blot (Fig 3.8) containing DNA isolated from the NCS13 isolates containing Tn*917*, indicating that the observed phenotypes were due to a single insertion of the Tn*917* transposon. No band was visible in the lane containing DNA isolated from the parent NCS13 strain (Fig. 3.8).

3.3.7 Binding of rGBS-PGK to lipoteichoic acid.

The glycolytic enzymes α-enolase and GAPDH have previously been demonstrated to interact with lipoteichoic acid on the surface of *L. crispatus* and *S. gordonii* (34, 35). I therefore hypothesized that PGK may also bind to the GBS surface through interaction with lipoteichoic acid. To determine if GBS-PGK binds to lipoteichoic acid, lipoteichoic acid isolated from group A streptococcus was fixed to wells of a 96 well plate and incubated with increasing concentrations of rGBS-PGK. The relative amount of rGBS-PGK remaining in the wells was quantified using anti-rGBS-PGK antibodies (Fig. 3.9). At each concentration of rGBS-PGK the A_{405} value from wells containing immobilized lipoteichoic acid were compared to wells containing immobilized BSA (negative control). At rGBS-PGK concentrations of 7.5 μg/ml and higher, a significant ($p<0.01$) increase in the A_{405} measurements were observed (Fig. 3.9). These results demonstrate that GBS-PGK binds lipoteichoic acid in vitro, and may become attached to the GBS surface through interaction with lipoteichoic acid, as has previously been demonstrated for α-enolase and GAPDH (34, 35).

3.3.8 Effect of pH on surface expression of GBS-PGK.

Interaction of the glycolytic enzymes α-enolase and GAPDH with lipoteichoic acid has been demonstrated to occur preferentially at low pH (34, 35). I therefore hypothesized that surface attachment of GBS-PGK would increase at

low pH. The pH of the TH broth was measured and found to be 8.2; following overnight growth the pH was found to be 5.1, near the pI of GBS-PGK (4.9). To determine if PGK expression on the surface of GBS was affected by changes in pH, NCS13 was grown overnight in TH broth adjusted to pH 5.3, 6.1, 7.6, 8.2 and 9.1 and the amount of GBS-PGK expressed on the bacterial surface was quantified. After overnight growth, the pH of the growth media fell to pH 4.2, 4.4, 4.8, 5.1 and 5.3 respectively, all below or near the pI of GBS-PGK. No difference was seen in the amount of PGK expressed on the surface of NCS13 when grown in TH broth at pH, 7.6, 8.2 or 9.1 (Fig. 3.10). Reducing the starting pH to either 5.3 or 6.1 significantly ($p<0.01$) increased the amount of PGK expressed on the GBS surface (Fig. 3.10). This increased PGK expression on the GBS surface was accompanied by decreased amounts of PGK in the culture supernatant (Fig. 3.11). Image J analysis of the western blots assaying GBS-PGK secreted into the culture supernatant revealed that growth in pH 5.3, 6.1, 7.6, 8.2 and 9.1 resulted in GBS-PGK band intensities of 0%, 3.7%, 40.3%, 100% and 102.2 % respectively. These results indicate surface attachment of GBS-PGK is increased with decreasing pH, which supports the hypothesis that PGK is bound to the GBS surface through interaction with lipoteichoic acid.

To further characterize the effect of pH on attachment of PGK to the GBS surface, attachment of rGBS-PGK to the surface of NCS13 resuspended in TBS and PBS at pH 4 and pH 8 was assayed. Attachment of exogenous rGBS-PGK was significantly ($p<0.01$) increased at pH 4 compared to pH 8 in both PBS and TBS (Fig. 3.12). These results serve to confirm that surface attachment of GBS-

PGK is increased at low pH. This is in agreement agree with previous work demonstrating that glycolytic enzymes become attached to the surface of gram positive bacteria through a pH dependent charge interaction with lipoteichoic acid (34, 35).

Glycolytic enzymes attached to the bacterial surface through interaction with lipoteichoic acid have previously been demonstrated to be removed from the bacterial surface through incubation at high pH or high osmolarity (34, 35). To determine if PGK attached to the GBS surface would similarly be affected by pH and osmolarity, overnight cultures were resuspended in 50mM tris at pH4, pH8 and pH8 with the addition of 2M NaCl. Incubation for 1 h in any of these conditions did not result in a significant loss of PGK from the GBS surface (Fig. 3.13). These results indicate that PGK is firmly attached to the GBS surface and cannot be removed by incubation at high pH or osmolarity. These results are similar to GAPDH associated with the surface of group A streptococcus (16).

3.4 Discussion:

Mutagenesis using Tn*917*, followed by assaying the level of GBS-PGK on the bacterial surface using ELISA, was used to identify genes affecting expression of PGK on the GBS surface. The Tn*917* mutagenesis screen identified transposon insertions in one gene, *sag1003*, which significantly reduced expression of GBS-PGK on the bacterial surface (Fig. 3.4). Significantly more GBS-PGK was found secreted into the supernatant of NCS13*sag1003*::Tn*917* overnight cultures compared to the parent NCS13 strain, suggesting that disruption of *sag1003* reduces attachment of PGK to the GBS surface. While the increased amount of secreted GBS-PGK was found to be statistically significant, due to the low A_{405} values, it was not possible to accurately quantify the increase in secreted GBS-PGK. The Tn*917* mutagenesis screen also identified a second gene, *sag0980*, Tn*917* insertion into *sag0980* reduced surface expression of GBS-PGK (Fig. 3.4). However, this reduced expression was not statistically significant (p-value of 0.11).

Tn*917* insertion into a third gene, *sag0979*, was found to have different effects based on the exact location of the transposon. The *sag0979* gene is 1661 bases long. Tn*917* insertion at base 1650 (designated *sag0979*(1)) significantly ($p<0.01$) increased expression of GBS-PGK on the bacterial surface, while insertion at base 1215 (designated *sag0979*(2)) resulted in a non-significant (p-value of 0.08) decrease in expression of GBS-PGK on the bacterial surface. The reason for these different phenotypes is unclear. One possible explanation is that

the insertion designated *sag0979*(1), since the insertion was located near the end of the *sag0979* gene, did not abolish the function of the encoded protein. The phenotype arising from this insertion may then have been due to altered expression levels of *sag0979* or *sag0980*. A second possible explanation is that transposon insertion near the end of the *sag0979* gene altered C-terminal region of Sag0979, resulting in an altered function of the protein. However, since Tn*917* insertion into *sag0979* resulted in two opposite phenotypes, it is difficult to definitively determine the role Sag0979 plays in surface expression of GBS-PGK.

Using Tn*917* mutagenesis, I have identified two genes that appear to be involved in attaching PGK to the GBS surface. Interestingly, both of these genes encode predicted membrane proteins involved in ATP dependent efflux of antimicrobial peptides. The DAS transmembrane prediction server (http://www.sbc.su.se/~miklos/DAS/tmdas.cgi) was used to predict the presence of transmembrane regions within the Sag0980 and Sag1003 proteins; it was found that Sag0980 and Sag1003 had 6 and 8 transmembrane regions respectively. The presence of transmembrane regions support the prediction that these proteins are membrane proteins and demonstrates that portions of these two proteins are exposed to the outer surface of the bacteria. As membrane proteins with exposed outer surface components, these two proteins could act as binding ligands for GBS-PGK. Increased surface expression of an other glycolytic enzyme, GAPDH, has previously been linked to the presence of the MefE erythromycin efflux pump in *S. pneumoniae* (36), suggesting that glycolytic enzymes may attach to the bacterial surface through interaction with efflux pumps. However, we have not

demonstrated direct binding of GBS-PGK to either of these proteins so it is not known if GBS-PGK attaches directly to Sag0980 and Sag1003, or is attached through an intermediary.

Alternatively, it is possible that the Tn*917* insertions had a regulatory effect on attachment of PGK to some other surface component on the GBS surface. Three (*sag0979, sag0980, sag1003*) of the four GBS genes identified in the Tn*917* screen have homology to genes encoding proteins involved in peptide transport, suggesting surface attachment may be regulated by an unknown GBS peptide. Two (*sag0979, sag0980*) of these three genes are encoded next to five genes (*sag0973-sag0977*), which are predicted to encode proteins involved in resistance to the antimicrobial peptides nisin and bacitracin. In addition, disruption of *sag1003* resulted in increased sensitivity to both nisin and bacitracin (Fig. 3.6). These results suggest that GBS may produce an antimicrobial peptide, similar to nisin or bacitracin, which regulates attachment of PGK to the bacterial surface. This is supported by the observation that Tn*917* insertion in all four identified genes resulted in decreased bacterial cell density after overnight culture.

How antimicrobial peptides would regulate attachment of PGK to the GBS surface is not immediately obvious. However, there is a common link between attachment of glycolytic enzymes to the surface of gram positive bacteria and antimicrobial peptide resistance. Previously published results have demonstrated glycolytic enzymes can attach to the bacterial surface through interaction with lipoteichoic acid (34, 35). In these publications, it was demonstrated that GAPDH and α-enolase were attracted to the negative charge on the lipoteichoic acid. I

have demonstrated that rGBS-PGK also binds to lipoteichoic acid isolated from group A streptococcus in vitro (Fig. 3.9), suggesting that GBS-PGK may interact with lipoteichoic acid to become attached to the GBS surface. One of the mechanisms used by GBS to resist killing by antimicrobial peptides is the incorporation of D-alanine residues into the lipoteichoic acid (37, 38). Incorporation of D-alanine residues into the lipoteichoic acid, which is mediated by the *dlt* operon (38, 39), reduces the negative charge on the lipoteichoic acid. This addition of positively charged D-alanine residues is thought to repel the positively charged antimicrobial peptide. It is conceivable that incorporation of D-alanine residues into the lipoteichoic acid, in addition to repelling antimicrobial peptides, would also prevent interaction with GBS-PGK. In *Clostridium difficile* and *Streptococcus gordonii*, transcription of the *dlt* operon is up-regulated in the presence of antimicrobial peptides in a dose dependent manner (40, 41). I propose that the genes identified in my Tn*917* mutagenesis screen are part of a second mechanism for antimicrobial peptide resistance. Disruption of these genes would result in increased amounts of antimicrobial peptide in and immediately surrounding the GBS cell. As a result, disruption of the genes identified in the Tn*917* mutagenesis screen may result in an increase in the D-alanine addition to the lipoteichoic acid to compensate. This increase in the incorporation of D-alanine residues into the lipoteichoic acid may prevent the interaction between lipoteichoic acid and GBS-PGK.

While my results demonstrate that rGBS-PGK binds to lipoteichoic acid in vitro (Fig. 3.9), it is not entirely clear if GBS-PGK becomes attached to the GBS

surface through interaction with lipoteichoic acid. Consistent with previous work demonstrating glycolytic enzymes binding to lipoteichoic acid on the bacterial surface (34, 35), growth in low pH media resulted in increased expression of GBS-PGK on the bacterial surface (Fig. 3.10) and decreased expression of GBS-PGK in the bacterial supernatant (Fig. 3.11). Similarly, higher attachment of exogenously added rGBS-PGK occurred at pH 4 compared to pH 8 (Fig. 3.12). These results demonstrate that GBS-PGK attaches to the GBS surface in a pH dependent manner, consistent with the hypothesis that surface attachment of GBS-PGK is mediated by interaction with lipoteichoic acid. However, unlike α-enolase and GAPDH of *L. crispatus* (34), GBS-PGK was not released from the GBS surface by a 1 h incubation at pH 8 or in 2M NaCl (Fig. 3.13). These results suggest that while initial interaction with the GBS surface may occur through a pH dependent interaction with lipoteichoic acid, GBS-PGK may eventually become firmly attached to the GBS surface through a mechanism that is not sensitive to pH or osmolarity. A similar firm attachment to the bacterial surface has previously been demonstrated for GAPDH attached to the group A streptococcal surface (16), which has been found to bind to the M protein (42).

While my results identified genes that affect attachment of GBS-PGK to the GBS surface, the results have implications beyond surface expression of glycolytic enzymes. I have identified one gene, *sag1003*, which provides protection against nisin and bacitracin. While resistance to antimicrobial peptides has been linked to the presence of an efflux pump in *Staphylococcus aureus* (37, 43), this is the first time an efflux pump has been linked to antimicrobial peptide

resistance in GBS. Also, disruption of all four identified genes resulted in reduced expression of the β-hemolysin (Fig. 3.7), a known GBS virulence factor. This result suggests that the identified genes may be involved in regulating expression of β-hemolysin. Taken together with the observation that NCS13*sag1003*::Tn*917* was more sensitive to nisin and bacitracin, these results suggest a link between antimicrobial peptides and expression of the GBS β-hemolysin. A similar link has been established between antimicrobial peptides and expression of virulence factors in group A streptococcus (44). It has been demonstrated that expression of the β-hemolysin is down regulated by the GBS CsrRS two component system (45). In group A streptococcus, expression of the CsrRS two component system is increased in the presence of antimicrobial peptides (44). If the CsrRS two component system from GBS responds to the same environmental stimuli as the CsrRS system from group A streptococcus, expression of the β-hemolysin would be decreased by the presence of antimicrobial peptides.

In conclusion, I have identified four GBS genes that, when disrupted by Tn*917* insertion, affect attachment of GBS-PGK on the GBS surface. In addition, disruption of *sag1003* results in increased sensitivity to the antimicrobial peptides nisin and bacitracin. Finally, disruption of the genes identified in the Tn*917* screen reduces expression of the β-hemolysin. While I have not ruled out the possibility of polar effects due to Tn*917* insertion, these results suggest an interesting correlation between surface expression of GBS-PGK and known

virulence characteristics of GBS, such as expression of the β-hemolysin(46-50) and resistance to antimicrobial peptides (37-39).

Table 3.1. Oligonucleotide primers used in this chapter.

Primer name	Sequence	Reference
RTP-1	CTAAACACTTAAGAGAATTG	Gutierrez *et al*. (1996)
RTP-2	TAGGCCTTGAAACATTGGTT	Gutierrez *et al*. 1996
ermBF	TGCGTCTGACATCTATCTGATTG	Fuller *et al*. (2002)
ermBR	TTATCTGGAACATCTGTGGTATGG	Fuller *et al*. (2002)

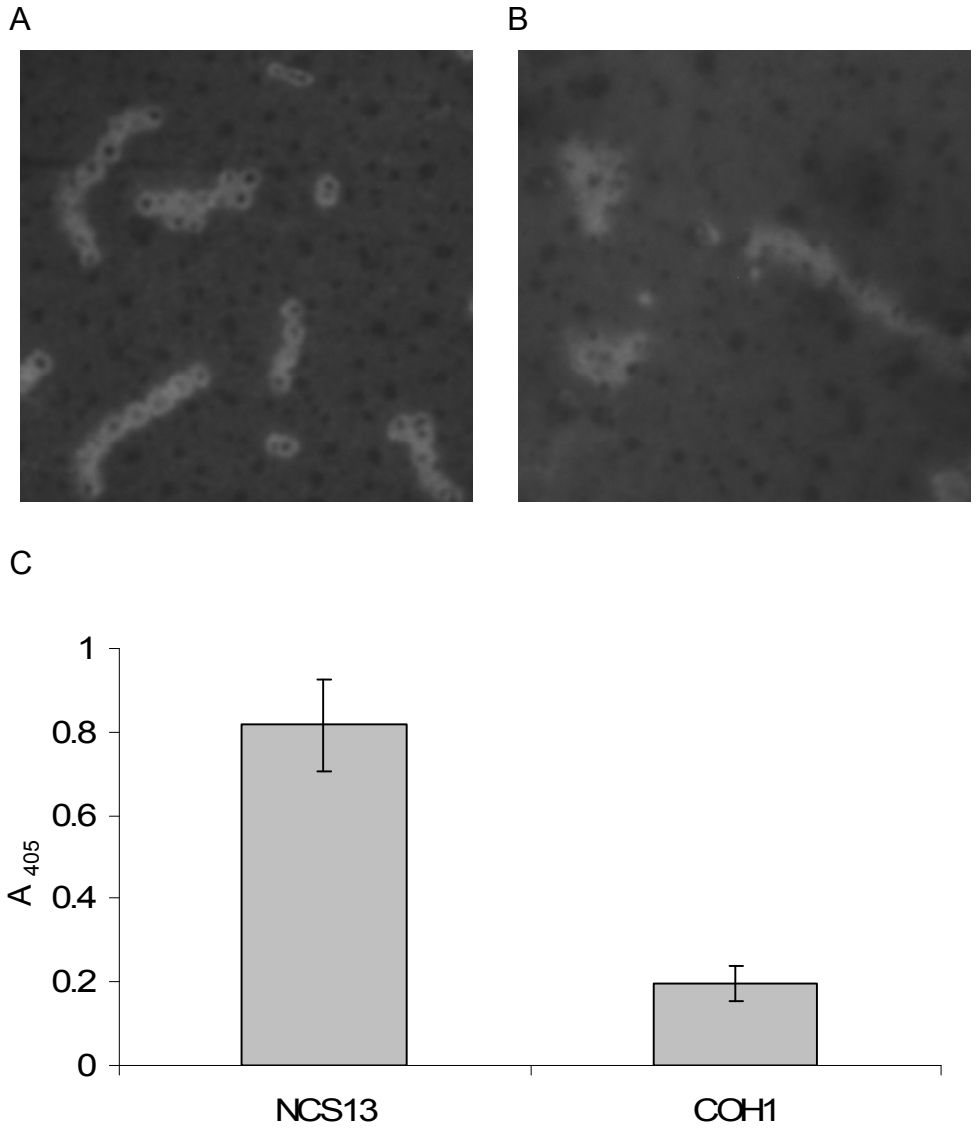

Figure 3.1. Development of a high throughput ELISA assay for measuring surface expression of GBS-PGK. Cultures of NCS13 (A) and COH1 (B) were grown overnight and fixed to glass slides. Slides were probed with anti-rGBS-PGK followed by anti-rabbit-FITC conjugate antibodies and viewed using a fluorescent microscope. Images were captured using 1.5s exposure. Figures are representative of experiments performed in duplicate. (C) Cultures of NCS13 and COH1 were grown overnight and fixed to wells of a 96 well plate. Wells were probed with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies, developed with 4-nitrophenol phosphate and the A_{405} was measured. Data points from ELISA experiments represent the average A_{405} value from 25 individual experiments, performed as positive and negative controls for the Tn*917* screen.

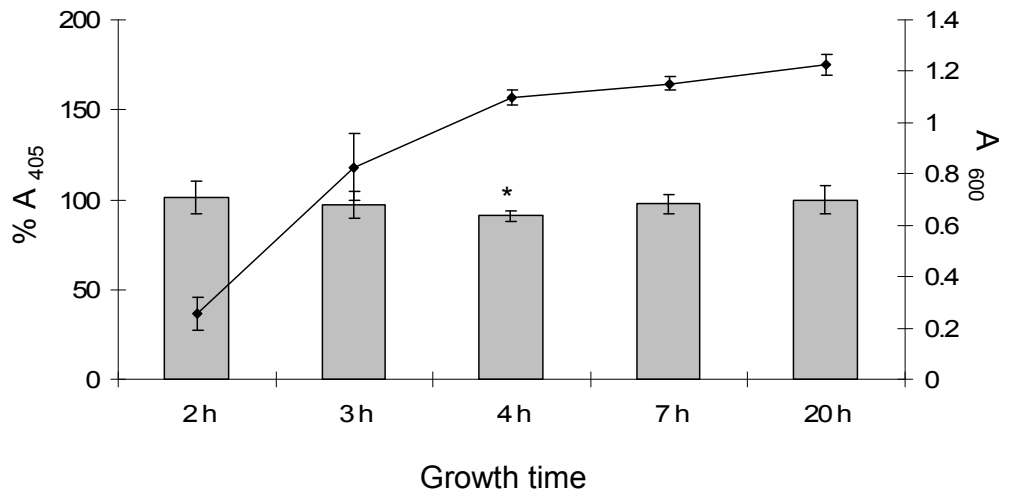

Figure 3.2. Effect of growth phase on surface expression of GBS-PGK. GBS strain NCS13 was grown for 2, 3, 4, 7 and 20 h and the A_{600} was measured to determine the bacterial density. Cells were washed 1× and resuspended to a constant bacterial density. Bacteria were fixed to wells of a 96 well polystyrene plate and probed with anti-rGBS-PGK followed by ant-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed for 30 min with 4-nitrophenol phosphate and the A_{405} was measured. Experiment was performed duplicate in triplicate. The bars represent the average % A_{405} values from all six replicates, while the line graph represents the average A_{600} values; error bars represent one standard deviation. A_{405} values were compared the value obtained from 20 h growth to determine statistical significance; (*) indicates statistical significance ($p<0.05$).

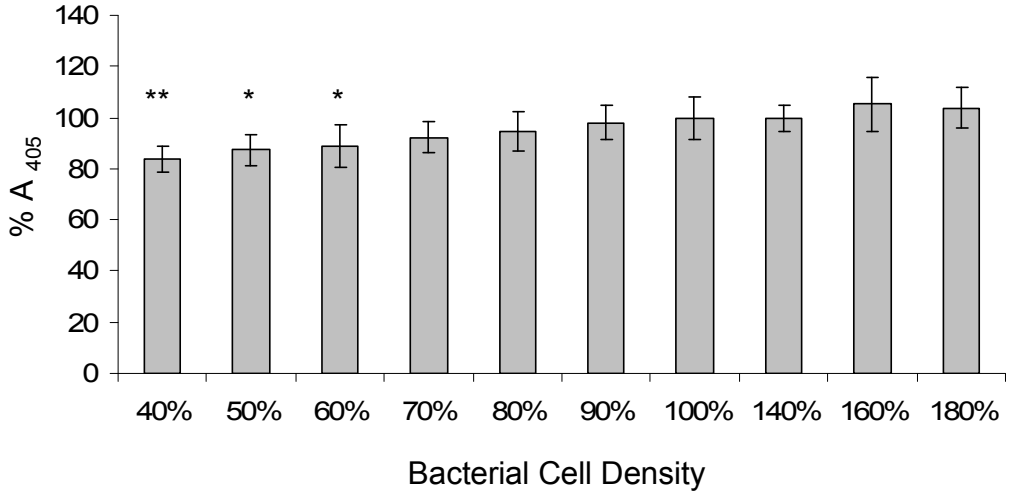

Figure 3.3. Effect of bacterial cell density on the quantification of surface expressed GBS-PGK using ELISA. The GBS strain NCS13 was grown for 20 h at 35°C and resuspended to bacterial concentrations of 40-180% of the original concentration. Bacterial cells were fixed to wells of a 96 well plate and probed with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed for 30 min with 4-nitrophenol phosphate and the A_{405} was measured. Experiment was performed duplicate in triplicate. A_{405} values were compared to the average A_{405} value obtained using 100% bacterial cell density to determine the % A_{405}. Data points represent the average % A_{405} value from all six experiments and error bars represent one standard deviation. A_{405} values were compared to the A_{405} values obtained from wells containing 100% bacterial cell density using the students t-test to determine statistical significance; (*) represents a p-value <0.05, (**) represents a p-value <0.01.

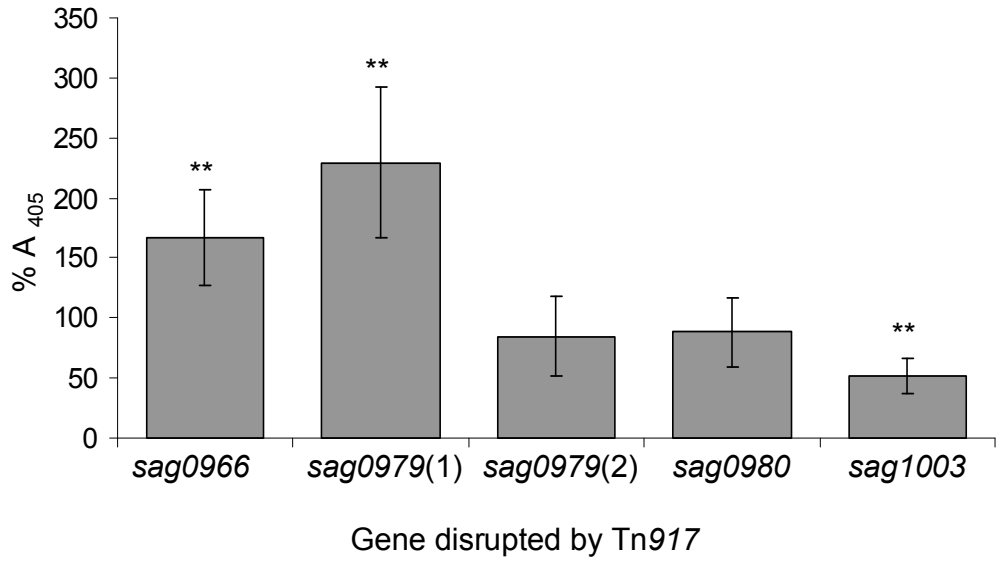

Figure 3.4. Expression of PGK on the surface of Tn*917* mutants. GBS strain NCS13 was transformed with the plasmid pTV1-Ok carrying the transposon Tn*917*. Transposon integration into the GBS genome was achieved through growth at 35°C in the presence of erythromycin. Colonies arising from the transposon integration event were assayed for expression of PGK on their surface using anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed using 4-nitrophenol phosphate and the A_{405} was measured. A_{405} values from wells containing mutant GBS strains were compared to measurements from wells containing the parent NCS13 strain to determine the $\%A_{405}$. Experiments were performed in duplicate; error bars represent one standard deviation. (**) indicates statistical significance ($p<0.01$).

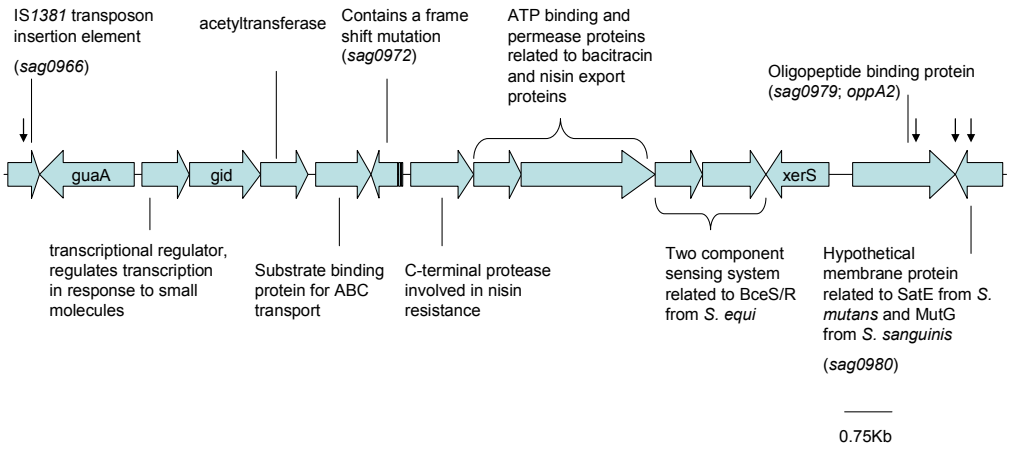

Figure 3.5. Map of genes near those identified by the Tn917 mutagenesis screen. BLAST search analysis of genes surrounding those identified using Tn*917* mutagenesis was performed. Upstream of the *oppA2* gene (*sag0979*) is an operon (*sag0973-sag0977*) that is predicted to mediate resistance to the antimicrobial peptides nisin and bacitracin. In addition, *sag0972* encodes a small peptide (57 amino acids) that may encode an antimicrobial peptide similar to nisin.

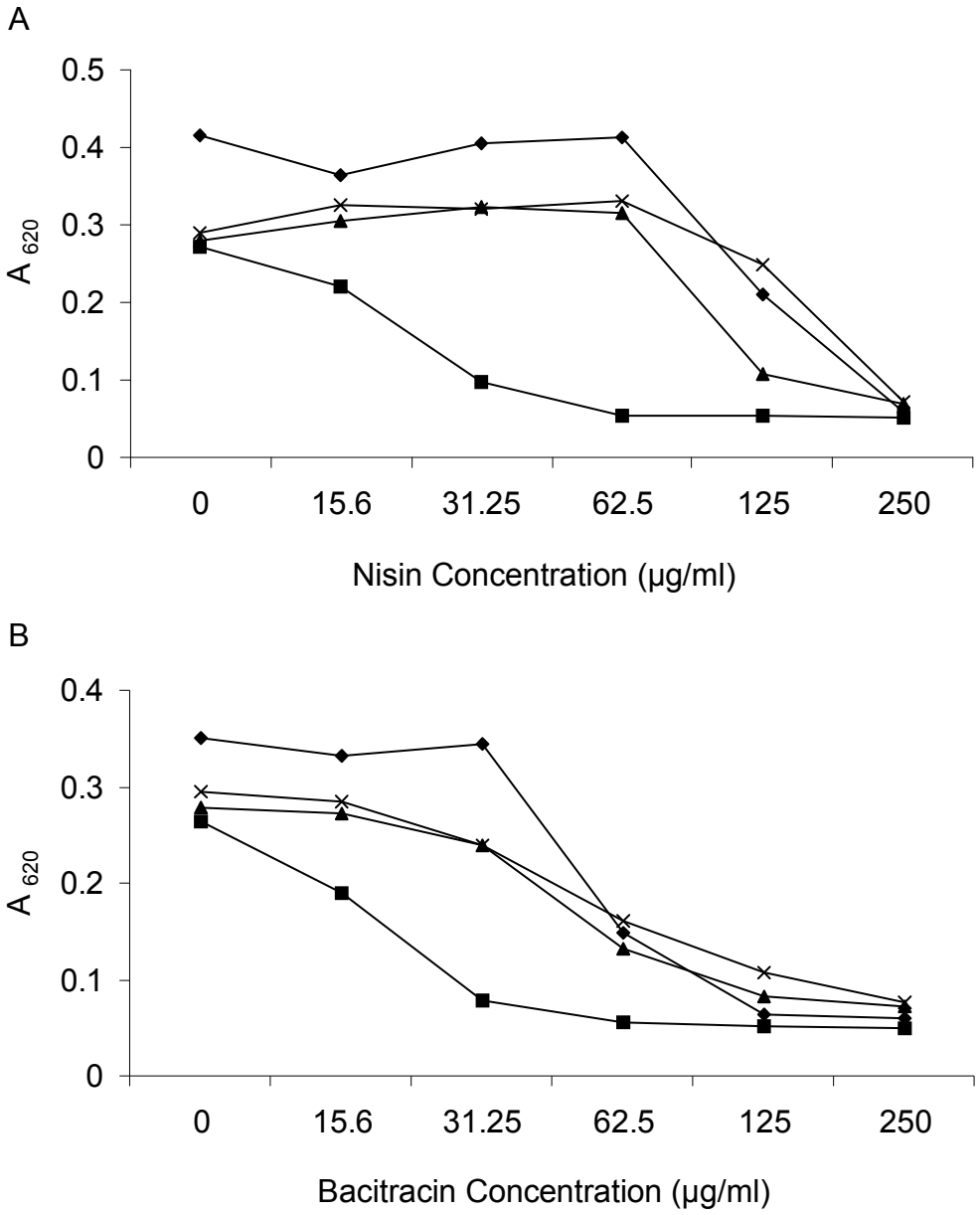

Figure 3.6. Sensitivity of NCS13 and Tn917 mutants to antimicrobial peptides. Overnight cultures of NCS13 (♦), NCS13*sag0979*(2)::Tn*917* (×), NCS13*sag0980*::Tn*917* (▲) and NCS13*sag1003*::Tn*917* (■) were used to inoculate Todd Hewitt broth containing increasing concentrations of either nisin or bacitracin (0-250 µg/ml). Cultures were grown 24 h at 35°C and the A_{620} was measured. Experiments were performed in triplicate, data points represent the average value from all three measurements.

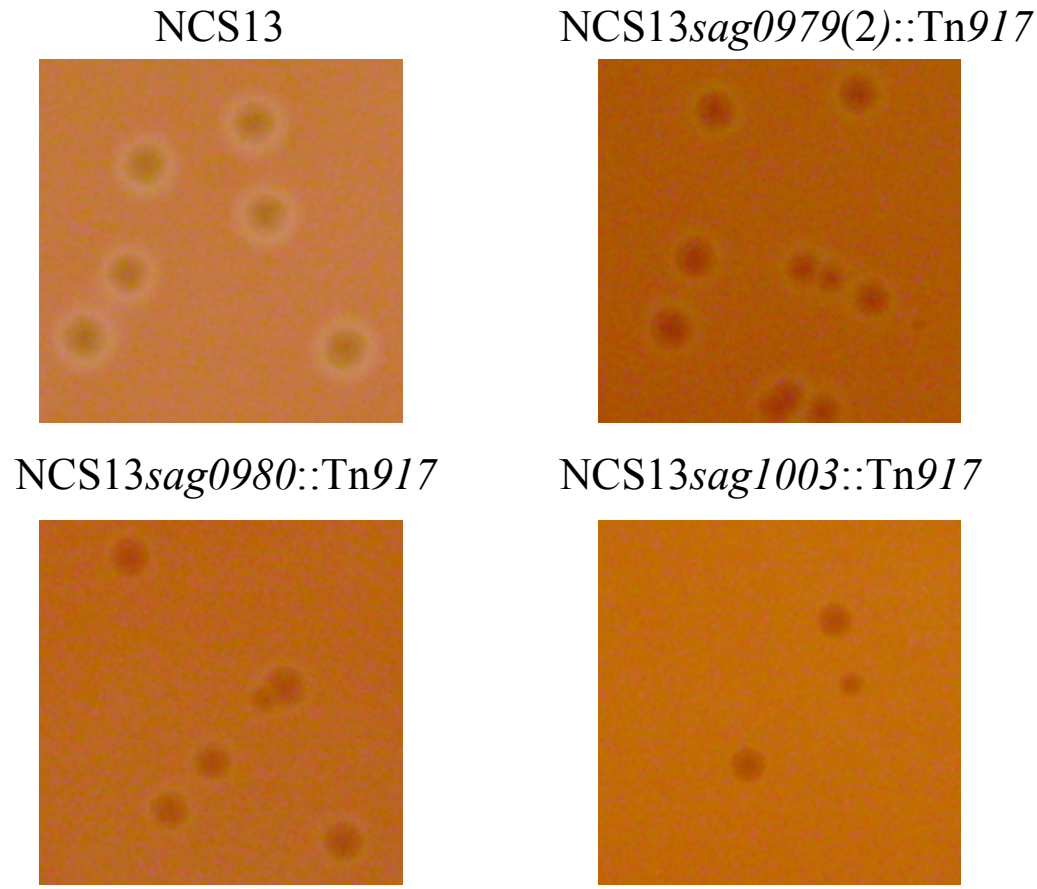

Figure 3.7. Effect of Tn*917* insertions on hemolysis. NCS13 strains containing a Tn*917* insertion in *sag0979*, *sag0980* and *sag1003* along with the parent NCS13 strain were grown overnight on sheep blood agar plates. The following day the bacterial plates were placed on a light-box and pictures of the colonies were taken to assess levels of hemolysis.

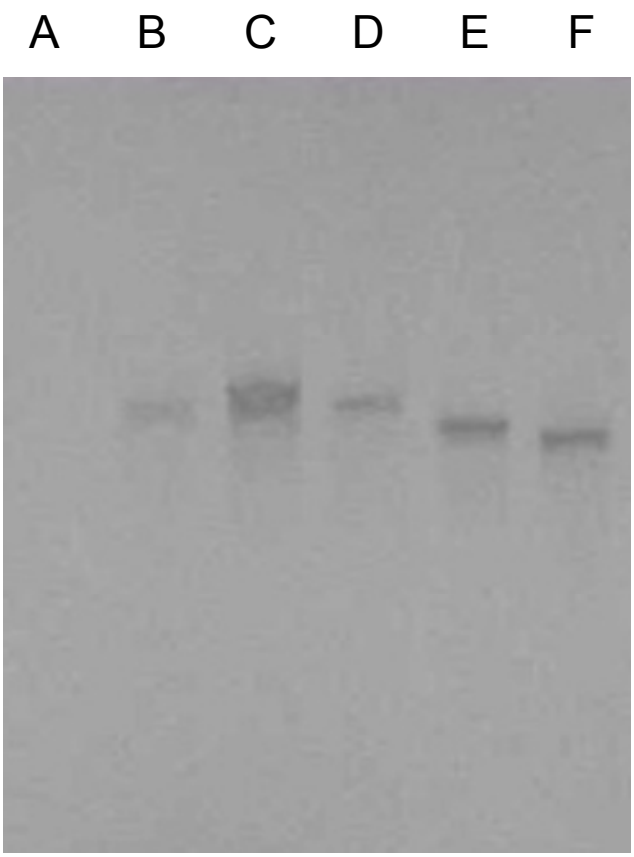

Figure 3.8. Southern blot analysis of Tn*917* mutants. Genomic DNA was isolated from overnight cultures of A) NCS13 B) NCS13*sag0979*(1)::Tn*917*, C) NCS13*sag0966*::Tn*917* D) NCS13*sag1003*::Tn*917*, E) NCS13*sag0980*::Tn*917* and F) NCS13*sag0979*(2)::Tn*917*. Genomic DNA (1 μg) was digested with *Hind* III for 5 h at 37°C and resolved on a 0.7% agarose gel. Resolved DNA was transferred and fixed to a nylon membrane. The membrane was probed with a digoxigenin labeled probe directed against the Tn*917* transposon. The membrane was then probed with anti-digoxigenin alkaline phosphatase conjugate antibodies and developed with SigmaFast BCIP/NBT.

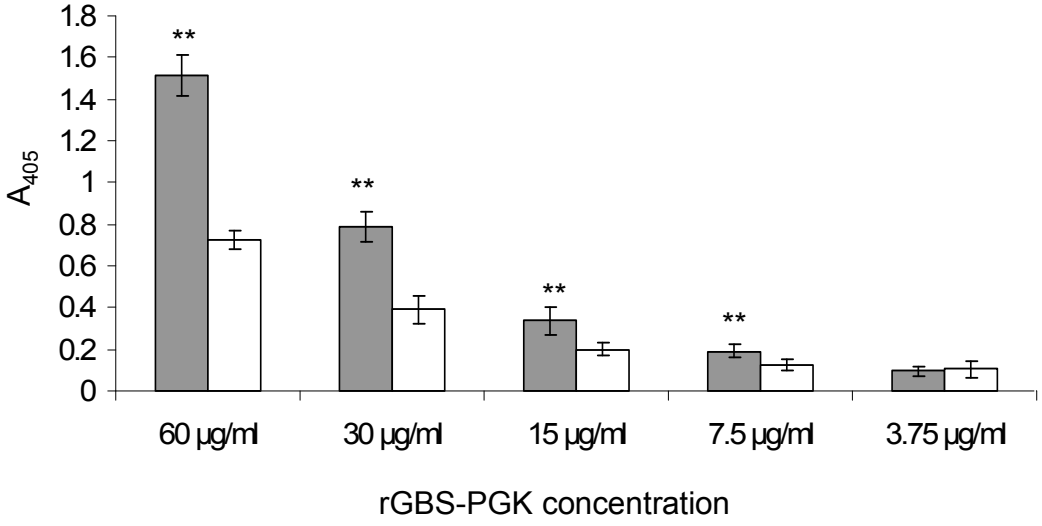

Figure 3.9. Interaction of rGBS-PGK with lipoteichoic acid measured using ELISA. Lipoteichoic acid isolated from group A streptococcus (shaded bars) and BSA (open bars) were fixed to wells of a 96 well plate (0.1 µg/well), and the wells were blocked overnight with 5% BSA 0.1% tween 20 in TBS. Wells were incubated with decreasing concentrations of rGBS-PGK (60-0 µg/ml in blocking buffer), the amount of rGBS-PGK remaining in the wells was quantified using anti-PGK followed by anti-rabbit-alkaline phosphatase conjugate antibodies, developed with 4-nitrophenol phosphate and the A_{405} was measured. Experiments were performed duplicate in triplicate. Data points represent the average value for each concentration of rGBS-PGK. Error bars represent one standard deviation. A_{405} values from wells containing lipoteichoic acid were compared to wells containing BSA for each rGBS-PGK concentration to determine statistical significance, (**) indicates $p<0.01$.

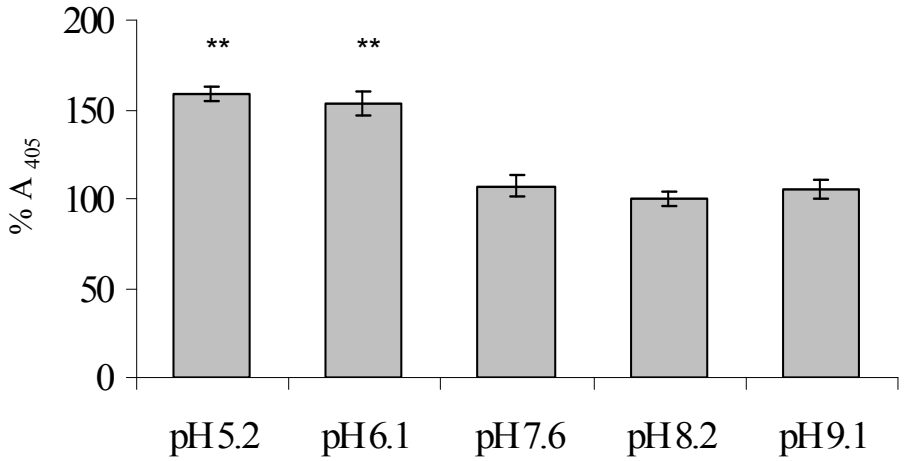

Figure 3.10. Effect of pH on surface GBS-PGK. NCS13 was grown overnight in TH broth at pH 5.2, 6.1, 7.6, 8.2 and 9.1. Bacterial cells were washed and fixed to a 96 well plate. Wells were probed with anti-rGBS-PGK followed by anti-rabbit alkaline phosphatase conjugate antibodies, developed with 4-nitrophenol phosphate and the A_{405} was measured. The A_{405} readings were compared to growth at pH 8.2 to determine the $\%A_{405}$ for the various adjusted pH's. Data points represent the average $\%A_{405}$ value from experiments performed triplicate in triplicate; error bars represent one standard deviation. (**) indicates statistical significance ($p< 0.01$).

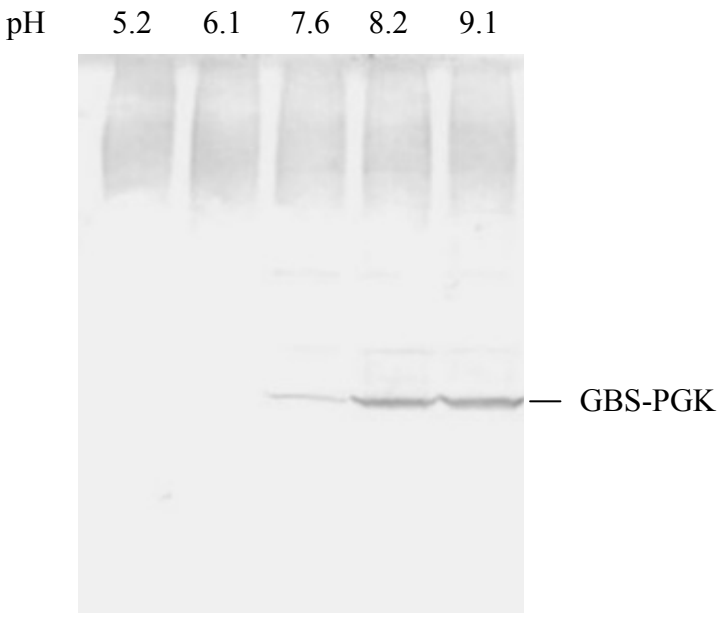

Figure 3.11. Effect of pH on secretion of GBS-PGK. NCS13 was grown overnight in TH broth at pH 5.2, 6.1, 7.6, 8.2 and 9.1. Supernatant proteins from overnight cultures were collected and separated via 10% SDS-PAGE. Separated proteins were electrophoretically transferred to a nitrocellulose membrane and the membrane was probed with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Membranes were developed with SigmaFast BCIP/NBT. Figure is representative of experiments performed in triplicate

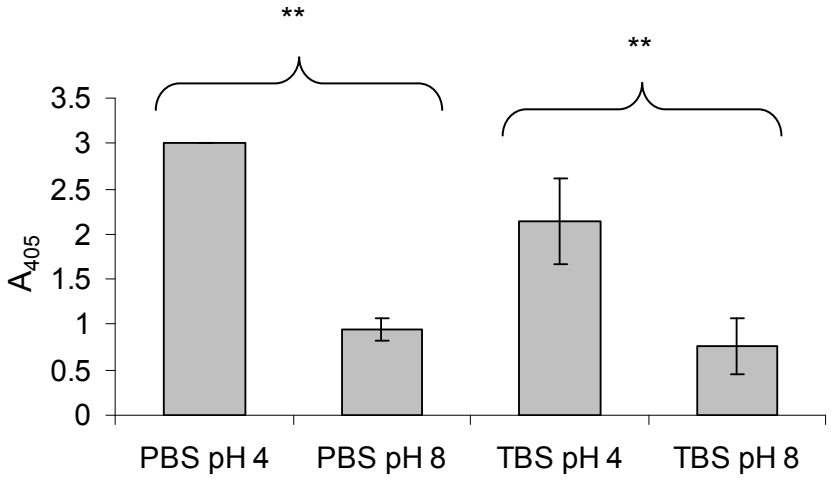

Figure 3.12. Effect of pH on attachment of rGBS-PGK to the GBS surface. Overnight cultures of NCS13 were resuspended in either PBS or TBS. Resuspended bacterial cultures were supplemented with rGBS-PGK (10 μg/ml) and incubated at 35°C for 1 h. Cells were washed 5× and fixed to a 96 well polystyrene plate. Wells were probed with anti-poly-histidine followed by anti-mouse IgG alkaline phosphatase conjugate antibodies, developed using 4-nitrophenol phosphate and the A_{405} was measured. Data points represent the average A_{405} values from experiments performed triplicate in triplicate; error bars represent one standard deviation. (**) indicates statistical significance ($p<0.01$)

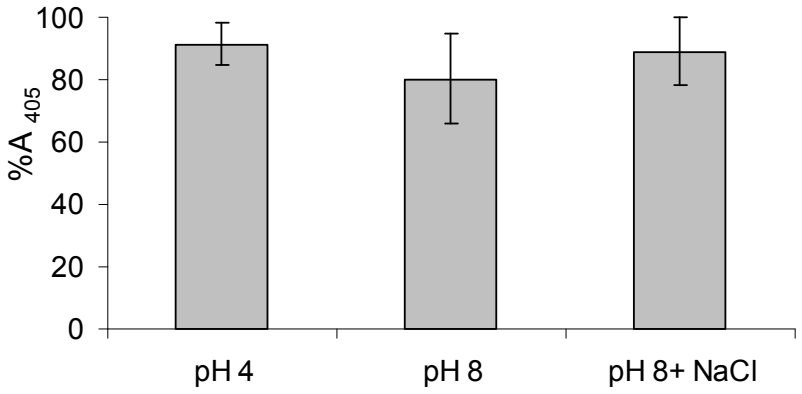

Figure 3.13. Effect of pH and osmolarity on release of PGK from the GBS surface. Overnight cultures of NCS13 were resuspended in 50 mM tris adjusted to pH 4, pH 8 or pH 8 supplemented with 2M NaCl. Bacterial cultures were incubated at room temperature for 1 h, washed 3× and resuspended in 1 culture volume of TBS. Washed bacterial cultures were fixed to wells of a 96 well polystyrene plate. Wells were probed with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies and developed using 4-nitrophenol phosphate. The A_{405} was measured and compared to those obtained from the original overnight culture of NCS13 to determine the $\%A_{405}$. Data points represent the average $\%A_{405}$ obtained from experiments performed triplicate in triplicate; error bars represent one standard deviation.

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Chapter 4:

The SecA2 Locus is Required for Expression of Phosphoglycerate Kinase on the Surface of Group B Streptococcus

4.1 Introduction:

Streptococcus agalactiae (Group B streptococcus; GBS) is the leading cause of invasive neonatal bacterial disease in North America (1-5). Most infants developing GBS disease first acquire the bacterium through aspiration of contaminated fluid, prior to or during birth (6). Following colonization of the lung, GBS invades the lung epithelial cells and eventually gains access to the bloodstream. During the ensuing bacteremia, GBS gains access to and invades the infant's blood brain barrier resulting in meningitis. To cause bacteremia and meningitis GBS must survive a variety of host defenses and invade barrier tissues designed to prevent bacterial passage. Survival and dissemination of GBS in the human body is mediated by a number of surface expressed and secreted proteins (7-10). One interesting collection of potential virulence proteins are surface expressed glycolytic enzymes (11, 12).

Glycolytic enzymes have well established metabolic functions, and are essential for survival, but have also been identified on the surface (13-18) and in the culture supernatant (17, 19-21) of gram positive bacteria. Two well studied examples of these include α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition to their role in glycolysis, α-enolase and GAPDH are thought to contribute to bacterial virulence through their ability to bind host proteins. α-enolase has been demonstrated to contribute to *Streptococcus pneumoniae* virulence through its ability to bind plasminogen (22, 23), while GAPDH has been demonstrated to contribute to group A streptococcal (GAS) virulence through its ability to bind uPAR/CD87 (16) and C5a (15). In

contrast to α-enolase and GAPDH, the function of phosphoglycerate kinase (PGK) present on the GBS surface (14, 24) is unknown. GBS-PGK has previously been demonstrated to bind actin, fibronectin and plasminogen (21) indicating that surface expressed PGK could contribute to GBS virulence. However, due to its essential role in metabolism, it has not yet been possible to definitively determine the function of surface expressed GBS-PGK.

Due to its essential role in glycolysis, the generation of a *pgk* knock-out mutant is not a feasible approach to determine the function of PGK on the GBS surface. An alternative approach would be to prevent transport or attachment of PGK to the GBS surface, generating a mutant GBS strain expressing PGK in the cytoplasm but not on the bacterial surface. Similar to α-enolase and GAPDH, PGK does not contain any known secretion or surface attachment signal sequences. As a result, it is unknown how PGK becomes expressed on the GBS surface. In *Listeria monocytogenes*, deletion of the *secA2* gene resulted in loss of α-enolase expression on the bacterial surface (25).

The objective of this work was to determine if SecA2 plays a role in surface expression of GBS-PGK.

4.2 Materials and Methods:

4.2.1 Oligonucleotide primers, bacterial strains and growth conditions.

Oligonucleotide primers used in this chapter are listed in table 4.1. The GBS strain NEM316 and knockout mutants NEM316Δ*secA2*, NEM316Δ*srr1* and NEM316Δ*gtfA,B* were kindly provided by Michel-Yves Mistou, and were used to determine the role of the SecA2 secretion system on surface expression of GBS-PGK. The gene deletions in NEM316Δ*secA2*, NEM316Δ*srr1* and NEM316Δ*gtfA,B* were confirmed by PCR amplification of the deleted regions (Fig. 4.1) using the previously published oligonucleotides primer srr1-O5, srr1-O8, secA2-O13, secA2-O16, gtfA,B-O17 and gtfA,B-O20 (26). GBS strains were grown in Todd-Hewitt (TH) broth (Difco Laboratories; Becton Dickinson, Mississauga, ON, CA), or on sheep blood agar (Dalynn Biologicals, Calgary, AB, CA) at 35°C.

4.2.2 Production and purification of recombinant GBS-PGK (rGBS-PGK).

Recombinant GBS-PGK (rGBS-PGK) was produced using the *Escherichia coli* strain M15 as previously described (21). The *pgk* gene was PCR amplified from genomic DNA isolated from NCS13 using the oligonucleotides primers pgk-pstF and pgk-hinR, ligated into the expression plasmid pQE30 and transformed into *Escherichia coli* strain M15. Overnight cultures were used to

seed 100 ml expression cultures in Luria-Bertani (LB) broth containing 80 μg/ml carbenicillin and 25 μg/ml kanamycin. After 1 h incubation at 35°C expression of rGBS-PGK was induced by the addition of 2 mM isopropyl β-D-1 thiogalactopyranoside (IPTG). After 4 h incubation at 35°C, cells were harvested by centrifugation. Purification of rGBS-PGK was performed using the Qiaexpressionist kit (Qiagen, Mississauga, ON, CA) under native conditions.

4.2.3 Expression of GBS-PGK on the surface of NEM316, NEM316ΔsecA2, NEM316Δsrr1 and NEM316ΔgtfA,B.

The GBS strain NEM316, and the knockout mutant strains NEM316Δ*secA2*, NEM316Δ*gtfAB* and NEM316Δ*srr1*, were assayed for surface expressed PGK using an ELISA similar to previously described for quantification of lipoteichoic acid on the GBS surface (27). Colonies of NEM316, NEM316Δ*secA2*, NEM316Δ*gtfAB* and NEM316Δ*srr1* were grown for 20 h in Todd Hewitt (TH) broth at 35°C, washed 1× and resuspended in one culture volume of tris buffered saline (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, CA) by overnight incubation at 4°C. Wells were washed 1× with TBS and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in TBS for 1 h at room temperature. After blocking the wells were washed 3× with TBS and incubated 1 h with anti-rGBS-PGK antibodies (1:300 in blocking buffer) (21).

Wells were washed 3× with TBS and incubated 1 h with anti-rabbit IgG-alkaline phosphatase conjugate antibodies (1:200 in blocking buffer; Sigma-Aldrich). Wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate (Sigma-Aldrich) for 30 min at room temperature before stopping the reaction with 25 µl NaOH (3N). The absorbance at 405 nm (A_{405}) was measured using an Athos LP400 microplate reader (Bio-Rad Laboratories Ltd. Mississauga, ON, CA).

Experiments were performed triplicate in triplicate, for each plate the A_{405} values obtained were compared to the average A_{405} reading from the parent NEM316 strain to determine the $\%A_{405}$. Data points from the mutant NEM316 strains were compared to the parent NEM316 strain using the student's t-test to determine statistical significance. A p-value < 0.05 was considered statistically significant.

4.2.4 Attachment of exogenously added rGBS-PGK to the surface of NEM316, NEM316ΔsecA2, NEM316Δsrr1 and NEM316ΔgtfA,B.

Attachment of exogenously added rGBS-PGK to the surface of NEM316, NEM316Δ*secA2*, NEM316Δ*srr1* and NEM316Δ*gtfA,B* was also assayed using ELISA. Parent and mutant strains of NEM316 were grown 20 h at 35°C in the presence of rGBS-PGK (0 or 2 µg/ml), washed 3× and resuspended in one culture volume of TBS. One hundred microliters of washed bacterial cultures were fixed to wells of a 96 well polystyrene plate (Maxi-sorp) by overnight incubation at

4°C. Wells were then washed 1× with TBS and incubated 1 h with blocking buffer (5% BSA in TBS). After blocking the wells were washed 3× with TBS and incubated 1 h with anti-poly histidine antibodies (diluted 1:300 in blocking buffer; Sigma-Aldrich). Wells were washed 3× with TBS and incubated 1 h with anti-mouse-IgG-alkaline phosphatase conjugate antibodies (1:200 in blocking buffer; Sigma-Aldrich). Wells were washed 3× with TBS and developed with 100 µl 4-nitrophenol phosphate for 30 min at room temperature before stopping the reaction with 25 µl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader.

Experiments were performed triplicate in triplicate. For each plate the A_{405} values obtained were compared to the average A_{405} value from the parent NEM316 strain to determine the $\%_{A405}$. Data points from the mutant NEM316 strains were compared to the parent NEM316 strain using the student's t-test to determine statistical significance.

4.2.5 Secretion of PGK by NEM316, NEM316ΔsecA2, NEM316Δsrr1 and NEM316ΔgtfA,B.

The presence of GBS-PGK secreted into the supernatant of parent and mutant NEM316 cultures was assayed using Western blot. Overnight NEM316 cultures (10 ml) were centrifuged at 3000×g for 10 min and the supernatants were collected. Culture supernatants were passed through a 0.22 µM filter Millipore: Billerica, MA, USA) and concentrated 10× using a centrifugal filtration unit with

10 kDa cutoff (Amicon, Millipore). The concentrated supernatant proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a 0.45 μM nitrocellulose membrane (Bio-Rad Laboratories). Nitrocellulose membranes were washed 1× 15 min with TBS and blocked for 30 min with 5% BSA, 0.1% tween 20 in TBS. After blocking membranes were washed 1× 15 min with TBS and incubated overnight at 4°C with anti-rGBS-PGK antibodies (1:1250 in blocking buffer). Membranes were then washed 3× 15 min with TBST (0.1% tween 20 in TBS), 2× 15min with TBS and incubated 1 h with anti-rabbit-IgG-alkaline phosphatase conjugate antibodies (1:10 000 in blocking buffer) at room temperature. Membranes were washed 3× 15 min with TBST, 2× 15 min with TBS and developed with SigmaFast BCIP/NBT (Sigma-Aldrich) for 5 min before stopping the reaction with 3 changes of distilled water. Membranes were analyzed using Image J to determine the band intensities corresponding to secreted GBS-PGK.

4.2.6 Effect of sodium azide on secretion of GBS-PGK.

Overnight cultures of NEM316 along with the knockout mutant NEM316Δ*secA2* were split into three 10 ml aliquots. Two of the aliquots were centrifuged at 3000×g and the supernatants were removed. The cell pellets were resuspended in 10 ml fresh TH supplemented with sodium azide (0 or 50 mM). The third aliquot was left in the original growth media to act as a positive control. The bacterial cultures were incubated for 2 h at 35°C, the cultures were

centrifuged 3000×g and the supernatants were collected. Culture supernatants were passed through a 0.22 μm filter and concentrated 10× using a centrifugal filtration unit with 10 kDa cutoff. The concentrated supernatant proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a 0.45 μM nitrocellulose membrane (Bio-Rad Laboratories). Nitrocellulose membranes were washed 1× 15 min with TBS and blocked for 30 min with 5% BSA, 0.1% tween 20 in TBS. After blocking membranes were washed 1× 15 min with TBS and incubated overnight at 4°C with anti-rGBS-PGK antibodies (1:1250 in blocking buffer). Membranes were then washed 3× 15 min with TBST (0.1% tween 20 in TBS), 2× 15min with TBS and incubated 1 h with anti-rabbit-IgG-alkaline phosphatase conjugate antibodies (1:10 000 in blocking buffer) at room temperature. Membranes were washed 3× 15 min with TBST, 2× 15 min with TBS and developed with SigmaFast BCIP/NBT for 5 min before stopping the reaction with 3 changes of distilled water.

4.3 Results:

4.3.1 Expression of GBS-PGK on the surface of NEM316, NEM316ΔsecA2, NEM316Δsrr1 and NEM316ΔgtfA,B.

Expression of another glycolytic enzyme, α-enolase, on the surface of *L. monocytogenes* has been previously demonstrated to be dependent on the SecA2 section system (25). I therefore hypothesized that GBS SecA2 secretion system may be necessary for surface expression of the GBS-PGK. To determine if the SecA2 secretion system is involved in the surface expression of GBS-PGK, I assayed expression of PGK on the surface of the GBS strains NEM316 and NEM316Δ*secA2* (26). Significantly ($p<0.01$) less GBS-PGK was found on the surface of NEM316Δ*secA2* compared to the parent NEM316 strain (Fig 4.2A), suggesting the SecA2 secretion system is involved in transport of GBS-PGK to the bacterial surface. It was not clear from these results, whether the observed effect was due to loss of the SecA2 secretion motor itself, or from a loss of the fimbrial protein Srr1, the only protein known to be secreted by GBS SecA2, from the bacterial surface. To determine the role of Srr1 in surface expression of GBS-PGK, I assayed expression of GBS-PGK on the surface of NEM316Δ*srr1*, as well as NEM316Δ*gtfA,B* (26). The GtfA and GtfB proteins are cytoplasmic proteins that are responsible for glycosylation of Srr1, stabilizing the protein and targeting it for secretion via the SecA2 secretion system (26). Surprisingly, significantly less GBS-PGK was found on the surface of NEM316Δ*srr1* and NEM316Δ*gtfA,B* than either the parent NEM316 strain ($p<0.001$) or NEM316Δ*secA2* ($p<0.01$)

(Fig. 4.2A). On average NEM316Δ*secA2,* NEM316Δ*srr1* and NEM316Δ*gtfA,B* had A_{405} measurements of 67%, 32% and 34% respectively compared to the parent NEM316 strain (Fig. 4.2A). These results indicate that SecA2, Srr1, GtfA and GtfB play a role in surface expression of GBS-PGK.

4.3.2 Attachment of exogenously added rGBS-PGK to the surface of NEM316, NEM316ΔsecA2, NEM316Δsrr1 and NEM316ΔgtfA,B.

While NEM316ΔsecA2, NEM316Δ*srr1* and NEM316Δ*gtfA,B* were found to have reduced PGK expression on their surface compared to NEM316, it was unclear if this was due to decreased secretion or decreased surface attachment of the protein. To assay attachment of PGK to the surface of these GBS strains, NEM316, NEM316Δ*secA2*, NEM316Δ*srr1* and NEM316Δ*gtfA,B* were grown overnight in the presence of exogenously added rGBS-PGK. The amount of rGBS-PGK attached to the GBS surface was assayed using antibodies directed against the histidine tag (Fig. 4.2B). Similar amounts of rGBS-PGK bound to the surface of the NEM316 and NEM316Δ*secA2* strains, while significantly less ($p<0.01$) rGBS-PGK bound to the surface of the NEM316Δ*srr1* and NEM316Δ*gtfA,B* strains. On average NEM316Δ*srr1* and NEM316Δ*gtfA,B* had A_{405} values of 50% and 55% respectively compared to the parent NEM316 strain. These results indicate that Srr1, GtfA and GtfB but not SecA2 play a role in attaching PGK to the GBS surface.

4.3.3 Secretion of PGK by NEM316, NEM316ΔsecA2, NEM316Δsrr1 and NEM316ΔgtfA,B.

While Srr1 and GtfA,B appear to play a role in attaching PGK to the GBS surface, the reduced attachment does not completely explain the loss of surface expression. In addition, SecA2 was found to play a role in surface expression but not surface attachment of GBS-PGK. I hypothesized that these genes may also play a role in transport of PGK across the GBS membrane. To determine the role of SecA2, Srr1 and GtfA,B in secretion of GBS-PGK, the supernatant from overnight cultures of NEM316, NEM316Δ*secA2*, NEM316Δ*srr1* and NEM316Δ*gtfA,B* were assayed for the presence of PGK using Western blot (Fig. 4.3). Western blot analysis of GBS-PGK secreted into the supernatant of overnight cultures revealed visibly less GBS-PGK in the supernatant of all three mutant strains compared to the parent NEM316. Quantification of the western blot band intensities, using ImageJ, demonstrated band intensities for the NEM316Δ*secA2*, NEM316Δ*srr1* and NEM316Δ*gtfA,B* strains were 62%, 36% and 21% respectively compared to the parent NEM316 strain. These results indicate that SecA2, Srr1, GtfA and GtfB all play a role in transport of GBS-PGK across the bacterial membrane.

4.3.4 Effect of sodium azide on secretion of GBS-PGK.

While secretion of GBS-PGK was found to be reduced in NEM316Δ*secA2* compared to the parent NEM316 strain, relatively high amounts of GBS-PGK was still secreted (Fig. 4.3). This indicates that SecA2 is not the sole secretion mechanism used to express GBS-PGK on the GBS surface. In *Streptococcus parasanguinis*, the signal peptide of Fap1, a protein similar to Srr1, was found to target proteins for secretion through SecA in addition to SecA2 (28). I hypothesized that GBS-PGK may be secreted by SecA in addition to SecA2. To determine if GBS-PGK was also secreted by SecA, overnight cultures of NEM316 and NEM316Δ*secA2* were resuspended in fresh TH broth in the presence or absence of the SecA inhibitor sodium azide. After a 2 h incubation at 35°C the levels of GBS-PGK secreted into the supernatant were analyzed using Western blot (Fig. 4.4). GBS-PGK secretion by NEM316 and NEM316Δ*secA2* strains were visibly reduced in the presence sodium azide (Fig. 4.4). No secretion of GBS-PGK by NEM316Δ*secA2* was detectable in the presence of sodium azide. These results suggest that in addition to secretion by SecA2, GBS-PGK can be secreted by SecA.

4.4 Discussion:

Expression of glycolytic enzymes on the surface of gram positive bacteria has been predicted to occur through a two step process of secretion followed by attachment of the secreted proteins to the bacterial surface (11). My results demonstrate that SecA2 is partially responsible for secretion of GBS-PGK as deletion of the *secA2* gene reduced expression of GBS-PGK on the surface of NEM316 (Fig. 4.2A) and in the culture supernatant (Fig. 4.3). These results agree with previous work demonstrating that SecA2 of *L. monocytogenes* is required for surface expression of the glycolytic enzyme α-enolase (25). Unlike previous work in *L. monocytogenes*, deletion of the *secA2* gene did not result in a complete loss of GBS-PGK on the surface of NEM316 (Fig. 4.2A). Since the addition of sodium azide abolished secretion of GBS-PGK in the NEM316Δ*secA2* strain (Fig. 4.4) it seems likely that the SecA secretion system is also involved in secretion of GBS-PGK. A similar secretion profile has been demonstrated for FimA from *Streptococcus parasanguis* (29).

Surprisingly, the NEM316Δ*srr1* and NEM316Δ*gtfA,B* strains were found to have less GBS-PGK on their surface and in the culture supernatant following overnight growth than either the NEM316 or NEM316Δ*secA2* strains (Fig. 4.2A and 4.3). Given the similar phenotypes (Fig. 4.2A and 4.3) and the observation that Srr1 is unstable in the NEM316Δ*gtfA,B* strain (26), it seems likely that the phenotype from both mutant strains was due to the absence of Srr1. Since deletion of the *secA2* gene resulted in a complete loss of Srr1 on the surface of NEM316 (26), it is unlikely that the effect of the deletions of *srr1* and *gtfA,B* was

due to loss of Srr1 on the NEM316 surface. These results suggest that, while GBS-PGK can be secreted using either the SecA or SecA2 motor proteins, its secretion is almost entirely dependent on the presence of Srr1 in the bacterial cytoplasm. The requirement for Srr1 in the cytoplasm suggests that Srr1 may act as a chaperone protein targeting GBS-PGK for secretion. This is the first indication that the Srr1 protein may have a cytoplasmic function.

I have previously demonstrated that GBS-PGK likely attaches to the bacterial surface, first through interaction with lipoteichoic acid followed by a firm interaction with a second GBS surface ligand. Deletion of the *srr1* and *gtfA,B* genes resulted in significantly reduced amounts of exogenously added rGBS-PGK that would attach to the NEM316 surface (Fig. 4.2B). These results suggest that expression of this second GBS-PGK binding ligand on the GBS surface also requires Srr1 in the bacterial cytoplasm. Because NEM316Δ*secA2* does not express Srr1 on its surface (26) but still bound similar amounts of rGBS-PGK (Fig. 4.2B), the Srr1 protein itself is likely not involved in attaching PGK to the GBS surface. These results suggest that in addition to acting as a chaperone protein targeting GBS-PGK for secretion, Srr1 also targets a second ligand to the GBS surface responsible for attaching GBS-PGK to the bacterial surface. Alternatively, loss of Srr1 in the GBS cytoplasm may affect the expression or modification of lipoteichoic acid. This modulation of lipoteichoic acid expression may affect attachment of GBS-PGK to the bacterial surface.

In conclusion, my results indicate that the SecA2 locus is likely involved in secretion of GBS-PGK. In particular, deletion of the *srr1* and *gtfA,B* genes

result in almost a complete loss of GBS-PGK secretion. These results suggest that PGK may be targeted for secretion through interaction with Srr1 in the GBS cytoplasm. Characterizing the interaction between GBS-PGK and Srr1 may identify mutations within *pgk* that would prevent surface expression of GBS-PGK. Srr1 in the bacterial cytoplasm also appears to play a role in surface attachment of GBS-PGK, possibly through mediating secretion of a ligand responsible for attaching GBS-PGK to the bacterial surface. Characterizing the difference in surface protein expression between NEM316 and NEM316Δ*srr1* may identify the second GBS ligand responsible for attaching PGK to the GBS surface.

Table 4.1. Oligonucleotide primers used in this chapter

Primer Name	Primer sequence	Reference
pgk-pstF	TTCCTGCAGTTAATTTTCAGTCAATGC	Boone *et al*. (2011)
pgk-hinR	TTCAAGCTTTTTTCAGTCAATGCTGCCAAACC	Boone *et al*. (2011)
srr1-O5	AGCTGGTACCGGTTACAGGCGGTATCTAAGG	Mistou *et al*. (2009)
srr1-O8	GGATGGATCCAATTCTAGCACCGTATGCCTG	Mistou *et al*. (2009)
secA2-O13	AGCTGAATTCTGAGGAAGTTACAGATAGCGT	Mistou *et al*. (2009)
secA2-O16	GGATGGATCCCTTTGGATGGTAAAAGTCTGC	Mistou *et al*. (2009)
gtfA,B-O17	AGTGAGAATTCGGGAGAGTCTGCACGTAGTCAAA	Mistou *et al*. (2009)
gtfA,B-O20	AATAGCGGATCCCTTCATCTGTGTACCAA	Mistou *et al*. (2009)

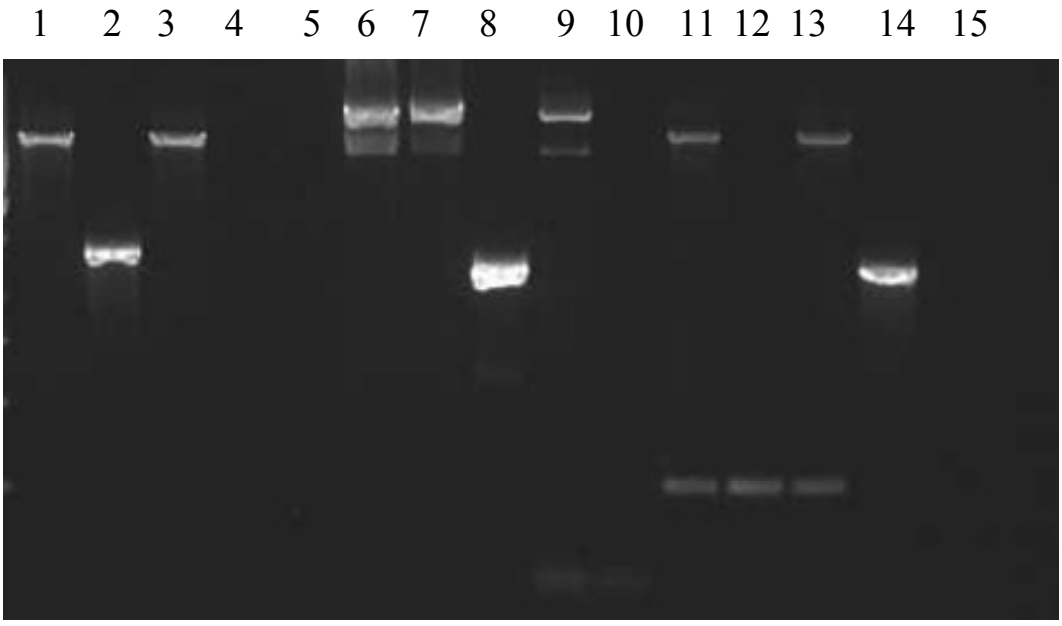

Figure 4.1. PCR amplification of gene deletions in NEM316 GBS strains. Genomic DNA isolated from NEM316 (lanes 1, 6, 11), NEM316Δ*secA2* (lanes 2, 7, 12), NEM316Δ*srr1* (lanes 3, 8, 13) and NEM316Δ*gtfA,B* (lanes 4, 9, 14) was subjected to PCR amplification using primer pairs O13 and O16 (lanes1-5), O5 and O8 (lanes 6-10) or O17 and O20 (lanes 11-15). Sterile water was used as a negative PCR control (lanes 5, 10 and 15). PCR products were resolved on a 1% agarose gel. The presence of a PCR band of smaller size than seen in lanes corresponding to the parent NEM316 strain indicates an internal region of the gene had been removed. The absence of a PCR product in lanes 4 and 12 was a result of loss of a primer binding site due to an internal deletion in the adjacent gene.

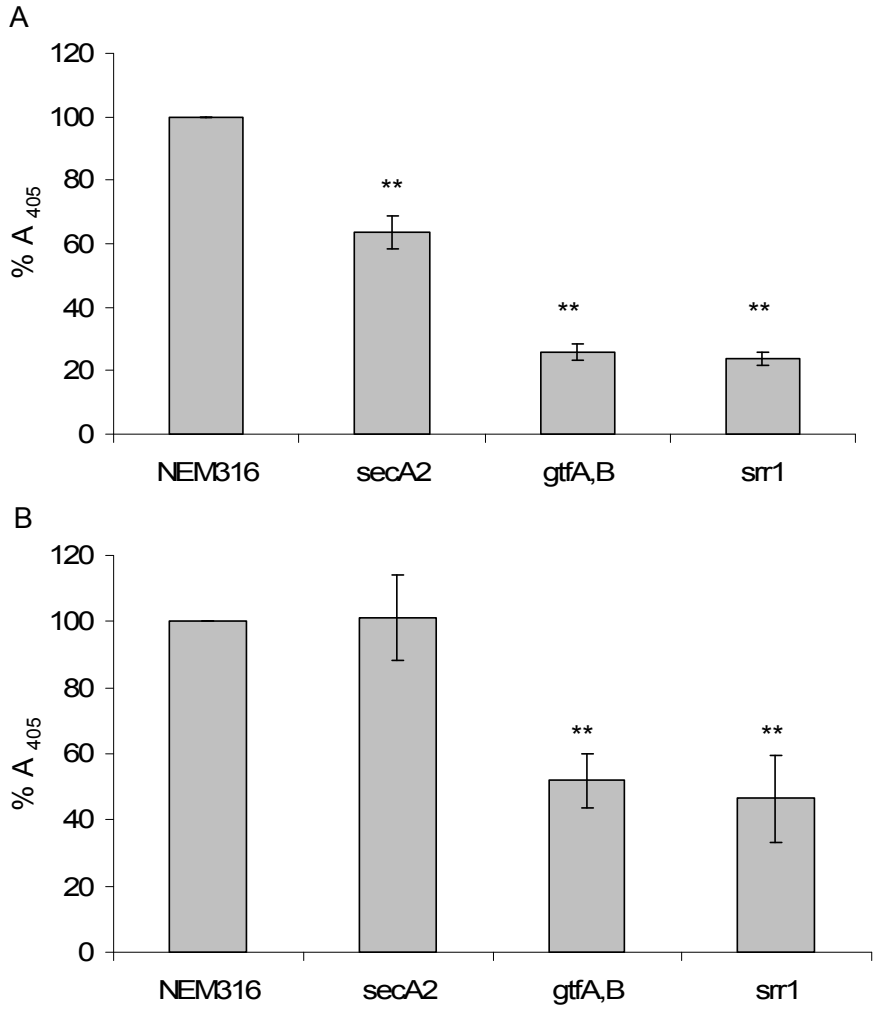

Figure 4.2. Presence of GBS-PGK on the surface and attachment of rGBS-PGK to the surface of GBS strains NEM316, NEM316Δ*secA2*, NEM316Δ*gtfA,B* and NEM316Δ*srr1*. A) Cultures were grown overnight in Todd Hewitt broth, washed and fixed to a 96 well plate. Wells were probed with anti-rGBS-PGK followed by anti-rabbit-alkaline phosphatase conjugate antibodies. B) Cultures were grown overnight in TH broth supplemented with 2 μg/ml rGBS-PGK, washed and fixed to a 96 well plate. Wells were probed with anti-poly-histidine followed by anti-mouse-alkaline phosphatase conjugate antibodies. Wells were developed for with 4-nitrophenol phosphate and the A_{405} was measured. A_{405} readings from the various mutant NEM316 strains were compared to the parent NEM316 strain to determine the $\%A_{405}$. Data points represent the average %A405 value obtained from experiments performed triplicate in triplicate; error bars represent one standard deviation. ** indicates statistical significance ($p<0.01$).

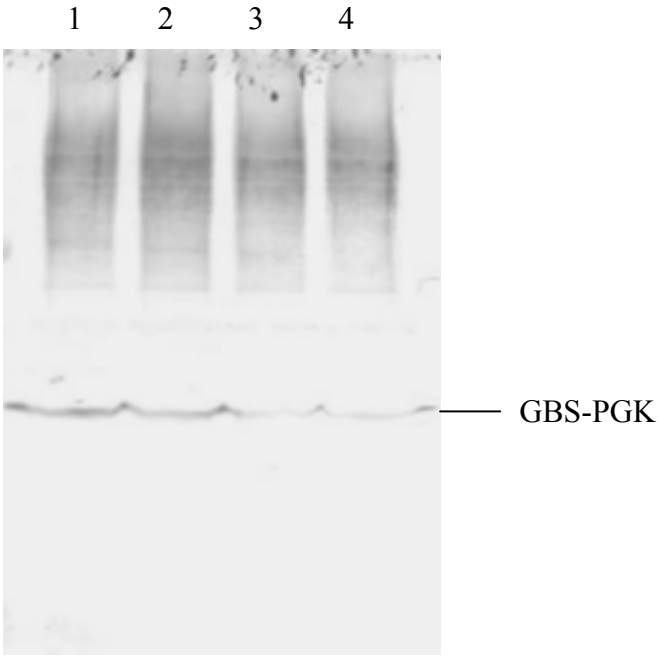

Figure 4.3. Role of SecA2 in secretion of PGK by GBS. NEM316 (1), NEM316Δ*secA2* (2), NEM316Δ*srr1* (3) and NEM316Δ*gtfA,B* (4) were grown overnight in TH broth. Cultures were centrifuged and the supernatant was filter sterilized, concentrated 10× and separated via 10% SDS-PAGE. Separated proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-rGBS-PGK followed by anti-rabbit alkaline phosphatase conjugate antibodies. Membrane was developed using SigmaFast BCIP/NBT. Figure is representative of the experiment performed in triplicate.

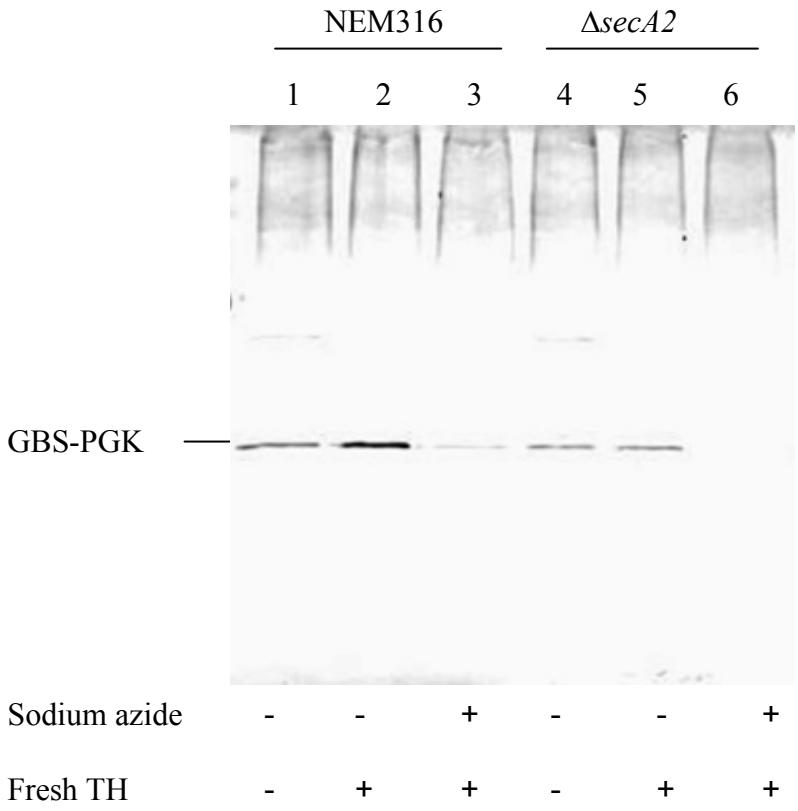

Figure 4.4. Effect of sodium azide on secretion of GBS-PGK. NEM316 (1-3) and NEM316ΔsecA2 (4-6) were grown overnight, centrifuged and resuspended in fresh TH broth (2 and 5) or fresh TH with 50 mM sodium azide (3 and 6). Cells were centrifuged and the supernatants were filter sterilized, concentrated 10× and separated via 10% SDS-PAGE along with supernatant proteins from overnight cultures (1 and 4). Separated proteins were electrophoretically transferred to a nitrocellulose membrane. Membrane was probed with anti-rGBS-PGK followed by anti-rabbit alkaline phosphatase conjugate antibodies. Membrane was developed using Sigma Fast BCIP/NBT. Figure is representative of the experiment performed in triplicate

4.5. References:

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Chapter 5.

Identification of the Actin and Plasminogen Binding Regions of Group B Streptococcal Phosphoglycerate Kinase.

A version of this chapter is in preparation for submission to the Journal of Biological Chemistry.

5.1 Introduction:

Group B streptococcus (GBS) is the leading bacterial cause of neonatal pneumonia, sepsis and meningitis in North America (1-3). In addition, GBS is a major cause of disease in the adult population (4-6). Surface expressed and secreted components are crucial for GBS virulence (7) as they mediate adherence to host cells (8-14), crossing of host barrier tissues (15-17) and immune evasion (18). Glycolytic enzymes, in addition to being present in the cytoplasm, have been identified as surface expressed and secreted proteins of gram positive bacterial pathogens (19-22) and are believed to contribute to virulence (22, 23). Two well studied examples of these are α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both of which interact with numerous host proteins (24-36). In contrast to α-enolase and GAPDH, the function of phosphoglycerate kinase (PGK) on the GBS surface (20, 37) has not been determined. PGK from GBS, and other sources, has been demonstrated to bind actin (31, 38) and plasminogen (38-40), indicating that it may play a role in GBS virulence.

Plasminogen is a 92 kDa protein circulating in the plasma at a concentration of approximately 180 μg/ml (41). Recruitment of plasminogen to the bacterial surface is a virulence strategy used by many pathogenic bacteria (41), which contributes to colonization (42, 43) and bacterial dissemination (41). Activation of plasminogen recruited to the GBS surface occurs via the host plasminogen activators urokinase (uPA) or tissue type plasminogen activator

(tPA) and allows the bacterium to dissolve fibrin clots and degrade extracellular matrix proteins contributing to bacterial dissemination (26, 41, 44-46). I have previously demonstrated that GBS-PGK binds plasminogen (38). I hypothesize that PGK expressed on the surface of GBS (20, 37, 38) may be involved in recruiting plasminogen to the GBS surface.

GBS-PGK has also been demonstrated to bind the eukaryotic cytoskeleton protein actin (38). While the interaction between GBS-PGK and actin has not been established as a virulence characteristic, I hypothesize that surface expressed GBS-PGK may contribute to adhesion as actin has been identified on the surface of a number of eukaryotic cells including: lymphocytes, monocytes, endothelial cells and L cells (47-52). Also, as a secreted GBS protein (38), PGK may gain access to the cytoplasm of eukaryotic cells through the pore forming β-hemolysin (53-56) to interact directly with the actin cytoskeleton. Transfection of HeLa cells with a plasmid carrying the phosphoglycerate kinase gene has previously been demonstrated to result in disruption of the actin cytoskeleton (57). Based on these observations, I hypothesize that GBS-PGK gaining access to the host cell cytoplasm through the β-hemolysin may cause localized disruptions of the actin cytoskeleton. If these disruptions occur following GBS adherence to the cell surface, they may contribute to GBS internalization. In addition, PGK gaining access to the host cell cytoplasm following internalization may cause actin cytoskeleton disruptions that impair intracellular trafficking and contribute to intracellular survival.

The extracellular location of GBS-PGK, along with its actin and plasminogen binding ability, suggests that GBS-PGK may contribute to GBS virulence. Similar to α-enolase and GAPDH from other streptococcal species, confirming the role of PGK in GBS virulence has been hampered by its role in glycolysis. Due to an essential role in metabolism, traditional knock-out mutagenesis is not possible. Site directed mutagenesis, creating a glycolytically active enzyme that has lost the ability to bind host proteins, has previously been used to demonstrate a role for α-enolase and GAPDH in virulence of other streptococcal species (26, 58). A similar approach may be necessary to determine the role of actin and plasminogen binding by surface expressed GBS-PGK in GBS virulence. The objective of this chapter was to identify point mutations within the *gbs-pgk* gene that abolish GBS-PGK binding to actin and plasminogen without affecting the glycolytic activity.

5.2 Materials and Methods:

5.2.1 Production and Purification of full length and truncated rGBS-PGK molecules.

Primers used for generation of PGK and PGK truncations are listed in table 5.1. The full length PGK gene was PCR amplified from NCS13 genomic DNA using primers PGK-*bam*F and PGK-*pst*R. The truncated PGK molecule spanning amino acids 83-303 (TJB 1) was PCR amplified using primers PGK1-*bam*F and PGK3-*hin*R. The truncated PGK molecule spanning amino acids 83-165 (TJB 2) was PCR amplified using primers PGK1-*bam*F and PGK4-*hin*R. The truncated PGK molecule spanning amino acids 166-303 (TJB 3) was PCR amplified using primers PGK2-*bam*F and PGK3-*hin*R. The truncated PGK molecule spanning amino acids 1-165 (TJB 4) was PCR amplified using primers PGK-*bam*F and PGK4-*hin*R. The truncated PGK molecule spanning amino acids 166-398 (TJB 5) was PCR amplified using primers PGK2-*bam*F and PGK-*hin*R. The resulting amplicons were ligated into the expression plasmid pQE 30 and transformed into chemically competent *Escherichia coli* M15 carrying the plasmid pREP4. Full length and truncated rGBS-PGK molecules were expressed and purified as previously described (38). Expression of the N-terminal hexahistidyl tagged recombinant GBS-PGK (rGBS-PGK) and PGK truncations were induced by the addition of 2 mM isopropyl β-D-1 thiogalactopyranoside (IPTG). The expressed proteins were purified according to manufacturer's

instructions on a nickel affinity column under native conditions using the Qiaexpressionist system (Qiagen; Mississauga, ON, CA).

5.2.2. Interaction of anti-rGBS-PGK antibodies with rGBS-PGK and truncated rGBS-PGK molecules.

Decreasing concentrations of full length rGBS-PGK along with the truncated rGBS-PGK molecules TJB 1, TJB 2, TJB 3 and TJB 4 (10-0.3125ng/well) were resuspended in 0.1 M sodium carbonate solution (pH 9.5) and immobilized to wells of a 96 well polystyrene plate (Maxi-sorp; NUNC, Thermo Fischer Scientific, Nepean, ON, CA) via overnight incubation at room temperature. Wells were washed 1× 10 min with tris buffered saline (TBS) and incubated for 1 h with blocking buffer (5% BSA, 0.1% tween 20 in TBS) at room temperature. Wells were washed 3× 10 min with TBS and incubated with anti-rGBS-PGK antibodies (1:300 in blocking buffer) (38) for 1 h at room temperature. Wells were washed 3× 10 min with TBS and incubated with anti-rabbit-IgG alkaline phosphatase conjugate antibodies (1:200 in blocking buffer; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Wells were washed 3× 10 min with TBS and developed for 30 min at room temperature with 100µl 4-nitrophenol phosphate (Sigma-Aldrich); development was stopped with 25 µl NaOH (3N). The absorbance at 405nm (A_{405}) was measured using an Athos LP400 microplate reader (Bio-Rad Laboratories Ltd. Mississauga, ON, CA).

5.2.3 Interaction of full length and truncated rGBS-PGK with actin and plasminogen.

Actin and plasminogen binding by full length and truncated rGBS-PGK was assayed using ELISA similar to previously described (38). Actin (1 μg/well; Sigma-Aldrich) and plasminogen (0.1 μg/well; Sigma-Aldrich) were resuspended in 0.1 M sodium carbonate solution (pH 9.5) and immobilized to wells of a 96 well polystyrene plate (Maxi-sorp) via 8 h incubation at room temperature. Wells were washed 1× 10 min with TBS and incubated for 16 h with blocking buffer (5% BSA, 0.1% tween 20 in TBS) at room temperature. Wells were washed 3× 10 minutes with TBS and incubated with full length and truncated rGBS-PGK molecules (100 μl; 15 μg/ml) for 1 h at room temperature. Wells were washed 3× 10 min with TBS and incubated with anti-rGBS-PGK antibodies (1:300 in blocking buffer) for 1 h at room temperature. Wells were washed 3× 10 min with TBS and incubated with anti-rabbit-IgG alkaline phosphatase conjugate antibodies (1:200 in blocking buffer) for 1 h at room temperature. Wells were washed 3× 10 min with TBS and developed for 30 min at room temperature with 100 μl 4-nitrophenol phosphate; development was stopped with 25 μl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader.

Experiments were performed in triplicate and the A_{405} measurements were compared to those obtained from standard curves containing decreasing amounts full length or truncated rGBS-PGK to determine the amount of full length and truncated rGBS-PGK molecules remaining in the wells.

5.2.4. Peptide Mapping.

To map the actin and plasminogen binding regions of GBS-PGK, a peptide mapping procedure similar to previously described (27) was used. The PGK region corresponding to amino acids 83-303 (TJB 1) were divided into 21 peptides (1-21), each peptide was 20 amino acids in length and overlapped the previous peptide by 10 amino acids (Peptide 2.0). To more precisely map the binding regions a second round of peptide mapping was performed. The peptides found to bind actin or plasminogen were further divided into 39 peptides (22-60), each peptide was 15 amino acids in length and overlapped the previous peptide by 12 amino acids. All peptides were ordered as crude peptides and their amino acid sequences are listed in table 5.2. Peptides were fixed to a nitrocellulose membrane (15 μg/spot) using the biodot apparatus (BioRad Laboratories Ltd.). The membrane was washed with TBS (15 min), incubated 30 min with blocking buffer (5% BSA, 0.1% tween 20 in TBS), washed with TBS (15 min) and incubated with either actin or plasminogen (20 μg/ml in blocking buffer) for 16 h at 4°C. As a negative control, duplicate membranes containing peptides were incubated 16 h at 4°C with blocking buffer. The membrane was washed 15 min with TBS and incubated with either anti-actin (Clone C4; Millipore, Billerica, MA, USA) or anti-plasminogen (Clone 3E6; Sigma-Aldrich) antibodies (1:1000 in blocking buffer) for 2 h at 4°C. The membrane was washed 3× 15 min with TBST (0.1% tween 20 in TBS), 2× 15 min with TBS before incubating with anti-

mouse-IgG alkaline phosphatase conjugate (1:10 000 in blocking buffer; Sigma-Aldrich) for 1 h at room temperature. Membranes were washed 3× 15 min with TBST and 2× 15 min with TBS before developing with SigmaFast BCIP/NBT (Sigma-Aldrich) for 5 min. Membrane development was stopped with three changes of distilled water.

5.2.5. Modeling GBS-PGK and visualization of the actin and plasminogen binding domains.

To predict the protein structure, the amino acid sequence of GBS-PGK was submitted to the I-TASSER site (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) as previously described (59-61). The predicted model structure of GBS-PGK was viewed using the RasMol program and regions of GBS-PGK identified to bind actin or plasminogen in the dot blot were highlighted to determine their locations within the GBS-PGK molecule.

5.2.6. Site directed mutagenesis.

The *pgk* gene was PCR amplified from NCS13 genomic DNA using the primers PGK-*pst*F and PGK-*hin*R, cloned into the plasmid pUC19 and transformed into chemically competent *E. coli* DH5α. The plasmid was recovered using the Qiaspin miniprep kit (Qiagen) and subjected to mutagenesis using the GeneTailor Site-Directed Mutagenesis System (Invitrogen; Carlsbad,

CA, USA) following manufacturers instructions. The plasmid was first methylated for 1 hour at 37°C, followed mutagenesis through PCR using the oligonucleotides primers listed in table 1. Amplification was carried out using the following cycles: 94°C for 2 minutes; 20 cycles of 94°C 30sec, 55°C 30sec, 68° 4min; 68°C 10 minutes. Following the mutagenesis reaction, the plasmid was transformed into One-Shot® MAX Efficiency® DH5α^{TM}-T1^{R} (Invitrogen). Plasmids containing the mutagenized *pgk* gene were recovered using the Qiaspin miniprep kit (Qiagen) and the *pgk* gene was sequenced to confirm the presence of the desired mutation. The mutant *pgk* genes were cloned into the expression plasmid pQE-30 using the primers PGK-*bam*F and PGK-*pst*R. To confirm the presence of the desired mutation pQE-30 plasmids containing the mutated PGK gene were sequenced using the primers pQE-30-Fseq and pQE-30-Rseq. Mutated rGBS-PGK molecules were produced and purified as N-terminal hexahistidyl recombinant proteins using the Qiaexpressionist system as described above.

5.2.7. Glycolytic activity of mutant and non-mutant rGBS-PGK molecules.

Mutated and non-mutated rGBS-PGK was assayed for enzymatic activity according to the enzymatic assay provided with PGK isolated from bakers yeast (Sigma-Aldrich) (62). Enzymatic activity was determined by adding 2.5 μg to a 3 ml reaction mix containing 50 mM potassium phosphate, 0.83 mM glyceraldehyde-3-phosphate, 0.3 mM β-nicotinamide adenine dinucleotide, 0.2 mM adenosine 5`-diphosphate, 4.2 mM magnesium sulfate, 133 mM glycine and

1 unit glyceraldehyde-3-phosphate dehydrogenase. The absorbance at 340nm (A_{340}) was measured for five min and the change in absorbance (ΔA_{340}) was used to calculate the enzymatic activity using the following formula:

$$\text{Units/mg enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(3)}{(6.22 \times [\text{PGK}])}$$

3 = total volume (in milliliters) of assay

6.22 = millimolar extinction coefficient of β-NADH at 340 nm.

[PGK] = amount of PGK used in milligrams

Commercial PGK (0.06 units; Sigma-Aldrich) isolated from *Saccharomyces cerevisiae* was used as a positive control and potassium phosphate (100 mM) was used as a negative control.

5.2.8. Interaction of anti-rGBS-PGK antibodies with rGBS-PGK and mutated rGBS-PGK molecules.

Decreasing concentrations (10-0 ng/well) of full length rGBS-PGK along with the mutated rGBS-PGK molecules that were found to be glycolytically active (PGK-M1, PGK-M3, PGK-M5, PGK-M6, PGK-M7 and PGK-M8) were resuspended in 0.1 M sodium carbonate solution (pH 9.5) and immobilized to wells of a 96 well polystyrene plate (Maxi-sorp) via overnight incubation at room temperature. Wells were washed 1× 10 min with TBS and incubated for 1 h with blocking buffer (5% BSA, 0.1% tween 20 in TBS) at room temperature. Wells were then washed 3× 10 min with TBS followed by incubated with anti-rGBS-

PGK antibodies (1:300 in blocking buffer) for 1 h at room temperature. Further washing, 3× 10 min with TBS, was performed and the wells were incubated with anti-rabbit-IgG alkaline phosphatase conjugated antibodies (1:200 in blocking buffer) for 1 h at room temperature. Finally, wells were washed 3× 10 min with TBS and developed for 30 min at room temperature with 100 µl 4-nitrophenol phosphate; development was stopped with 25 µl NaOH (3N). The absorbance at A_{405} was measured using an Athos LP400 microplate reader.

5.2.9. Binding of mutant rGBS-PGK to immobilized actin and plasminogen.

Non-mutated rGBS-PGK, along with mutant rGBS-PGK molecules, that were found to be enzymatically active, were assayed for binding to actin and plasminogen using a similar ELISA as described above. Actin (1 µg/well) and plasminogen (0.1 µg/well) were immobilized to wells of a 96 well polystyrene plate (Maxi-sorp); 96 well polystyrene plates with no protein immobilized were used to control for non-specific binding of rGBS-PGK with BSA. Wells were incubated overnight with blocking buffer (5% BSA, 0.1% tween-20 in TBS), washed 1× with TBS and incubated with the rGBS-PGK molecules (15 µg/ml in blocking buffer) for 1 h. As an added control, plates containing either actin or plasminogen were again incubated with blocking buffer to control for non-specific binding of the anti-rGBS-PGK antibodies. Wells were washed 3× with TBS and incubated with anti-rGBS-PGK (1:300 in blocking buffer) for 1 h. Wells were washed 3× with TBS and incubated with anti-rabbit IgG alkaline

phosphatase conjugate antibodies (1:200 in blocking buffer). Wells were washed 3× with TBS and developed for 30 min at room temperature with 100 µl 4-nitrophenol phosphate (Sigma-Aldrich); development was stopped with 25 µl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader.

Experiments were performed triplicate in triplicate and the A_{405} measurements from the control plates were subtracted from the A_{405} measurements from the experimental plates to provide a final A_{405} measurement. These A_{405} measurements were compared to those obtained from standard curves containing decreasing amounts of mutated and non-mutated rGBS-PGK to determine the amount of rGBS-PGK molecules remaining in the wells.

5.2.10. Binding of actin and plasminogen to immobilized rGBS-PGK and mutant rGBS-PGK molecules.

Binding of actin and plasminogen to immobilized rGBS-PGK was also assayed. Non-mutated rGBS-PGK, along with mutant rGBS-PGK molecules that were found to be enzymatically active, were fixed to wells of a 96 well polystyrene plate (0.5, 0.25 and 0.125 µg/well) for eight hours at room temperature and the wells were incubated overnight with blocking buffer (5% BSA, 0.1% tween 20 in TBS). Wells were washed with TBS and incubated with either actin or plasminogen (20 µg/ml or 0 µg/ml in blocking buffer) for 1h. Wells were washed 3× with TBS and incubated with either anti-actin (Millipore; clone C4) or anti-plasminogen (Sigma-Aldrich; clone 3E6) antibodies (1:300 in

blocking buffer). Wells were washed 3× with TBS and incubated with either anti-rabbit IgG alkaline phosphatase conjugate or anti-mouse IgG alkaline phosphatase conjugate antibodies (1:200 in blocking buffer). Wells were washed 3× with TBS and developed for 30 min at room temperature with 100 µl 4-nitrophenol phosphate before stopping the reaction with 25 µl NaOH (3N). The A_{405} was measured using an Athos LP400 microplate reader.

Experiments were performed triplicate in duplicate. To control for non-specific binding of the antibodies to the various rGBS-PGK molecules, the A_{405} measurements from wells incubated with 0 µg/ml actin or plasminogen were subtracted from wells incubated with 20 µg/ml actin or plasminogen.

5.2.11. Statistical analysis.

Statistical analysis was performed for the binding of mutated and non-mutated rGBS-PGK molecules with actin and plasminogen. The data was displayed graphically, the data points represent the average value of all experiments and the error bars represent one standard deviation. Binding data for mutant rGBS-PGK was compared to binding by non-mutated rGBS-PGK using the student's t-test and a p value < 0.05 was considered statistically significant.

5.3. Results:

5.3.1. Interaction of full length and truncated rGBS-PGK with actin and plasminogen.

To identify the actin and plasminogen binding region of GBS-PGK, full length and truncated rGBS-PGK molecules were assayed for binding to actin and plasminogen immobilized to 96 well plates. It was not possible to assess binding of TJB 4 to either actin or plasminogen as the anti-rGBS-PGK antibodies did not bind this truncated form of rGBS-PGK (Fig. 5.1). Also, the truncation TJB 5 was unstable and could not be purified at a high enough concentration to assay binding actin or plasminogen. However, the truncation TJB 1 was found to bind similar amounts of actin and plasminogen compared to the full length rGBS-PGK molecule (Fig. 5.2). No quantifiable binding to either actin or plasminogen was detected for TJB 2 or TJB 3. These results suggest that the major actin and plasminogen domain of GBS-PGK is located between amino acids 83-303 and involves amino acids on either side of amino acids 165-166.

5.3.2. Peptide mapping.

The middle region of PGK (TJB 1) was further assayed for binding to both actin and plasminogen using dot blots of peptides spanning this region. In the initial round of peptide mapping, actin and plasminogen were found to bind

similar regions of GBS-PGK. Dark spots corresponding to actin binding peptides 8, 13 and 14 along with faint spots corresponding to actin binding peptides 4, 5, 12 and 15 were observed (Fig. 5.3A). Dark spots corresponding to plasminogen binding peptides 4, 5, 8, 13 and 14 along with a faint spot corresponding to plasminogen binding to peptide 18 were observed (Fig. 5.4A). These peptides were further targeted in a second round of peptide mapping. In the second round of peptide mapping, dark spots corresponding to actin binding to peptides 32, 33, 39, 45, 47, 48 and 49 was observed, while faint spots corresponding to actin binding peptides 34, 38, 50, 57 and 60 could also be observed (Fig. 5.3B). Dark spots corresponding to plasminogen binding peptides 39, 42, 43, 49, 50 and 57 was observed, while faint spots corresponding to plasminogen binding peptides 27, 32, 33, 34, 35, 38, 49 and 50 to plasminogen could also be observed (Fig. 5.4B). These results suggest that the actin binding site of GBS-PGK may involve amino acids 154-165, 194-205, 225-236 and 291-305 while the plasminogen binding site of GBS-PGK may involve amino acids 123-132, 194-205, 207-221, 228-236 and 291-305.

5.3.3. Modeling GBS-PGK and visualization of the actin and plasminogen binding domains.

To better understand their location within the GBS-PGK molecule, the potential actin and plasminogen binding sites of GBS-PGK, as identified by the peptide mapping experiments, were highlighted on the I-TASSER model GBS-

PGK structure (Fig. 5.5). Two of the identified binding sites, amino acids 194-202 and amino acids 225-233, were found to be localized to internal regions of the GBS-PGK molecule; the binding of these peptides to actin and plasminogen is likely due to non-specific hydrophobic interactions and do not represent true binding sites for either actin or plasminogen. Four of the identified binding sites, including amino acids 126-130, 154-165 and 202-205 and 207-221, were found to localize close together within the cleft between the two domains of the GBS-PGK molecule. Finally, the amino acids 291-305 were located on the outer surface of GBS-PGK.

5.3.4. Site directed mutagenesis.

With the exception of amino acids 194-202 and 225-233, all of the identified actin and plasminogen regions of GBS-PGK were targeted for site directed mutagenesis. Since I have previously demonstrated that the interaction between rGBS-PGK and plasminogen could be inhibited by the lysine analogue 6-aminocaproic acid (Fig. 2.2B) (38), lysine residues within the identified potential binding sites were targeted. Within the amino acid sequences identified as potential actin and plasminogen binding regions of GBS-PGK, four lysine rich motifs were identified. The first lysine rich motif was found to span amino acids 126-130 (KKESK), the second motif was found to span amino acids 204-208 (KVSDK), the third motif was found to span amino acids 218-221 (KADK) and the fourth motif was found to span amino acids 302-306 (KSIAK). In addition,

the region spanning amino acids 154-165 contained a positively charged region spanning amino acids 156-159 (HRAH) that was found to localize near the lysine residues 126, 127 and 130. Since these charged residues could potentially enhance binding to actin and plasminogen, they were also targeted for mutation. Because PGK was not identified in a screen identifying plasminogen binding proteins on the surface of *S. pneumoniae* (24), the GBS-PGK protein sequence (63) was compared to the PGK protein from *S. pneumoniae* using BLAST. This comparison revealed that the PGK protein from *S. pneumoniae* contained a glutamic acid to proline mutation at amino acid 133, near the lysine rich motif spanning amino acids 126-130. Based on this observation, the glutamic acid residues at amino acids 133 and 134 were also targeted for mutation.

A total of eight mutant rGBS-PGK molecules, summarized in table 5.3, were generated targeting the four regions of GBS-PGK identified as potential actin and plasminogen binding sites. PGK-M1 contained mutations converting the lysine residues at amino acids 126, 127 and 130 to alanine. PGK-M2 contained mutations converting the lysine residues at amino acids 204 and 208 to alanine. PGK-M3 contained mutations converting the lysine residues at amino acids 218 and 221 to alanine. PGK-M4 contained mutations converting the histidine residues at amino acids 156 and 159 along with the arginine residue at amino acid 157 to alanine. PGK-M5 contained a mutation converting the lysine residue at amino acid 130 to glutamic acid. PGK-M6 contained the glutamic acid to proline mutation at amino acid residue 133 seen in the *S. pneumoniae* PGK molecule. PGK-M7 contained mutations converting the lysine residues at amino

acids 126, 127 and 130 along with the glutamic acid residues at amino acids 133 and 134 to alanine. PGK-M8 contained mutations converting the lysine residues at amino acids 302 and 306 to alanine. Mutant rGBS-PGK molecules were assayed for enzymatic activity. PGK-M2 and PGK-M4 were found to have very little enzymatic activity (27% and 17% respectively; Fig. 5.6). These mutant proteins were not assayed for binding to actin or plasminogen as they would not be usable for future experiments replacing the genomic *pgk* gene. In contrast, PGK-M1, PGK-M3, PGK-M5, PGK-M6, PGK-M7 and PGK-M8 were found to have enzymatic activities of 95%, 69%, 112%, 112%, 101% and 113% respectively compared to non mutated rGBS-PGK (Fig. 5.6). These six mutant rGBS-PGK molecules were assayed for binding to actin and plasminogen.

5.3.5. Binding of rGBS-PGK and mutant rGBS-PGK to immobilized actin and plasminogen.

Binding of the mutant rGBS-PGK molecules to actin and plasminogen that had been fixed to 96 well plates was assayed using ELISA. The mutant PGK molecules had similar binding to the anti-rGBS-PGK antibodies as rGBS-PGK (Fig. 5.7), further supporting the hypothesis that the mutations did not affect the overall folding of the protein. With the exception of PGK-M3, which appeared to have significantly increased ($p<0.01$) binding to both actin and plasminogen, all the mutant PGK molecules demonstrated significantly reduced ($p<0.01$) binding to both actin and plasminogen (Fig. 5.8). The amount of rGBS-PGK, PGK-M1,

PGK-M3, PGK-M5, PGK-M6, PGK-M7 and PGK-M8 retained after incubation in wells containing immobilized actin was found to be 1.86 ng, 0.44 ng, 2.53 ng, 0.45 ng, 0.21 ng, 0.63 ng and 0.12 ng respectively. The amount of rGBS-PGK, PGK-M1, PGK-M3, PGK-M5, PGK-M6, PGK-M7 and PGK-M8 retained after incubation in wells containing immobilized plasminogen was found to be 4.92 ng, 1.42 ng 6.87 ng, 0.98 ng, 0.80 ng, 2.94 ng and 1.11 ng respectively. These results suggest that the actin and plasminogen binding sites of GBS-PGK are similar and involve residues 126-134, 204, 208, 302 and 306.

5.3.6. Binding of actin and plasminogen to immobilized rGBS-PGK and mutant rGBS-PGK molecules.

To further characterize the effect of the six mutations, decreasing amounts of the rGBS-PGK molecules (0.5, 0.25, 0.125 μg) were fixed to wells of a 96 well plate and assayed for binding of both actin and plasminogen. At all three concentrations, all the mutant rGBS-PGK molecules demonstrated significantly reduced ($p<0.05$) binding to both actin and plasminogen (Fig. 5.9 and 5.10). Actin binding by PGK-M1, PGK-M5 and PGK-M6 were found to be reduced by 75%, as 0.5 μg of these constructs were found to retain similar amounts of actin as 0.125 μg rGBS-PGK. Actin binding by PGK-M3, PGK-M7 and PGK-M8 were found to be reduced by 50%, as 0.25 μg of these constructs retained similar amounts of actin as 0.125 μg rGBS-PGK and 0.5 μg retained similar amounts of actin as 0.25 μg rGBS-PGK.

Plasminogen binding by PGK-M5 was found to be reduced by 75% as 0.5 μg PGK-M5 retained similar amounts of plasminogen as rGBS-PGK. Plasminogen binding by PGK-M1, PGK-M3, PGK-M6 and PGK-M8 was found to be reduced by 50-75% as

0.5 μg of these constructs retained significantly less ($p<0.05$) plasminogen than 0.25 μg rGBS-PGK but significantly more ($p<0.05$) than 0.125 μg rGBS-PGK. Finally, plasminogen binding by PGK-M7 was found to be reduced by slightly less than 50% as 0.25 μg PGK-M7 bound significantly more ($p<0.05$) plasminogen than 0.125 μg rGBS-PGK but 0.5 μg PGK-M7 bound similar amounts of plasminogen as 0.25 μg rGBS-PGK.

5.4. Discussion:

Using truncated rGBS-PGK molecules, I determined that the actin and plasminogen binding ability of rGBS-PGK appeared to be located between amino acids 88-303 (Fig. 5.2). Peptide mapping of this middle region of GBS-PGK identified four lysine rich motifs that may mediate binding to actin and plasminogen. In addition, I identified a positively charged region near the lysine rich motif spanning amino acids 126-130 that may enhance binding to actin and plasminogen. A similar technique has previously been utilized to identify the plasminogen binding region of α-enolase from *S. pneumoniae* (27). Site directed mutagenesis revealed that mutation of the lysine residues 204 and 208 resulted in loss of enzymatic activity. Site directed mutagenesis of the histidine residues 156 and 159, along with the arginine residue 157, also resulted in loss of enzymatic acitivity. However, site directed mutagenesis of the other three lysine rich motifs (126-130, 218-221 and 302-306) resulted in the generation of enzymatically active rGBS-PGK molecules with altered binding to both actin and plasminogen.

Two mutant rGBS-PGK molecules, PGK-M1 and PGK-M5, were generated specifically targeting the lysine residues located within the lysine rich motif spanning amino acids 126-130. Both of these mutations were found to have significantly reduced binding to both actin and plasminogen (Fig. 5.8-5.10). While GBS-PGK has been identified as a surface expressed plasminogen binding protein (38), PGK was not identified in a previous screen to identify plasminogen binding proteins on the surface of *Streptococcus pneumoniae* (24). Comparison

of the amino acid sequences of GBS-PGK and PGK from *S. pneumoniae* revealed a glutamic acid to proline difference at amino acid 133 of PGK from *S. pneumoniae*. This glutamic acid residue was found to be located adjacent to the identified lysine rich motif spanning amino acids 126-130 of the model GBS-PGK structure (Fig 5.11). PGK-M6, representing the PGK protein from *S. pneumoniae*, was found to have significantly reduced binding to both actin and plasminogen. These results demonstrate that the glutamic acid residue at amino acid 133 is also important for binding to both actin and plasminogen and provide evidence that PGK expressed on the surface of other streptococcal species may not retain the ability to bind actin and plasminogen. Finally, a fourth mutant rGBS-PGK molecule, PGK-M7, was generated converting the lysine residues 126, 127 and 130 along with the glutamic acid residues 133 and 134 to alanine. While PGK-M7 was found to have significantly reduced binding to both actin and plasminogen compared to non-mutated rGBS-PGK, PGK-M7 binding to both actin and plasminogen was higher than PGK-M1. These results demonstrate that the overall charge of this region may be important for binding to actin and plasminogen as conversion of the glutamic acid residues to alanine partially restored the binding lost by converting the lysine residues to alanine.

A second actin and plasminogen binding site was also identified spanning amino acid residues 302-306 of GBS-PGK. PGK-M8, containing mutations converting the lysine residues 302 and 306 to alanine, demonstrated significantly reduced binding to both actin and plasminogen (Fig. 5.8-5.10). Similar to the lysine rich motif spanning amino acids 126-130, the lysine residues 302 and 306

were found to localize near two glutamic acid residues (amino acids 286 and 288). This suggests that the actin and plasminogen binding sites of GBS-PGK may involve motifs involving lysine residues that localize near glutamic acid residues. The requirement for negatively charged residues in plasminogen binding sites has previously been demonstrated for α-enolase from *S. pneumoniae* (27). Conversion of the first aspartic acid residue of the internal plasminogen binding site of α-enolase (FYDKERKVYD) to alanine was found to abolish binding to plasminogen (27).

I also identified a third potential actin and plasminogen site involving the lysine residues located at amino acids 218 and 221. These residues were targeted for mutagenesis to generate PGK-M3. While more PGK-M3 appeared to be retained in wells containing immobilized actin or plasminogen compared to rGBS-PGK (Fig. 5.8), twice as much immobilized PGK-M3 was required to retain the same amount of actin or plasminogen compared to rGBS-PGK (Fig. 5.9 and 5.10). One potential explanation for these results is that these lysine residues compose a minor binding site for actin and plasminogen. At high rGBS-PGK concentrations (ELISA assays containing immobilized actin or plasminogen), this minor binding site may compete with the major binding site for binding to actin and plasminogen. At low rGBS-PGK concentrations (ELISA assays containing immobilized rGBS-PGK), both binding sites may retain actin or plasminogen. A second potential explanation for these results is that substitution of these lysine residues with alanine resulted in structural changes to the rGBS-PGK molecule that affected both the ability of the protein to adhere to the 96 well plates and the

reactivity with the anti-rGBS-PGK antibodies. While it is difficult to predict the affect these amino acid substitutions would have on the GBS-PGK structure, the reduced enzymatic activity of PGK-M3 suggests that some structural changes may have occurred. Potentially, PGK-M3 did not immobilize to the wells as efficiently as non-mutated rGBS-PGK, but this was compensated for during the generation of the standard curves (Fig. 5.7) by increased reactivity with the anti-rGBS-PGK antibodies. The increased reactivity with the anti-rGBS-PGK antibodies would result in the apparent increased retention of PGK-M3 in wells containing immobilized actin or plasminogen (Fig. 5.8), while the reduced immobilization efficiency would result in reduced retention of actin or plasminogen in wells containing immobilized PGK-M3 (Fig. 5.9 and 5.10).

While I could not definitively determine the role of the lysine residues at amino acids 218 and 221, I have generated rGBS-PGK molecules with significantly reduced binding to both actin and plasminogen by targeting amino acids 126-134 and 302-306. Since these mutant rGBS-PGK molecules retained their glycolytic activity, it should be possible to replace the GBS genomic *pgk* gene with these mutant *pgk* genes to determine the role of actin and plasminogen binding by surface expressed GBS-PGK in GBS virulence. This method has previously been used to demonstrate the role of plasminogen binding by α-enolase from *S. pneumoniae* (26) and uPAR/CD87 binding by GAPDH from group A streptococcus (GAS) (58).

It has previously been determined that PGK expressed on GBS surface (20, 37), may recruit plasminogen to the GBS surface (38). Recruitment of

plasminogen to the bacterial surface is a virulence characteristic used by many pathogenic bacteria (41, 46, 64), including GBS (45, 46). Activation of the recruited plasminogen to plasmin contributes to bacterial dissemination through breakdown of extracellular matrix proteins resulting in intercellular junction cleavage (41, 44, 65). This is likely one of the mechanisms used by GBS to mediate paracellular invasion (66). In addition, recruitment of plasminogen to the bacterial surface has also been demonstrated to contribute to the attachment of GAS (43) and *Streptococcus suis* (67) to eukaryotic cells. GBS is thought to express at least two other potential plasminogen binding proteins, GAPDH and Skizzle, on its surface. Also, α-enolase has been described as a plasminogen binding protein on the surface of other streptococcal species and may contribute to plasminogen recruitment by GBS as well. To date, the contribution of these four proteins in recruiting plasminogen to the GBS surface has not been determined. In this work, I have identified point mutations within the *pgk* gene that result in a loss of plasminogen binding by rGBS-PGK. Using these mutant *pgk* genes, it should be possible to determine the role of surface expressed GBS-PGK in recruitment of plasminogen to the GBS surface. The observation that PGK-M6 bound significantly less plasminogen than rGBS-PGK suggests that while PGK may be involved in recruiting plasminogen to the GBS surface, PGK from other streptococcal species may not be capable of binding plasminogen.

In addition to plasminogen, rGBS-PGK has also been demonstrated to bind actin (38). While recruitment of plasminogen to the bacterial surface is a well established virulence characteristic, a role for actin binding by bacterial

surface proteins has not been established. However, binding to actin may play a role in adhesion to eukaryotic cells as actin has been identified on the surface of a number of eukaryotic cells (47, 48, 50, 51). Based on the localization of actin on the surface of some human cells, I hypothesize that actin binding by surface expressed GBS-PGK could contribute to attachment to these cells. Also, since GBS expresses a number of pore forming toxins (53-55), PGK secreted by GBS (38) may gain access to the host cell cytoplasm to interact with the actin cytoskeleton (56). Expression of GBS-PGK in the cytoplasm of eukaryotic cells has previously been demonstrated to cause disruptions of the actin cytoskeleton (57). I hypothesize that PGK secreted by GBS may gain access to the host cell cytoplasm through pore forming toxins, causing a localized disruption of the actin cytoskeleton, which may facilitate internalization or intracellular survival. I have generated mutations within the *pgk* gene that significantly reduces actin binding by rGBS-PGK (Fig. 5.8 and 5.9). Using these mutant *pgk* genes it should be possible to determine if actin binding by surface expressed GBS-PGK contributes to GBS virulence. Similar to plasminogen binding by GBS-PGK, PGK-M6 was found to have significantly reduced binding to actin. This result suggests that while surface expressed GBS-PGK may contribute to virulence through binding actin, PGK from other streptococcal species may not be capable of binding actin.

In conclusion, I have identified point mutations within the GBS *pgk* gene that significantly reduces binding to both actin and plasminogen by rGBS-PGK. The locations of these mutations were mapped to the model GBS-PGK protein (Fig. 5.11). Replacement of the genomic copy of GBS *pgk* gene with these

mutant *pgk* genes should result in a GBS strain expressing a PGK protein that is not capable of binding either actin or plasminogen. This mutant GBS strain could then be assayed for virulence using established methods to assay adhesion, internalization, intracellular survival, paracellular invasion, survival within human serum and in a mouse model to determine the contribution of actin and plasminogen binding by surface expressed GBS-PGK in to GBS virulence.

Table 5.1. Oligonucleotide primers used in this chapter.

Primer name	Primer sequence
PGK-bamF	TTCGGATCCGCTAAATTGACT
PGK-pstR	TTCCTGCAGTTATTTTCAGTCAATGC
PGK-pstF	CGCCTGCAGATGGCTAAATTGACTGTTAAAGACGTT
PGK-hinR	TTCAAGCTTTTTTCAGTCAATGCTGCCAAACC
PGK1-bamF	TTCGGATCCGCTAAACTTGGTCAAGATGTTG
PGK2-bamF	TTCGGATCCTCAGCAAACGTTGAAAAAGCT
PGK3-hinR	TTCAAGCTTTGATTTAGGACCGATGTCAAG
PGK4-hinR	TTCAAGCTTAATACCTACGTTTGATGCATG
PGK-M1	TTGAAGATGTTGACGGTGCGGCAGAATCTGCGAATGACGAAG AACTTGGTAAATACTGGG
PGK-M1ol	ACCGTCAACATCTTCAAAACGAGTGTTTTCAACC
PGK-M2	CTATTCTTGGTGGCTCAGCAGCTTCTGATGCGATTGGTGTTATCG
PGK-M2ol	TGAGCCACCAAGAATAGCTACGAATGG
PGK-M3	GGTGTTATCGAAAACCTTCTTGAAGCAGCTGATGCAGTTCTTATCGG TGGTGG
PGK-M3ol	AGAAGGTTTTCGATAACACCAATCTTA
PGK-M4	GATGCATTTGGTACAGCAGCCGCTGCTGCCGCATCAAACG
PGK-M4ol	TGCTGTACCAAATGCATCGTTAACGAAGATTCC
PGK-M5	TTGAAGATGTTGACGGTAAGAAAGAATCTGAGAATGACG AAGAACTTGGTAAATACTGGG
PGK-M5ol	ACCGTCAACATCTTCAAAACGAGTGTTTTCAACC
PGK-M6	GAATCTAAGAATGACCCAGAACTTGGTAAATACTGG
PGK-M6ol	GTCATTCTTAGATTCTTTCTTACCG
PGK-M7	TTGAAGATGTTGACGGTGCGGCAGAATCTGCGAATGACG CAGCACTTGGTAAATACTGGG
PGK-M7ol	ACCGTCAACATCTTCAAAACGAGTGTTTTCAACC
PGK-M8	GGTCTTGACATCGGTCCTGCATCAATCGCTGCATTTGATGCAGCACT TACTGG
PGK-M8ol	AGGACCGATGTCAAGACCAAGGAACCCTTC
pQE-30-Fseq	CGGATAACAATTTCACGAG
pQE-30-Rseq	GTTCTGAGGTCATTACTGG

Table 5.2 Peptides used in this chapter. Bold indicates binding to either actin or plasminogen.

Peptide	Sequence	Peptide	Sequence
PGK 1	aklgqdvvfpgvtrgaklee	PGK 31	vndafgtahrahasn
PGK 2	gvtrgakleeainaledgqv	PGK 32	**afgtahrahasnvgi**
PGK 3	ainaledgqvllventrfed	PGK 33	**tahrahasnvgisan**
PGK 4	**llventrfedvdgkkesknd**	PGK 34	**rahasnvgisanvek**
PGK 5	**vdgkkeskndeelgkywasl**	PGK 35	**asnvgisanvekava**
PGK 6	eelgkywaslgdgifvndaf	PGK 36	vgisanvekavagfl
PGK 7	gdgifvndafgtahrahasn	PGK 37	ailggerpfvailgg
PGK 8	**gtahrahasnvgisanveka**	PGK 38	**ggerpfvailggskv**
PGK 9	vgisanvekavagfllenei	PGK 39	**rpfvailggskvsdk**
PGK 10	vagflleneiayiqeavetp	PGK 40	vailggskvsdkigv
PGK 11	ayiqeavetperpfvailgg	PGK 41	lggskvsdkigvenl
PGK 12	**erpfvailggskvsdkigvi**	PGK 42	**skvsdkigvenllek**
PGK 13	**skvsdkigvienllekadkv**	PGK 43	**sdkigvenllekadk**
PGK 14	**enllekadkvligggmtytf**	PGK 44	igvenllekadkvli
PGK 15	**ligggmtytfykaqgieign**	PGK 45	**enllekadkvliggg**
PGK 16	ykaqgieignslveedkldv	PGK 46	lekadkvligggmty
PGK 17	slveedkldvakdlleksng	PGK 47	**adkvligggmtytfy**
PGK 18	**akdlleksngklilpvdske**	PGK 48	**vligggmtytfykaq**
PGK 19	klilpvdskeanafagytev	PGK 49	**gggmtytfykaqgie**
PGK 20	anafagytevrdtegeavse	PGK 50	**mtytfykaqgieign**
PGK 21	dtegeavsegflgldigpks	PGK 51	tfykaqgieignslv
PGK 22	edgqvllventrfed	PGK 52	kaqgieignslveed
PGK 23	qvLlventrfedvdg	PGK 53	ytevrdtegeavseg
PGK 24	lventrfedvdgkke	PGK 54	vrdtegeavsegflg
PGK 25	ntrfedvdgkkeskn	PGK 55	tegeavsegflgldi
PGK 26	fedvdgkkeskndee	PGK 56	eavsegflgldigpk
PGK 27	**vdgkkeskndeelgk**	PGK 57	**segflgldigpksia**
PGK 28	kkeskndeelgkywa	PGK 58	flgldigpksiakfd
PGK 29	skndeelgkywaslg	PGK 59	ldigpksiakfdeal
PGK 30	deelgkywaslgdgi	PGK 60	**gpksiakfdealtga**

Table 5.3. Summary of mutant rGBS-PGK molecules

Mutant number	Amino acid # and change	PGK enzyme activity
PGK-M1	126 K to A 127 K to A 130 K to A	95%
PGK-M2	204 K to A 208 K to A	27%
PGK-M3	218 K to A 221 K to A	69%
PGK-M4	156 H to A 157 R to A 159 H to A	17%
PGK-M5	130 K to E	112%
PGK-M6	133 E to P	112%
PGK-M7	126 K to A 127 K to A 130 K to A 133 E to A 134 E to A	101%
PGK-M8	302 K to A 306 K to A	113%

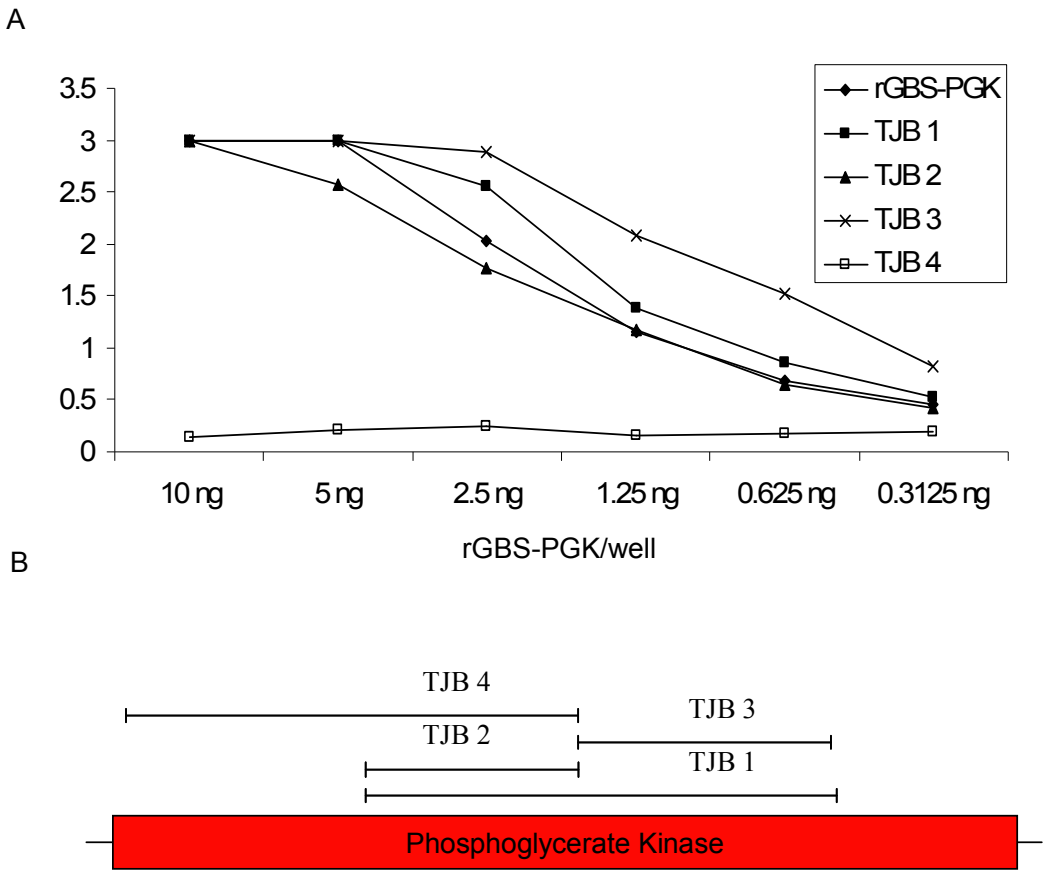


Figure 5.1. Reactivity of rGBS-PGK and truncations with anti-rGBS-PGK antibodies. A) decreasing concentrations of rGBS-PGK along with truncations TJB 1, TJB 2, TJB 3 and TJB 4 were fixed to wells of a 96 well plate. Wells were probed with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed with 4-nitrophenol phosphate and the A_{405} was measured. Data points represent the average value of experiments performed in duplicate. B) Location of the various truncated rGBS-PGK molecules within the PGK protein.

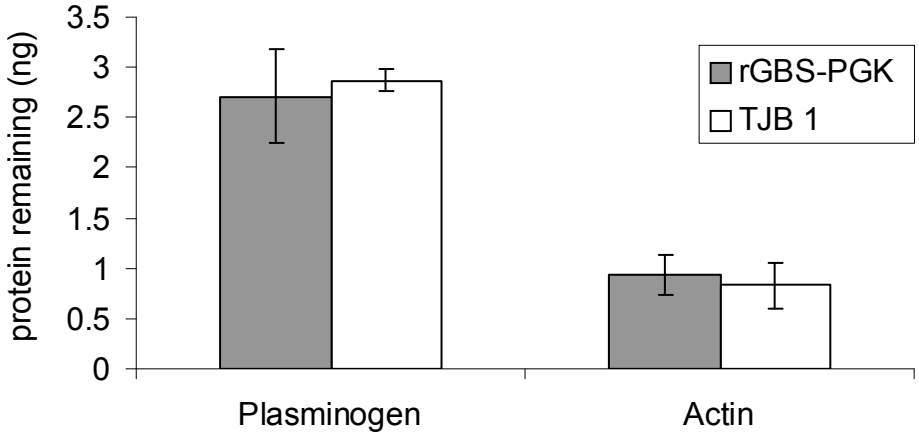


Figure 5.2. Binding of rGBS-PGK and TJB 1 to actin and plasminogen. Actin (1 μg/well) and plasminogen (0.1 μg/well) were fixed to wells of a 96 well polystyrene plate and blocked overnight with 5% BSA, 0.1% tween 20 in TBS. Wells were incubated with 15 μg/ml rGBS-PGK or TJB 1 for 1 h. Wells were probed with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed with 4-nitrophenol and the A_{405} was measured. A_{405} measurements were compared to standard curves to determine the amount of rGBS-PGK or TJB 1 remaining in the wells. Data points represent the average value from experiments performed triplicate in triplicate; error bars represent one standard deviation.

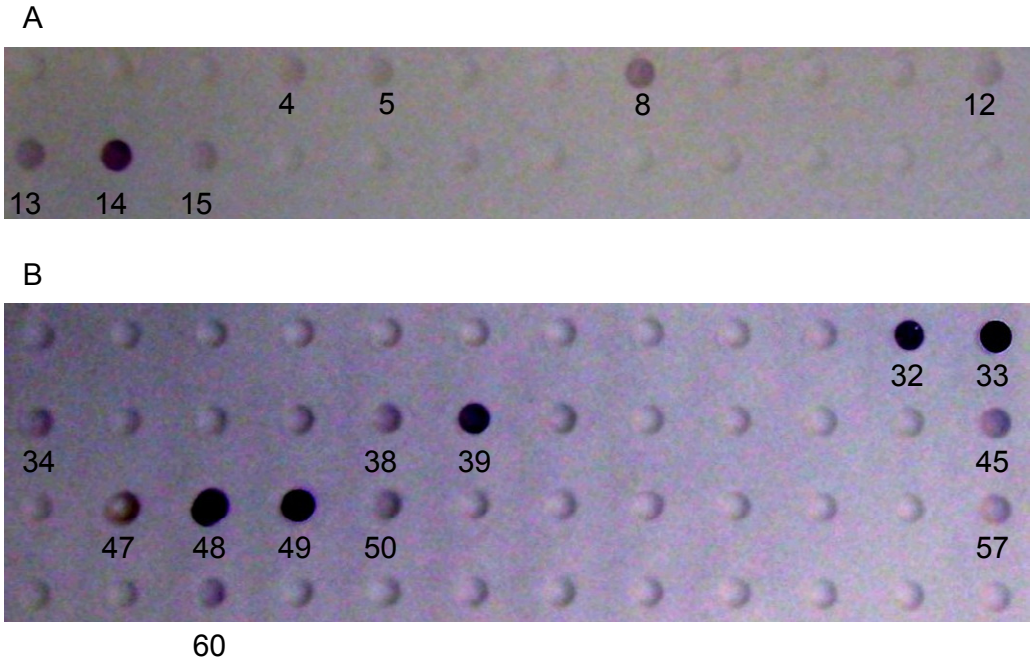


Figure 5.3. Binding of actin to peptides generated based on the amino acid sequence of GBS-PGK. (A) peptides 20 amino acids in length overlapping by 10 amino acids followed by (B) peptides 15 amino acids in length overlapping by 12 amino acids were ordered from Peptide 2.0. Peptides were resuspended in TBS and 15 μg/spot were fixed to a nitrocellulose membrane. The membrane was incubated with actin (20 μg/ml) and probed with anti-actin followed by anti-mouse alkaline phosphatase conjugate antibodies. Membrane was developed for 5 minutes with BCIP/NBT. Figure shown is representative of the experiment performed in triplicate.

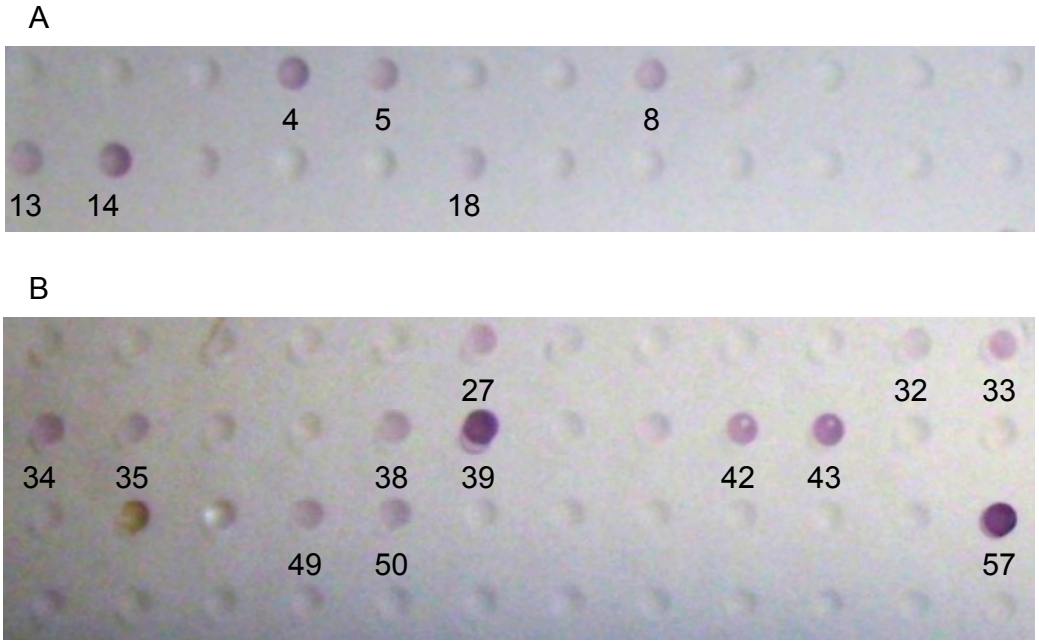
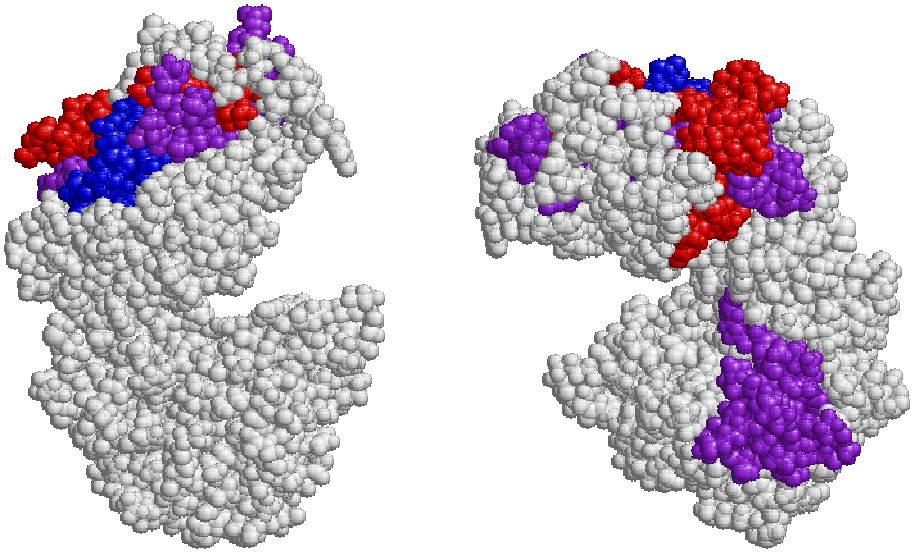


Figure 5.4. Binding of plasminogen to peptides generated based on the amino acid sequence of GBS-PGK. (A) peptides 20 amino acids in length overlapping by 10 amino acids followed by (B) peptides 15 amino acids in length overlapping by 12 amino acids were ordered from Peptide 2.0. Peptides were resuspended in TBS and 15 μg/spot were fixed to a nitrocellulose membrane. The membrane was incubated with plasminogen (20 μg/ml) and probed with anti-plasminogen followed by anti-mouse alkaline phosphatase conjugate antibodies. Membrane was developed for 5 minutes with BCIP/NBT. Figure shown is representative of the experiment performed in triplicate.

A



B

MAKLTVKDVDLKGKKVLVRVDFNVPLKDGVITNDNRITAALPTIKYIIEQGGRAILFSHLGR
VKEEADKEGKSLAPVAADLAAKLGQDVVFPGVTRGAKLEEAINALEDGQVLLVENTRFED**VD**
GKKESKNDEELGKYWASLGDGIFVNDAFGTAH**RAHASNVGI**SANVEKAVAGFLLENEIAYIQ
EAVETPERPFVAILGGSKV**SDKIGVIENLLEKADK**VLIGGG**MTYTFY**KAQGIEIGNSLVEED
KLDV**AKDLLEKSNGKLILPVDSKE**ANAFAGYTEVRDTEGEAV**SEGFLGLDIGPKSIA**KFDEA
LTGAKTVVWNGPMGVFENPDFQAGTIGVMDAIVKQPGVKSIIGGGDSAAAAINLGRADKFSW
ISTGGGASMELLEGKVLPGLAALTEK

Figure 5.5. Mapping of peptides, which bound actin or plasminogen, onto the model GBS-PGK structure. A) The amino acid sequence of GBS-PGK was submitted to iTASSER to generate a model structure. The model structure of GBS-PGK was visualized using RasMol. The regions of GBS-PGK identified to bind actin or plasminogen through the peptide mapping experiment are highlighted on the model GBS-PGK molecule. Amino acids highlighted in red indicate peptides that bound plasminogen. Amino acids highlighted in blue indicate peptides that bound actin. Amino acids highlighted in purple indicate peptides that bound both actin and plasminogen. B) Amino acids identified to bind actin or plasminogen were highlighted on the amino acid sequence of GBS-PGK. Bold residues indicate peptides that were found to bind plasminogen, while underlined residues represent peptides that were found to bind actin.

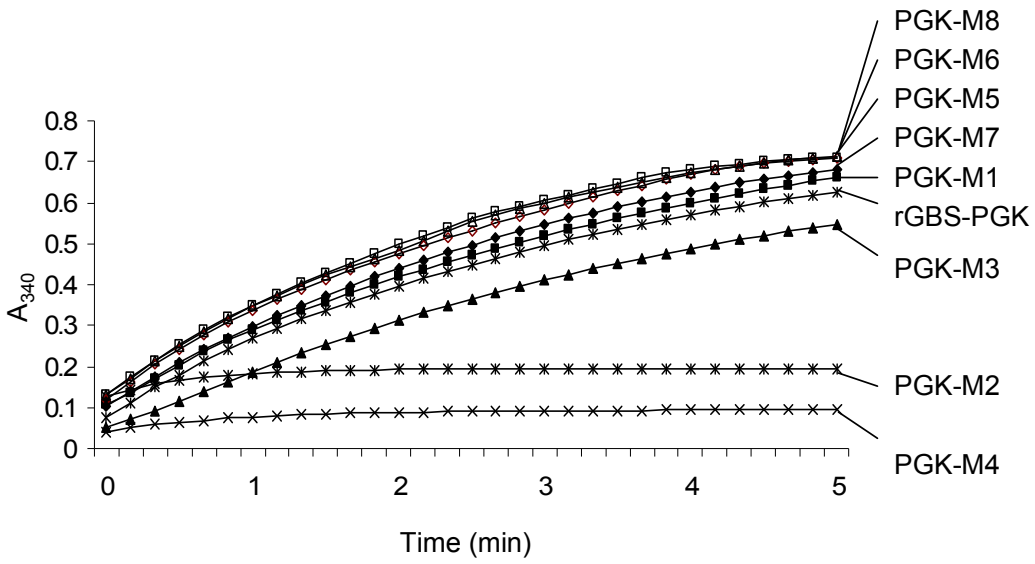


Figure 5.6. Enzymatic activities of wildtype and mutant rGBS-PGK. Wildtype and mutant rGBS-PGK was produced and purified under native conditions using the Qiagen Qiaexpressionest system. Purified proteins (2.5 μg) were added to 3 ml reaction mixtures containing 50 mM potassium phosphate, 0.83 mM glyceraldehyde-3-phosphate, 0.3 mM β-nicotinamide adenine dinucleotide, 0.2 mM adenosine 5'-diphosphate, 4.2 mM magnesium sulfate, 133 mM glycine and 1 unit glyceraldehyde-3-phosphate dehydrogenase. The absorbance at 340 nm (A_{340}) was measured for five min. Data points are representative of experiments performed in duplicate

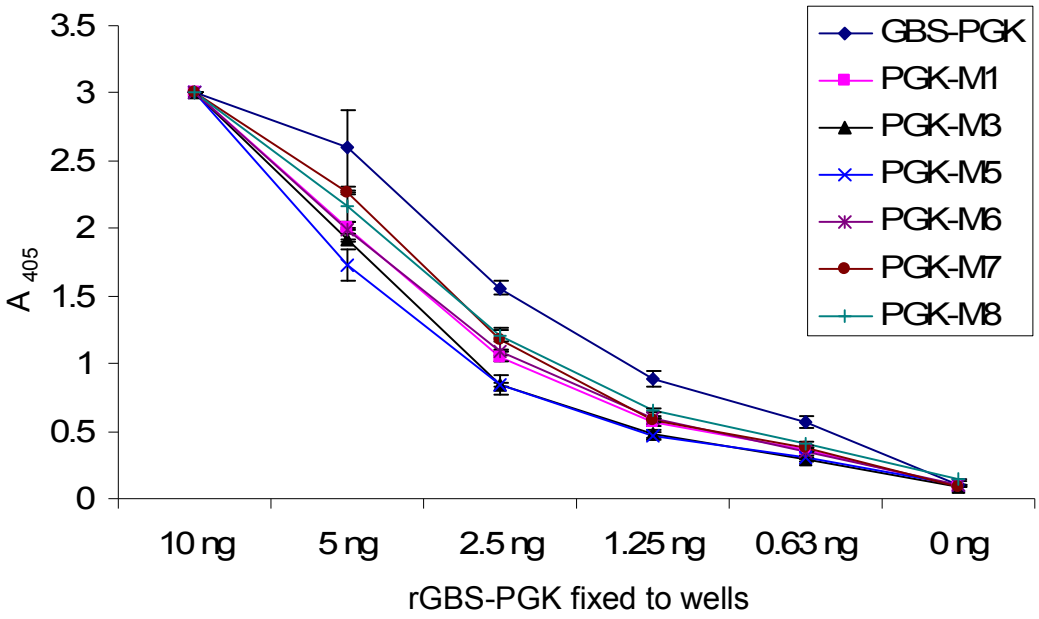


Figure 5.7. Interaction of anti-rGBS-PGK antibodies with mutated and non-mutated rGBS-PGK. Decreasing concentrations of mutated and non-mutated rGBS-PGK proteins were fixed to wells of a 96 well polystyrene plate. After overnight blocking the wells were incubated with anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed using 4-nitrophenol phosphate and the A_{405} was measured. Data points represent the average A_{405} values from experiments performed in triplicate; error bars represent one standard deviation.

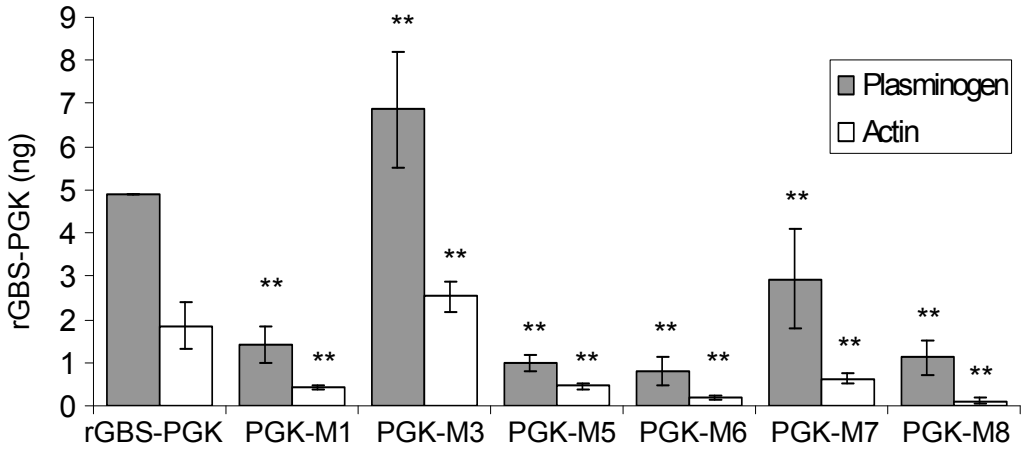


Figure 5.8. Binding of mutated and non-mutated rGBS-PGK molecules to actin and plasminogen fixed to wells of a 96 well plate. Actin (1 μg/well) and plasminogen (0.1 μg/well) were fixed to wells of a 96 well plate. After blocking overnight wells were incubated with rGBS-PGK or rGBS-PGK molecules containing mutations within the identified binding regions (15 μg/ml). The amount of rGBS-PGK remaining in the wells was assayed using anti-rGBS-PGK followed by anti-rabbit IgG alkaline phosphatase conjugate antibodies. Wells were developed with 4-nitrophenol phosphate and the A_{405} was measured. The A_{405} readings were compared to standard curves containing 0-10 ng rGBS-PGK proteins to determine the amount of rGBS-PGK protein remaining in each well. Data points represent the average values from experiments performed triplicate in triplicate; error bars represent one standard deviation. (**) indicates statistical significance, p-value < 0.01

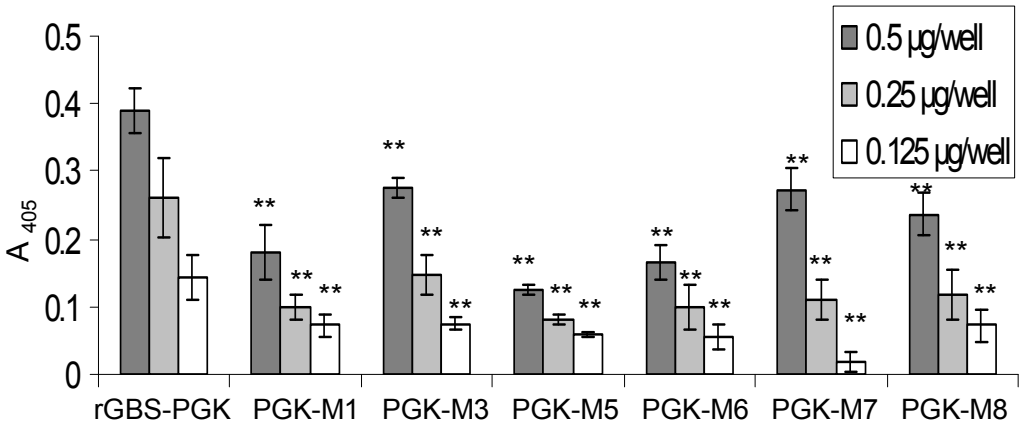


Figure 5.9. Actin binding to mutated and non-mutated rGBS-PGK molecules. rGBS-PGK molecules (1, 0.5, 0.25, 0.125 µg/well) were fixed to a 96 well polystyrene plate. After overnight blocking wells were incubated with actin (20 µg/ml). Plates were probed with anti-actin followed by anti-mouse IgG alkaline phosphatase conjugate antibodies. Plates were developed with 4-nitrophenol phosphate and the A_{405} was measured. Each data point represents the average A_{405} value of experiments performed triplicate in duplicate; error bars represent one standard deviation. Measurements from wells containing mutant rGBS-PGK molecules were compared to wells containing the same amount of rGBS-PGK to determine statistical significance; (**) indicates statistical significance p-value <0.01.

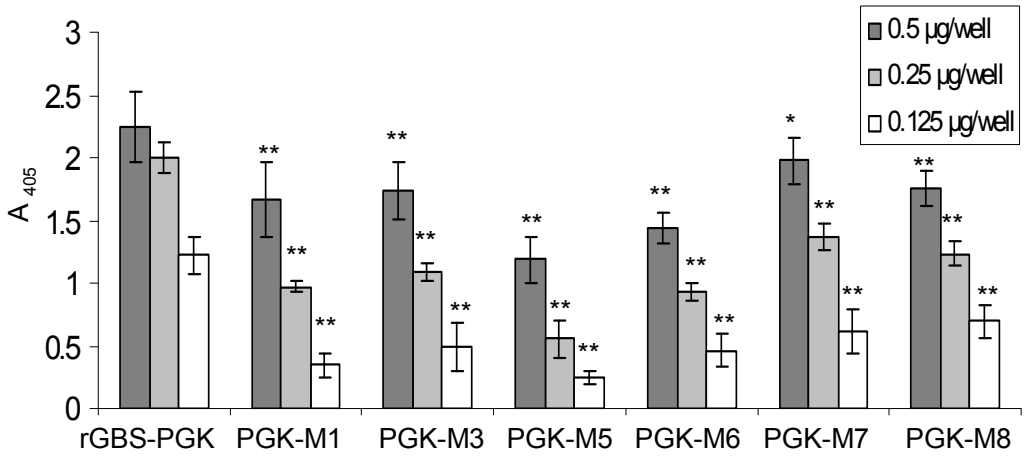


Figure 5.10. Plasminogen binding to mutated and non-mutated rGBS-PGK molecules. rGBS-PGK molecules (0.5, 0.25, 0.125 µg/well) were fixed to a 96 well polystyrene plate. After overnight blocking wells were incubated with plasminogen (20 µg/ml). Plates were probed with anti-plasminogen followed by anti-mouse IgG alkaline phosphatase conjugate antibodies. Plates were developed with 4-nitrophenol phosphate and the A_{405} was measured. Data points represent the average of experiments performed triplicate in duplicate; error bars represent one standard deviation. Measurements from wells containing mutant rGBS-PGK molecules were compared to measurements from wells containing the same amount of rGBS-PGK to determine statistical significance (*) indicates statistical significance p-value < 0.05, (**) indicates a p-value < 0.01.

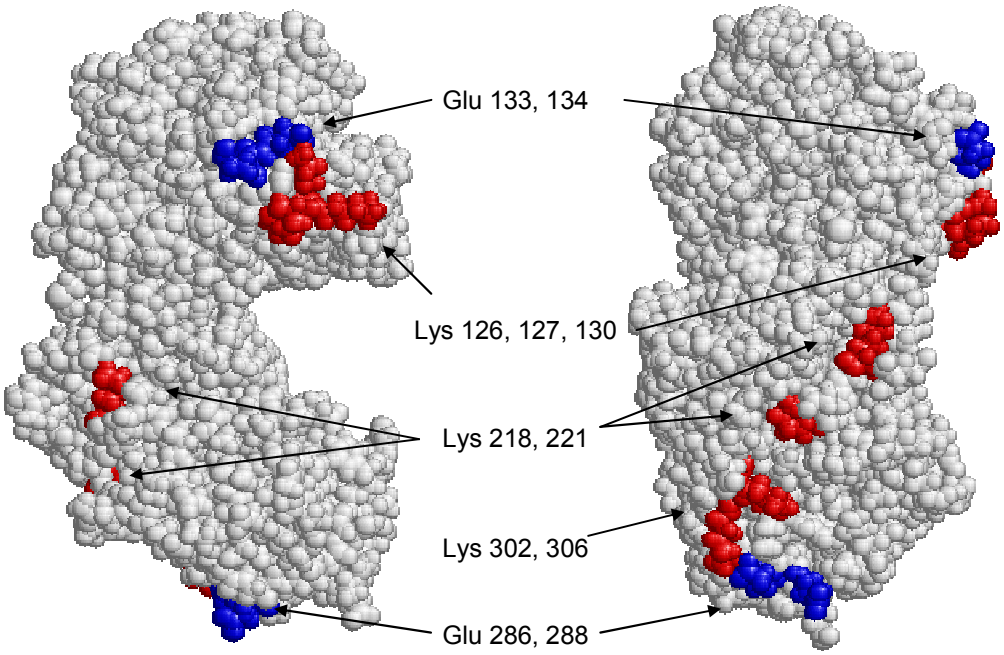


Figure 5.11. Mapping the actin and plasminogen binding sites onto the model GBS-PGK structure. The amino acid sequence of GBS-PGK was submitted to iTASSER to generate a model structure. The model structure of GBS-PGK was visualized using RasMol. The regions of GBS-PGK identified to affect binding to actin and plasminogen through the site directed mutagenesis experiment are highlighted on the model GBS-PGK molecule. Lysine residues are highlighted in red, while glutamic acid residues are highlighted in blue.

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Chapter 6:

Summary and Future directions:

Despite aggressive antimicrobial prophylaxis, group B streptococcus (GBS) remains a major cause of invasive disease in the neonatal population. While prophylaxis has reduced the prevalence of group B streptococcal early onset disease (EOD), it has had no effect on the occurrence of group B streptococcal late onset disease (LOD) (1). In addition, the majority of EOD cases are now occurring in infants from mothers who tested negative for GBS colonization (1). Finally, the prevalence of GBS in midgestation stillbirths appears to be as high as the prevalence of GBS-EOD before the introduction of antimicrobial prophylaxis (2). To date, reducing the rate of GBS induced stillbirths has not yet been addressed. These issues, in addition to concerns regarding antimicrobial resistance, highlight the need for improved therapies to prevent GBS infection in the neonatal population.

To cause invasive disease, GBS expresses a number of surface components that mediate the disease process. One surface expressed protein from GBS that has not been well characterized is phosphoglycerate kinase (PGK). The work presented in this thesis focused on identifying how PGK becomes expressed on the GBS surface and identifying the potential roles for surface expressed GBS-PGK in GBS virulence. As a surface protein of GBS, PGK is a potential target for the development of a vaccine to prevent GBS infections in both the neonatal and adult populations. A better characterization of the PGK molecule to identify regions that contribute to GBS virulence may facilitate the development of a vaccine targeting GBS-PGK.

6.1 Expression of PGK on the GBS surface:

PGK has previously been identified on the GBS surface (3, 4). The data presented in chapter two of this thesis confirms these previous publications and demonstrates that, like other surface expressed glycolytic enzymes(5-7), GBS-PGK can be found secreted into the growth media (Fig. 2.6). Since it is not possible to generate a *pgk* knockout, to determine the function of PGK on the GBS surface it will be necessary to prevent transport of PGK to the GBS surface. At the outset of my project, it was unknown how PGK and other glycolytic enzymes were transported to the surface of gram positive bacteria (8, 9). As a result it was not known how to prevent expression of PGK on the GBS surface. The work presented in chapters three and four of this thesis characterizes surface expression of GBS-PGK, in an attempt to generate a mutant GBS strain that does not transport PGK to its surface.

My initial attempt to identify genes involved in surface expression of GBS-PGK utilized a Tn*917* transposon mutagenesis screen. This screen identified four genes (*sag0966, sag0979, sag0980* and *sag1003*) that affect attachment of PGK to the GBS surface. Taken together with my other results, these genes appear to have a regulatory effect on surface attachment of GBS-PGK. Three of the four genes identified in the screen (*sag0979*, *sag0980* and *sag1003*) are predicted to be involved in peptide transport. Also, three of the identified genes (*sag0966*, *sag0979* and *sag0980*) were found to flank five other genes (*sag0973*-*sag0977*) predicted to be involved in sensing of and resistance to

antimicrobial peptides. These results led to the hypothesis that the genes identified in the Tn*917* screen may be involved in antimicrobial peptide resistance, and that surface expression of GBS-PGK is somehow regulated in response to the presence of antimicrobial peptides. Consistent with this hypothesis, disruption of the genes identified in the Tn*917* screen resulted in reduced bacterial growth. Disruption of one of the genes (*sag1003*) resulted in increased sensitivity to both nisin and bacitracin, confirming the role of Sag1003 in antimicrobial peptide resistance. Disruption of the other three genes did not affect sensitivity to either nisin or bacitracin. While these results may suggest that Sag0966, Sag0979 and Sag0980 are not involved in antimicrobial peptide resistance, it is also possible that they serve a unique function required for resistance to the antimicrobial peptide produced by NCS13. Regulation of PGK attachment to the GBS surface in response to an antimicrobial peptide is not entirely surprising based on the observation that lipoteichoic acid may be one of the ligands responsible for attaching PGK to the GBS surface (Fig. 3.9) and that modification of lipoteichoic acid is one of the major mechanisms utilized by GBS for resistance to antimicrobial peptides (10-12).

Previous work has demonstrated that α-enolase and GAPDH can be bound to the bacterial surface through a pH dependent charge interaction with lipoteichoic acid (13, 14). My results demonstrate that rGBS-PGK is also capable of binding to lipoteichoic acid from group A streptococcus (GAS) in vitro (Fig. 3.9). Consistent with these results, my experiments demonstrate that binding of GBS-PGK to the GBS surface preferentially occurs at low pH (Fig. 3.10-3.12).

These results suggest that PGK may become attached to the GBS surface through interaction with lipoteichoic acid. Based on this model of PGK attachment to the GBS surface, antimicrobial peptide resistance mechanisms could inhibit surface attachment of GBS-PGK. One of the main methods that GBS resists killing by antimicrobial peptides is through the addition of D-alanine to the lipoteichoic acid (11, 12). This provides resistance to antimicrobial killing by increasing the charge on the bacterial surface, repelling the positively charge AMP. This alteration of the lipoteichoic acid may also affect interaction with GBS-PGK. D-alanine incorporation into the lipoteichoic acid, has been demonstrated to be up regulated in the presence of antimicrobial peptides in *Clostridum difficile* (15) and *Streptococcus gordonii* (16). This increased D-alanine incorporation may prevent the interaction between GBS-PGK and lipoteichoic acid on the GBS surface.

While my results demonstrate that GBS-PGK binds lipoteichoic acid, attachment of PGK to the GBS surface does not appear to be entirely dependent on lipoteichoic acid. Previous work has demonstrated that enzymes interacting with the bacterial surface through interaction with lipoteichoic acid can be removed from the bacterial surface by high pH or high osmolarity (13, 14). My results demonstrate that PGK is firmly attached to the GBS surface and cannot be removed by incubation at pH 8 or in 2M NaCl (Fig. 3.13). These results are more consistent with results from GAPDH binding to the surface of GAS (17), which has been demonstrated to occur through interaction with the M-protein (18). The presence of a second ligand for attaching PGK to the GBS surface is supported by my results assaying attachment of rGBS-PGK to the surface of the mutant

NEM316 strains (Fig. 4.2B). Attachment of rGBS-PGK with the surface of NEM316 and NEM316Δ*secA2* were found to be similar and significantly higher than rGBS-PGK binding to NEM316Δ*srr1* and NEM316Δ*gtfA,B*, suggesting that Srr1 may be necessary for expression of the second GBS-PGK binding ligand on the GBS surface. Since NEM316Δ*secA2* was found to have no Srr1 expression on the bacterial surface (19), but still bound rGBS-PGK on the surface (Fig. 4.2B), it is unlikely that Srr1 itself is the second ligand responsible for attaching GBS-PGK to the bacterial surface.

Finally, my research led me to address how PGK becomes transported across the GBS membrane. In a previous paper it was determined that SecA2 is responsible for surface expression of the glycolytic enzyme α-enolase on the surface of *Listeria monocytogenes* (20). To determine if SecA2 is also required for expression of PGK on the GBS surface, I assayed strains of NEM316 containing deletions of the *secA2*, *srr1* and *gtfA,B* genes. My results demonstrate that deletion of the *secA2* gene resulted in decreased amounts of PGK on the GBS surface and in the culture supernatant (Fig 4.2 and 4.3). Surprisingly NEM316 strains containing deletions of *srr1* and *gtfA,B* expressed even less PGK on their surface and in the culture supernatants, compared to the strain containing a deletion in the *secA2* gene (Fig 4.2 and 4.3). PGK secretion could also be inhibited by the SecA inhibitor sodium azide (Fig 4.4). Based on these results I have concluded that PGK can be secreted by either SecA or SecA2, but Srr1 is necessary to target PGK for secretion.

My results allow me to propose a model for expression of PGK on the GBS surface that encompasses secretion, surface attachment and to some extent regulation of surface attachment (Fig 6.1). My results suggest that PGK is transported across the GBS membrane through both SecA and SecA2. PGK is targeted for secretion through interaction with Srr1 in the GBS cytoplasm. Once transported across the GBS membrane PGK binds to the GBS surface through interaction first with lipoteichoic acid and finally with a second ligand requiring Srr1 for expression. Interaction with lipoteichoic acid is likely inhibited by the presence of antimicrobial peptides, via the addition of D-alanine to the lipoteichoic acid. Both the SecA2 locus and lipoteichoic acid have previously been demonstrated to be involved in expression of glycolytic enzymes on the surface of other gram positive bacteria (13, 14, 20). As a result, I expect that my model will not only be applicable to surface expressed GBS-PGK, but may also be applicable to studying expression of other glycolytic enzymes on the surface of other gram positive pathogens as well.

6.2 Potential virulence functions of GBS-PGK:

A second method that has been previously used to determine the function of surface expressed glycolytic enzymes is to use site directed mutagenesis to prevent binding to host proteins (21, 22). To gain a better understanding of potential functions for GBS-PGK in GBS virulence, I identified host proteins that would interact with rGBS-PGK. The experiments documented in chapter two of

this thesis demonstrate that rGBS-PGK binds actin, fibrin, fibrinogen, fibronectin and plasminogen. These results suggest that surface expressed and secreted GBS-PGK may have multiple functions contributing to GBS adhesion, dissemination and survival within the host. The actin binding ability of rGBS-PGK suggests that surface expressed GBS-PGK could contribute to adhesion as actin has been identified on the surface of a variety of human cells (23-27). The actin binding ability of secreted GBS-PGK may also contribute to internalization and survival within human cells as expression of GBS-PGK in the cytoplasm of HeLa cells has been shown to result in disruption of the actin cytoskeleton (28). I have also demonstrated that rGBS-PGK binds plasminogen; plasminogen binding by bacterial pathogens has previously been demonstrated to contribute to adhesion (29, 30) and bacterial dissemination (29-33). Fibronectin binding ability of ScpB has been previously demonstrated to contribute to internalization within A549 and Hep2 cells (34); presumably, fibronectin binding by surface expressed GBS-PGK may have a similar function. Finally, my results demonstrate that rGBS-PGK binds fibrinogen and fibrin. Fibrinogen binding has previously been demonstrated to play a role in adhesion and internalization of GBS (35, 36). In addition, GBS is known to cleave fibrinogen and coat its surface with the fibrin like product to interfere with complement mediated opsonization (37). The fibrinogen and fibrin binding ability of rGBS-PGK suggest that surface expressed GBS-PGK may contribute to this process, facilitating survival within the bloodstream.

The work presented in chapter five of this thesis identified the actin and plasminogen binding sites of GBS-PGK, in an effort to generate a mutant GBS

strain expressing a PGK protein that does not bind actin or plasminogen. Surprisingly, the actin and plasminogen binding sites of GBS-PGK were similar. Both the actin and plasminogen binding sites of GBS-PGK mapped to amino acids 126-134, 302 and 306. As a result I have generated rGBS-PGK molecules that have significantly reduced binding to both actin and plasminogen (Fig. 5.8-5.10). While I have generated rGBS-PGK proteins with reduced binding to both actin and plasminogen, the binding has not yet been abolished. It will likely be necessary to generate an rGBS-PGK molecule containing mutations in both binding sites to abolish binding to actin and plasminogen. Because actin and plasminogen binding were mediated by the same amino acids in GBS-PGK, it will likely not be possible to generate an rGBS-PGK molecule that lacked binding to only one of these host proteins. As a result, it may not be possible to differentiate between the role of actin binding and plasminogen binding by surface expressed GBS-PGK.

6.3 Future Directions:

The overarching goal of my research was to determine the role surface expressed GBS-PGK plays in GBS virulence. While understanding the function of surface expressed GBS-PGK is far from complete, my results lay the foundation for future research identifying the role of surface expressed PGK in GBS virulence. My results focusing on determining how GBS-PGK becomes surface expressed provide a possible method to prevent expression of PGK on the

GBS surface. In addition, my research focusing on identifying host proteins bound by GBS-PGK has provided some potential virulence functions of GBS-PGK. Finally my research identifying the actin and plasminogen binding sites of GBS-PGK should allow future work to determine the role of actin and plasminogen binding by surface expressed GBS-PGK in GBS virulence.

The results from the Tn*917,* 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 one of the mutant NCS13 strains was more sensitive to antimicrobial peptides may provide some insight into how GBS resists killing by the human innate immune system. In addition, the observation that all of the mutant NCS13 strains had reduced β-hemolysis raises some interesting questions regarding regulation of virulence in GBS. While these results first need to be confirmed using directed mutagenesis targeting the genes identified in the Tn*917* screen, they provide interesting avenues for further research into GBS virulence.

6.3.1 Regulation of GBS virulence in response to antimicrobial peptides.

The results of the Tn*917* screen suggest that attachment of GBS-PGK to the GBS surface is somehow regulated in response to antimicrobial peptides. It seems likely that this regulation is mediated by the addition of D-alanine residues into the lipoteichoic acid. The loss of β-hemolysis in the Tn*917* mutants suggest

that factors regulating PGK attachment to the GBS surface also affect expression of GBS virulence factors. These results raise a number of interesting questions for future research.

The first research question arising from my Tn*917* mutagenesis screen is "Does GBS produce an antimicrobial peptide?" My hypothesis that surface expression of GBS-PGK is regulated in response to an antimicrobial peptide requires that NCS13 produces an antimicrobial peptide. BLAST search analysis of the genes surrounding those identified in the Tn*917* mutagenesis screen identified a gene that may potentially encode this antimicrobial peptide. The *sag0972* gene contains a frame-shift mutation resulting in premature truncation of the protein to 57 amino acids, the same length as the gene encoding the nisin precursor protein (38). I hypothesize that the truncated protein encoded by *sag0972* may be the antimicrobial peptide that regulates surface expression of GBS-PGK. Mass spectrometer analysis of proteins secreted by overnight cultures of NCS13 may be able to determine if Sag0972 is produced as well as identify any post-translational modifications. If this is successful it should be possible to assay purified Sag0972 for antimicrobial activity as well as for its effect on surface expression of GBS-PGK and expression of the β-hemolysin. An alternate approach would be to delete the *sag0972* gene and assay the mutant NCS13 strain for surface expression of GBS-PGK and β-hemolysis. If my hypothesis is correct, addition of purified Sag0972 should reduce surface expression of GBS-PGK and decrease expression of the β-hemolysin while deletion of *sag0972* should result in

increased expression of PGK on the bacterial surface as well as increased β-hemolysis.

A second research question arising from the results of my Tn*917* screen is "Does modification of the lipoteichoic acid through the addition of D-alanine residues affect surface expression of GBS-PGK?" My hypothesized model for regulating surface attachment of rGBS-PGK relies on PGK binding to lipoteichoic acid being inhibited by the addition of D-alanine residues into the lipoteichoic acid. Incorporation of D-alanine residues into the lipoteichoic acid has been previously demonstrated to be one of the mechanisms used by GBS to resist killing by antimicrobial peptides (10-12). The genes responsible for the incorporation of D-alanine residues are located within the six gene *dlt* operon (*sag1787-sag1792*) (39). Deletion of *dltA* (*sag1790*) has previously been demonstrated to abolish D-alanine incorporation into the lipoteichoic acid (10). Based on my model for regulation of surface attachment of GBS-PGK, deletion of *dltA* should result in increased surface expression of GBS-PGK and reduced β-hemolysis. An alternate approach would be to express the *dlt* operon from a plasmid. Based on my model for regulation, over expression of the *dlt* operon should result in decreased surface expression of GBS-PGK and increased expression of the β-hemolysin.

The third research question arising from the results of my Tn*917* mutagenesis screen is "How does GBS sense and respond to this antimicrobial peptide?" Based on my hypothesized model for surface attachment of GBS-PGK, attachment is regulated by the incorporation of D-alanine into the lipoteichoic

acid. One two component system that controls D-alanine incorporation into the lipoteichoic acid is DltR/S (10). However, this two component system appears to sense the level of D-alanine incorporation into the lipoteichoic acid, not the presence of antimicrobial peptides (10). Either this two component system responds to more than one stimulus, or it does not sense the presence of antimicrobial peptides. Another potential sensing system is the CiaR/H two component system, which has been demonstrated to contribute to antimicrobial peptide resistance in GBS (40). However, microarray analysis of GBS strains containing a deletion in the *ciaR* gene, did not demonstrate differences in expression levels of genes contained in the *dlt* operon. Located near the genes identified in the Tn*917* screen, are two genes (*sag0976* and *sag0977*) that appear to encode a two component system responsible for sensing and regulating gene expression in response to antimicrobial peptides. Blast search analysis of these two genes demonstrated similarity to the BceR/S two component system of *Streptococcus equi*. Based on this similarity, I hypothesize that these two genes may be involved in sensing and responding to antimicrobial peptides. Deletion of these two genes, followed by assaying surface expression of GBS-PGK and β-hemolysis should determine if these two genes are involved in regulating expression of either of these two proteins. If my hypothesis is correct deletion of these two genes should result in increased sensitivity to antimicrobial peptides, such as nisin, bacitracin and Sag0972. These mutant strains should also have increased surface expression of GBS-PGK and increased expression of the β-hemolysin.

The fourth research question arising from the results of my Tn*917* mutagenesis screen is "What other genes are regulated in response to antimicrobial peptides?" My results suggest that expression of the β-hemolysin, a known GBS virulence factor, is regulated in response to antimicrobial peptides. A similar gene regulation in group A streptococcus has been demonstrated as the major virulence two component system CsrRS has been found to respond to the human antimicrobial peptide LL-37 (41). It would be interesting to determine if virulence genes in GBS are also regulated in response to antimicrobial peptides. Over the course of answering the first three research questions, the necessary bacterial strains and purified peptides will be produced to answer this question. These would include the NCS13Δ*sag0966*, NCS13Δ*sag0972*, NCS13Δ*sag0976*, NCS13Δ*sag0977*, NCS13Δ*sag0979*, NCS13Δ*sag0980* and NCS13Δ*sag1003* strains of GBS and the purified Sag0972 peptide. Using micro-array to characterize the changes in gene expression of these seven GBS strains compared to the parent NCS13 strain when grown in the presence and absence of the Sag0972 peptide should provide insight into which genes are regulated in response to antimicrobial peptides and the relative contribution of the various identified genes. Considering the CsrR/S system has been found to regulate expression of virulence factors in GBS, it may also be interesting to generate GBS strains lacking this two component system to assay for response to antimicrobial peptides as well.

The results from my Tn*917* transposon mutagenesis screen have implications beyond surface expression of GBS-PGK. In addition to identifying a

mechanism used by GBS to resist killing by antimicrobial peptides, my results suggest a potential mechanism for regulation of GBS virulence factors in response to the human innate immune system. Admittedly, a number of experiments are required to confirm my hypotheses. However, if my hypotheses are correct, they may significantly improve our understanding of GBS virulence.

6.3.2 Secretion of GBS-PGK.

My results provide some interesting insights into how PGK is transported across the GBS membrane. Of particular interest, deletion of *srr1* gene nearly abolished secretion of GBS-PGK. These results led to the hypothesis that Srr1 may be a cytoplasmic chaperone protein that delivers GBS-PGK to the secretion machinery. If this hypothesis is true, then preventing interaction between GBS-PGK and Srr1 should prevent secretion of GBS-PGK. While my results suggest that GBS-PGK interacts with Srr1 to be targeted for secretion, direct interaction between GBS-PGK and Srr-1 has yet to be shown. To confirm my hypothesis it will be necessary to demonstrate direct interaction between GBS-PGK and Srr1, either through ELISA or yeast two hybrid assays. Once the direct interaction between GBS-PGK and Srr1 is confirmed, it should be possible to use error prone PCR or peptide mapping to identify amino acid residues within GBS-PGK required for interaction with Srr1. Once a GBS-PGK molecule that does not interact with Srr1 has been generated, replacement of the genomic *pgk* gene with

one containing these mutations should result in a GBS strain that expresses PGK in the bacterial cytoplasm but not on the bacterial surface. Assaying this GBS strain for virulence using established attachment and invasion assays should identify the role of extracellular GBS-PGK in GBS virulence. In addition, assaying survival of this GBS strain in human serum should determine if extracellular GBS-PGK contributes to GBS survival in the human host.

One potential complication to this is the requirement for the *gtfA* and *gtfB* genes for secretion of GBS-PGK. My results demonstrate that NEM316Δ*gtfA,B* also did not secrete PGK. This is likely due to the instability of Srr1 in the absence of glycosylation (19). Alternatively, glycosylation of Srr1 may be necessary for interaction with GBS-PGK or targeting the Srr1-PGK complex to the transport machinery. It will likely be necessary to express Srr1 in the presence of GtfA and GtfB, to ensure proper modification of the Srr1 protein, in order to characterize the interaction of GBS-PGK with Srr1.

6.3.3 Surface attachment of GBS-PGK.

While preventing secretion of GBS-PGK should provide the greatest insight into the role of PGK in GBS virulence, differentiating between the function of surface expressed and secreted PGK would require preventing attachment to the bacterial surface. My results suggest that PGK is attached to the GBS surface through binding to lipoteichoic acid as well as another surface component that requires Srr1 for expression. I hypothesize that this second

ligand, requiring Srr1 for expression, is responsible for the firm attachment of GBS-PGK to the GBS surface. If this is true, GBS-PGK attachment to the surface of NEM316Δ*srr1* should be more sensitive to high pH and high osmolarity, similar to previously described for α-enolase and GAPDH attachment to the *S. gordonii* and *L. crispatus* surface (13, 14). It is also possible that deletion of *srr1* altered expression of lipoteichoic acid on the bacterial surface. If this is the case, attachment of GBS-PGK to the surface of NEM316Δ*srr1* should be no more sensitive to osmolarity and pH than attachment to the surface of NEM316.

My results identified lipoteichoic acid as one of the GBS surface components responsible for attaching GBS-PGK to the GBS surface (Fig. 4.5). I hypothesize that lipoteichoic acid mediates the initial attachment of GBS-PGK to the GBS surface. As a result, inhibiting this interaction may prevent surface attachment of GBS-PGK. Characterizing the interaction between lipoteichoic acid and rGBS-PGK, using similar methods as I have used to identify the actin and plasminogen binding sites of GBS-PGK, should allow for the generation of a rGBS-PGK molecule that does not bind to lipoteichoic acid.

While my results suggest that expression of Srr1 is necessary for expression of a second ligand responsible for attaching PGK to the GBS surface, I have not identified this ligand. Characterizing surface expressed proteins of NEM316 compared to NEM316Δ*srr1*, similar to experiments performed in *Listeria monocytogenes* (20), may narrow down the list of potential PGK binding proteins on the GBS surface. Coupling this with a blot overlay assay, similar to those used to identify plasminogen binding proteins (42, 43), may identify the

protein responsible for attaching PGK to the GBS surface. Once this second ligand has been identified, it should be possible to identify the binding site within GBS-PGK responsible for this interaction using truncation and peptide mapping experiments.

Once the binding sites within GBS-PGK responsible for interaction with lipoteichoic acid and the second ligand have been determined, site directed mutagenesis could be used to generate an rGBS-PGK molecule that binds to neither. Replacing the genomic *pgk* gene with this mutant *pgk* gene should result in a GBS strain that expresses PGK in the cytoplasm as well as secreted into the growth media, but not attached to the bacterial surface. This mutant GBS strain could then be assayed for virulence using established attachment and invasion assays to determine the role of surface attached GBS-PGK in GBS virulence. In addition, assaying this GBS strain for survival in human serum could be used to determine if surface attached GBS-PGK contributes to GBS survival in the blood stream.

6.3.4 Host protein binding by GBS-PGK.

My results suggest that surface expressed GBS-PGK may contribute to GBS virulence through its ability to bind actin, fibrin, fibrinogen, fibronectin and plasminogen. However, it is not clear if PGK expressed on the GBS surface would retain these binding activities. It is also not clear the role these binding activities may have in GBS virulence considering that GBS has been

demonstrated to express other surface proteins with similar binding activities (33-36, 44, 45). In order to confirm a role for these binding activities of GBS-PGK in GBS virulence, it will be necessary to generate mutant GBS strains expressing PGK proteins containing mutations to abolish each of these binding activities. I have identified the actin and plasminogen binding domains of GBS-PGK and generated an rGBS-PGK protein with significantly reduced binding to both actin and plasminogen (Fig. 6.2). Replacing the genomic *pgk* gene with the mutant *pgk* genes I have generated should result in mutant GBS strains expressing a form of PGK on their surface that lacks the ability to bind actin or plasminogen.

In addition to actin and plasminogen, I have demonstrated that rGBS-PGK binds to fibrin, fibrinogen and fibronectin. Using similar truncation and peptide mapping techniques as used to identify the actin and plasminogen binding domains of rGBS-PGK, it should be possible to identify the fibrin, fibrinogen and fibronectin binding sites of GBS-PGK. Replacing the genomic *pgk* gene with these mutant *pgk* genes should result in mutant GBS strains expressing a form of PGK on their surface that lacks the ability to bind fibrin, fibrinogen or fibronectin. These mutant GBS isolates could then be assayed for virulence characteristics such as adhesion and invasion of eukaryotic cells, paracellular invasion, degradation of extracellular matrix proteins and fibrin clot dissolution in order to determine the role of host protein binding by surface expressed GBS-PGK on GBS virulence.

6.3.5 Prevention and treatment strategies.

Despite aggressive antimicrobial prophylaxis, GBS remains a major cause of invasive disease in the neonatal population (1). Currently, women are screened at 35-37 weeks gestation for the presence of GBS. Those women that are positive for colonization with GBS are given antimicrobial prophylaxis during labor. Presently, the majority of infants developing GBS-EOD were from mothers who were negative for GBS colonization (1). This observation highlights the need for either more effective screening methods or the introduction of universal prophylaxis to prevent GBS infection in the neonatal population. In addition to the failure of the current antimicrobial prophylaxis methods to completely prevent GBS-EOD, antimicrobial prophylaxis has had no effect on the prevalence of GBS-LOD (1). These observations, along with concerns regarding antimicrobial resistance, highlight the need for a more complete therapy to prevent GBS disease in the neonatal population. The research presented in this thesis may be useful in developing this therapy in the form a vaccine targeting PGK on the GBS surface.

As a surface protein of GBS (3, 4), PGK may be a candidate protein for the development of a vaccine to prevent invasive GBS disease. Antibodies against GBS-PGK have previously been demonstrated to provide protection against GBS infections in a mouse model (4). However, concerns about auto-immunity make developing vaccines to glycolytic enzymes difficult. Ideally, developing a peptide based vaccine to target a region of GBS-PGK that is not present in the human PGK protein should eliminate concerns about auto-

immunity. I have identified the actin and plasminogen binding domains of GBS-PGK. One of these binding sites is located between amino acids 126-134. The region of the PGK protein between amino acids 123-139 is not conserved between the GBS-PGK and human PGK proteins (Fig. 6.3). As a result, it may be possible to target this region of the GBS-PGK protein to develop a peptide vaccine to prevent invasive GBS disease in the neonatal population without the concern of an auto-immune response.

Antigenic shift to avoid immune clearance resulting from the vaccine is another concern regarding the long term efficacy of any vaccine. Since this region of the GBS-PGK protein is involved in binding to actin and plasminogen, processes I believe contribute to GBS virulence, any antigenic changes to avoid immune clearance should result in a less virulent GBS strain. In addition to being effective against GBS infections, targeting this region of GBS-PGK may provide protection against other bacterial infections. With the exception of the point mutation at amino acid residue 133, converting the glutamic acid residue to proline, the nine residue region that I have identified as an actin and plasminogen binding site of GBS-PGK is completely conserved within PGK proteins from other streptococcal species. As a result, I expect that this vaccine target would also provide some cross protection against other gram positive bacteria as PGK as glycolytic enzymes are expressed on the surface of numerous gram positive bacteria (9, 17, 30, 42, 46-49).

While antibodies to full length GBS-PGK have previously been demonstrated to provide protection against GBS infections (4), the level of

protection that would be provided by a vaccine targeting this small region of PGK is unknown. Future work determining whether this region of the PGK molecule will produce an immune response, whether this immune response will be effective against GBS infections, and whether this immune response will provide cross protection against other streptococcal species, is necessary to determine if a peptide spanning amino acids 122-139 of GBS-PGK would be a usable vaccine candidate to prevent GBS infections.

The research presented in this thesis also suggests a second therapeutic option for prevention of GBS disease. Based on the results of the Tn*917* mutagenesis screen, I have concluded that NCS13 regulates attachment of PGK to the GBS surface in response to an antimicrobial peptide. This conclusion implies that NCS13 produces an antimicrobial peptide. As discussed in section 6.3.1, I hypothesize that this antimicrobial peptide is encoded by the *sag0972* gene. Since antimicrobial peptides are typically effective against closely related bacterial strains (50), potentially Sag0972 could be used to treat infections caused by other strains of GBS. Once it has been determined that Sag0972 is expressed, and any post translational modifications, it should be possible to assay purified Sag0972 for antimicrobial action against other GBS strains. Potentially Sag0972 could be used to prevent transfer of GBS from mother to infant to prevent the occurrence of GBS-EOD. Since antimicrobial peptides typically have a narrow host range, the development of resistance should be less of a concern. As a result it may be possible to use Sag0972 for universal prophylaxis in all pregnant women similar to the bacteriophage lytic enzymes discussed in section 1.1.3 of this thesis.

6.4 Conclusions:

Due to the role of PGK in glycolysis, traditional knock-out mutagenesis has not been possible to use as a means to determine the function of surface expressed GBS-PGK. The work described in this thesis identified how PGK becomes expressed on the GBS surface as well as potential virulence functions for surface expressed and secreted GBS-PGK. While my work does not definitively demonstrate that surface expressed GBS-PGK is involved in GBS virulence, the host protein binding profile certainly demonstrates that surface expressed GBS-PGK could contribute to adhesion, internalization, paracellular invasion and survival within the host. In addition, my research lays the foundation for future research characterizing the function of surface expressed GBS-PGK. The discovery that secretion of GBS-PGK relies on the presence of Srr-1 suggests a possible method to prevent secretion of GBS-PGK, a functional equivalent of knock-out mutagenesis. Furthermore, I have identified point mutations within GBS-PGK that prevent binding to both actin and plasminogen without affecting the glycolytic activity. These mutant *pgk* genes should allow future research to determine the role of actin and plasminogen binding by surface expressed GBS-PGK in GBS virulence. The data presented in this thesis not only advances our understanding of PGK expressed on the GBS surface, but also our understanding of surface expressed glycolytic enzymes in general.

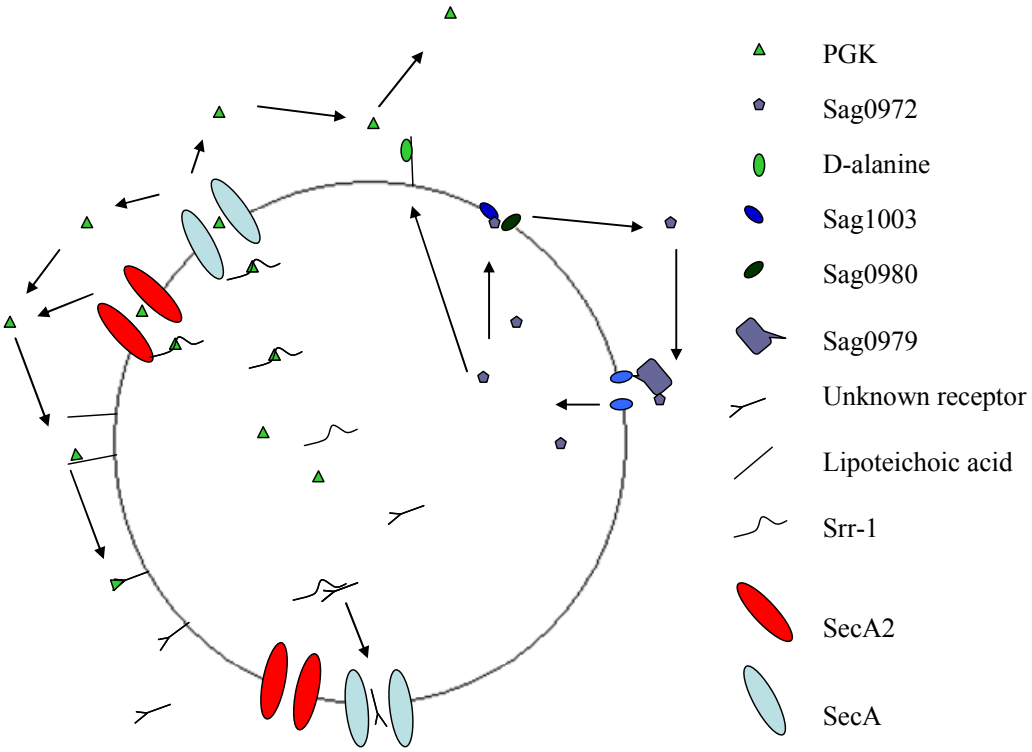

Figure 6.1. Model for secretion, surface attachment and regulation of surface expressed GBS-PGK. GBS-PGK is targeted for secretion across the GBS membrane through interaction with Srr-1 in the bacterial cytoplasm. Srr-1 delivers GBS-PGK to either SecA or SecA2 for secretion across the GBS membrane. Once secreted, GBS-PGK interacts with the GBS surface, initially through a pH dependent charge interaction with lipoteichoic acid. Following the interaction with lipoteichoic acid, GBS-PGK is transferred to a second surface ligand. GBS-PGK binding to this second ligand is not sensitive to pH or high salt concentrations. In addition to being sensitive to pH changes, GBS-PGK binding to the GBS surface is also affected by antimicrobial peptides. Presumably, this affect is due to the addition of D-alanine residues into the lipoteichoic acid. The presence of proteins involved in transport and resistance to antimicrobial peptides (Sag0979, Sag0980 and Sag1003) appear to increase binding of GBS-PGK to the GBS surface. This is likely due to the ability to neutralize and expel antimicrobial peptides from the bacterial cytoplasm and reducing the need for D-alanine incorporation into the lipoteichoic acid.

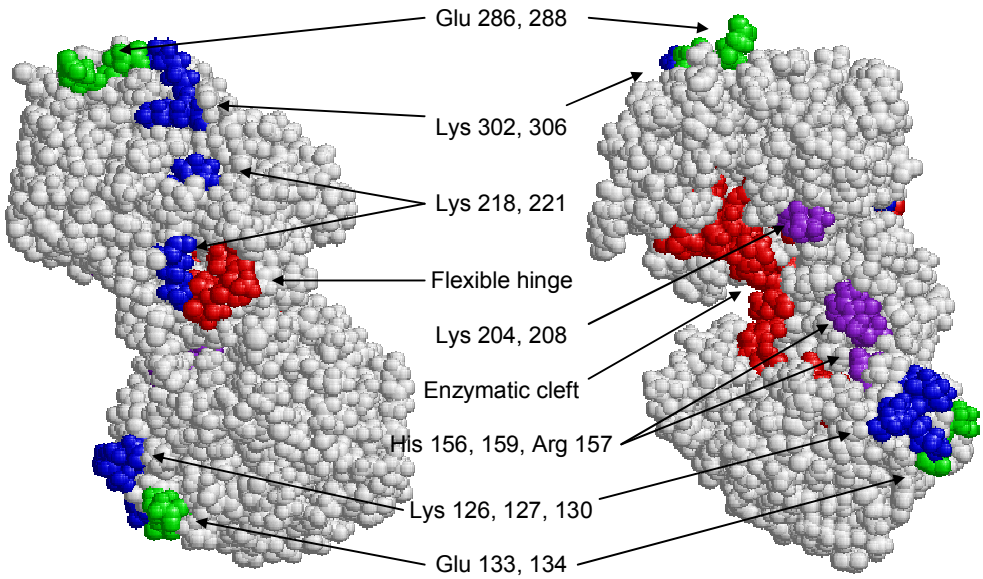

Figure 6.2. Mapping the amino acid residues necessary for glycolysis, actin binding and plasminogen binding onto the model GBS-PGK structure. The amino acid sequence was used to generate a model structure of the protein using iTASSER. The model structure was viewed using RasMol and the important amino acid residues were mapped onto the model structure. Colored in red are the amino acids previously found to be involved in the glycolytic function of GBS-PGK (51). Colored in purple are the amino acids mutated in this work that resulted in loss of enzymatic activity. Colored in blue are the lysine residues identified to be involved in binding to actin and plasminogen. Colored in green are the glutamic acid residues identified or predicted to be involved in binding actin and plasminogen.

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Human PGK 6    KLTLDKLDVKGKRVVMRVDFNVPMKNNQITNNQRIKAAVPSIKFCLDNGAKSVVLMSHLG  65
               KLT+  +D+KGK+V++RVDFNVP+K+  ITN+ RI AA+P+IK+ ++ G +++ L SHLG
GBS PGK   3    KLTVKDVDLKGKKVLVRVDFNVPLKDGVITNDNRITAALPTIKYIIEQGGRAI-LFSHLG  61

Human PGK 66   RPDGVPMPDKYSLEPVAVELKSLLGKDVLFLKDCVGPEVEKACANPAAGSVILLENLRFH  125
               R       +  SL PVA +L + LG+DV+F     G ++E+A      G V+L+EN RF
GBS PGK   62   RVKEEADKEGKSLAPVAADLAAKLGQDVVFPGVTRGAKLEEAINALEDGQVLLVENTRFE  121

Human PGK 126  VEEEGKGKDASGNKVKAEPAKIEAFRASLSKLGD-VYVNDAFGTAHRAHSSMVGV--NLP  182
                       D  G K      ++  + ASL   GD ++VNDAFGTAHRAH+S VG+  N+
GBS PGK   122  --------DVDGKKESKNDEELGKYWASL---GDGIFVNDAFGTAHRAHASNVGISANVE  170

Human PGK 183  QKAGGFLMKKELNYFAKALESPERPFLAILGGAKVADKIQLINNMLDKVNEMIIGGGMAF  242
               +   GFL++ E+ Y  +A+E+PERPF+AILGG+KV+DKI +I N+L+K ++++IGGGM +
GBS PGK   171  KAVAGFLLENEIAYIQEAVETPERPFVAILGGSKVSDKIGVIENLLEKADKVLIGGGMTY  230

Human PGK 243  TFLKVLNNMEIGTSLFDEEGAKIVKDLMSKAEKNGVKITLPVDFVTADKFDENAKTGQAT  302
               TF K    +EIG SL +E+   + KDL+ K+  NG K+ LPVD   A+ F    +  + T
GBS PGK   231  TFYKA-QGIEIGNSLVEEDKLDVAKDLLEKS--NG-KLILPVDSKEANAFAGYTEV-RDT  285

Human PGK 303  VASGIPAGWMGLDCGPESSKKYAEAVTRAKQIVWNGPVGVFEWEAFARGTKALMDEVVKA  362
                   +  G++GLD GP+S  K+ EA+T AK +VWNGP+GVFE   F  GT  +MD +VK
GBS PGK   286  EGEAVSEGFLGLDIGPKSIAKFDEALTGAKTVVWNGPMGVFENPDFQAGTIGVMDAIVK-  344

Human PGK 363  TSRGCITIIGGGDTATCCAKWNTEDKVSHVSTGGGASLELLEGKVLPGVDALS  415
                  G  +IIGGGD+A         DK S +STGGGAS+ELLEGKVLPG+ AL+
GBS PGK   345  -QPGVKSIIGGGDSAAAAINLGRADKFSWISTGGGASMELLEGKVLPGLAALT  396
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Fig 6.3. BLAST alignment of human PGK with GBS-PGK. The amino acid sequences of GBS-PGK and human PGK were aligned using BLAST. Boxed area represents the amino acid sequences from amino acids 123-139 of GBS-PGK.

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