VARIATION IN HOST-PATHOGEN INTERACTIONS AMONG GENETICALLY DIVERSE STRAINS OF GROUP B *STREPTOCOCCUS*

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ABSTRACT

VARIATION IN HOST-PATHOGEN INTERACTIONS AMONG GENETICALLY DIVERSE STRAINS OF GROUP B *STREPTOCOCCUS*

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Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is a highly diverse species that can be found asymptomatically colonizing the gastrointestinal and genital tracts of healthy adults, but is also capable of causing severe invasive disease. GBS is a leading cause of sepsis and meningitis in neonates and the only preventative measure is antibiotic therapy given to pregnant mothers during labor to prevent transmission. Although this method was effective at reducing case rates upon implementation, case rates have remained unchanged since the initial decline and some mothers remain persistently colonized by GBS. Due to the high level of diversity among strains it is important to understand how strains differ at the various stages of disease progression in order to have a more complete understanding of GBS pathogenesis. Here, I examined how genotypically diverse strains differ in their interactions with human cells. The examination of strains of the same serotype in ability to associate with decidual cells and lung epithelial cells revealed that strains within the same serotype, and even the same sequence type (ST) differed in attachment and invasion, but this variation was dependent on host cell type. More specifically, strains of the hypervirulent lineage, ST-17 associated with decidual cells significantly more than the other STs, but the opposite was true for lung epithelial cell attachment. Mechanisms of persistent colonization was explored by comparing antibiotic tolerance and macrophage survival between ST-17 and ST-12 strains, which persisted and was

eradicated after antibiotic prophylaxis, respectively. This study revealed that although the ST-17 strain was not tolerant to antibiotics, subinhibitory antibiotics enhanced phagocytic uptake of this strain where it was able to survive for an extended period of time. Additionally, intracellular survival of the ST-17 strain was dependent on acidification of the phagosome, whereas altered pH had no effect on survival of the ST-12 strain, suggesting GBS can use different mechanisms of survival. Moreover, serotype III GBS strains were better able to survive phagosomal stress compared to other serotypes. Lastly, transcriptome analysis of the ST-17 strain during intracellular survival revealed temporal gene expression responses to long term survival and identified a large number of factors important for intracellular survival. Through mutagenesis studies, the roles of NADH peroxidase (Npx) and cadmium resistance protein (CadD) in GBS intracellular survival was examined. These studies demonstrated that Npx promotes resistance to reactive oxygen stress through detoxification of hydrogen peroxide and CadD serves as a heavy metal efflux pump to confer resistance to intoxication by certain divalent metal cations. The work described here reveals new insights in GBS pathogenesis and helps identify key virulence factors that can serve as targets for alternative therapeutics and vaccine development.

This dissertation is dedicated to my husband, Kip, for his love and support. He has always been there for me and worked hard to support me while completing this work.

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KEY TO ABBREVIATIONS

AMP	Antimicrobial peptides
BafA	Bafilomycin A1
BCR	B cell receptor
β-h/c	β-hemolysin/cytolysin
CFU	Colony forming units
CPS	Capsular polysaccharide
DC	Dendritic Cell
EOD	Early-onset disease
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBS	Group B *Streptococcus*
HA	hyaluronan
IAP	Intrapartum antibiotic prophylaxis
ICP-MS	Inductively-coupled plasma mass spectrometry
Ig	immunoglobulin
LDH	Lactate dehydrogenase
LOD	Late-onset disease
MHC	Major histocompatibility complex
MLST	Multilocus sequence typing
MOI	Multiplicity of infection
NO	Nitric oxide

PBS	Phosphate buffered saline
PKC	Protein kinase C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sia	Sialic acid
ST	Sequence type
TFH	Follicular T helper cell
Th	T helper
THB	Todd-Hewitt Broth
TLR	Toll-like receptor
WT	Wild type

CHAPTER 1

OVERVIEW OF GROUP B STREPTOCOCCAL DISEASE AND HOST RESPONSE

Group B Streptococcus

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae,* is a gram positive encapsulated bacterium that was initially identified as the causative agent of bovine mastitis (1). Since then, it has also been shown to cause infections in humans as well as other animals. GBS infections can occur in neonates, the elderly, and immunocompromised adults. Additionally, GBS is a common asymptomatic colonizer of the human gastrointestinal and genitourinary tracts of healthy adults (2).

Incidence of GBS Infections

GBS is a leading cause of neonatal sepsis and meningitis and is an emerging pathogen in the elderly and immunocompromised adults. Moreover, GBS infection of the extraplacental membranes can lead to preterm birth or stillbirth. The Active Bacterial Core Surveillance report estimates a total of 26,500 cases of invasive GBS disease resulting in approximately 1,460 deaths annually in the United States (3).

GBS can cause a number of infections in adults including sepsis, pneumonia, skin and soft-tissue infections, and bone and joint infections. Although infection can occur at any age, risk of infection in adults increases with age. The average case rate of adult GBS infections of any age is 10 cases per 100,000 non-pregnant adults, but this increases to 25 cases per 100,000 adults at age 65 years or older (3).

Neonatal infections account for 58.6 cases per 100,000 population and can be divided in two classes of disease: early-onset (EOD) and late-onset (LOD). EOD typically presents as pneumonia and sepsis and occurs within the first few days of life (4, 5). LOD typically presents as bloodstream infections leading to meningitis and

occurs between one week and three months of age (6). Current rates of EOD are 0.24 cases per 1,000 live births and LOD rates are 0.27 cases per 1,000 live births in the United States (3).

Preventative measures against neonatal GBS disease involve intrapartum antibiotic prophylaxis (IAP) given to GBS colonized women or those in preterm labor to reduce the likelihood of transmission to the baby during birth. These measures have been very successful in reducing the incidence of EOD; however the prevalence of LOD remains the same and overall case rates have plateaued over the years (4). Moreover, some women remain persistently colonized even after IAP (7). Therefore, the identification and development of alternative preventative measures, such as vaccines and drug targets, are greatly needed.

Diversity among GBS strains

GBS can be classified into ten serotypes based on their capsular polysaccharide (CPS) (8). These ten antigenically distinct CPS types play a major role in GBS virulence, with types Ia, Ib, II, III, and V most often associated with disease. Serotype III is most often associated with cases of neonatal disease, whereas serotype V is most often associated with adult infections (2, 9). Structural and sequence comparisons of the ten types indicate that the variation in CPS type is due to horizontal gene transfer rather than gradual mutagenesis (10).

GBS isolates can be further characterized using a multilocus sequence typing (MLST) system that examines the allelic profile of seven conserved genes to group GBS strains into a number of sequence types (STs) (11). Several studies have shown

that ST-17, a serotype III GBS subgroup, are more often associated with neonatal disease, indicating that ST-17 strains may be more virulent than other GBS STs and has been referred to as a hypervirulent lineage (11–15).

The first genome sequence of GBS strain NEM316, published in 2002, revealed new insights of the pathogen's lifestyle, behaviors, and genetic diversity. The size of the GBS genome is approximately 2.1Mb and encodes approximately 2000 predicted protein coding sequences. The genome contains a large number of genomic islands, mainly pathogenicity islands, that contain genes responsible for mediating horizontal gene transfer, contributing to the genetic diversity of GBS (9).

Neonatal GBS Disease Pathogenesis and Maternal Asymptomatic Colonization

Colonization

In healthy adults, GBS is commonly found colonizing the outer mucus layer of the colon as well as the small intestine in addition to the genital tract. Although GBS is found in vaginal, rectal, and fecal samples, the site at which GBS predominantly colonizes is unknown (16). Overall colonization rates are the same between males and females; however, colonization rates greatly vary with site of isolation (16–19). In addition, vaginal colonization has been shown to be transient, but colonization of the lower gastrointestinal tract is more constant (20). It has been reported that up to 30% of women are vaginally colonized by GBS and approximately 50-70% of babies born to those women will become colonized (2).

The first step in neonatal GBS disease progression is asymptomatic colonization of vaginal epithelial cells. Heavy maternal colonization is a primary risk factor for EOD (21). The vaginal microbiota plays a key role in preventing disease through competitive exclusion, lowering the pH, and producing a number of antibacterial compounds, but is also very dynamic and can be greatly influenced by a number of factors including pH, hormone levels, and age (22). Therefore, GBS must employ a number of strategies to persist in this changing environment. Indeed, the Serine Rich Repeat proteins (Srr-1 and Srr-2), PilA, and CovR have all been identified in playing a role in colonization of vaginal epithelial cells (23, 24) and are discussed in more detail below.

Vertical transmission from mother to the newborn can result from invasive GBS that ascends the vaginal tract of a pregnant woman to infect through the extraplacental membranes to cause infection *in utero* or via aspiration of infected vaginal fluid while the baby passes through the birth canal and leads to EOD. The pathogenesis of LOD is not well understood (4, 5). After the baby inhales either infected amniotic or vaginal fluid, GBS infects the lungs where it then adheres to and invades lung epithelial cells. From the lungs, GBS can gain access to the bloodstream causing sepsis. In the most severe cases, GBS is able to breach the blood-brain barrier and cause meningitis (4). The severity of disease is attributed to susceptibility of the newborn and the ability of GBS to avoid immunologic clearance and adapt to the changing environments throughout disease progression.

Host Cell barriers to Infection

Throughout its dissemination through the body, GBS encounters a number of physical host cell barriers that it must be able to cross. GBS has been shown to attach to extracellular matrix components and adhere to and invade a variety of human cells *in vitro* including epithelial cells, endothelial cells, and cells of the extraplacental membranes, which includes the decidua, chorion, and amnion (25–29). A number of virulence factors have been identified that are involved in attachment and invasion of human cells, including surface proteins, pilus islands (PI), and secreted proteins.

There have been two distinct pilus loci, PI-1 and PI-2, identified in GBS; there are also two variants of PI-2: PI-2a and PI-2b. These PIs encode structurally different pili, each of which contain a backbone of PilB subunits with a PilA subunit at the end and a PilC protein at the base of each pilus (30, 31). Pili are involved in attachment, invasion, and biofilm formation (32). PilA was previously shown to mediate attachment to brain microvascular endothelial cells while PilB mediated invasion (33) Additionally, PilA but not PilB of PI-2a was found to play an important role in attachment to vaginal epithelial cells (23), but PI-1 does not mediate attachment to vaginal epithelial cells (34).

ScpB is a surface expressed protein that plays a dual role in pathogenesis: it cleaves and inactivates the human complement component C5a and serves as an invasin by binding to epithelial cells and fibronectin (35). BsaB is a recently defined adhesin that also promotes fibronectin binding (36). Fibrinogen binding is mediated by FbsA and FbsB; FbsA has been shown to play a role in adherence (37), while FbsB is an invasin (38). Laminin binding is mediated by the adhesin Lmb, which also plays a role in adherence to brain microvascular endothelial cells (39, 40). Srr-1 and Srr-2

mediate attachment to epithelial cells by interacting with human keratin 4 of the surface of the epithelium (24, 41). Although the two Srr loci function similarly, they share only 20% identity and Srr-2 is specific to ST-17 strains (42). LrrG is a surface expressed protein that adheres to epithelial cells, elicits protective immunity in a mouse model, and is highly conserved among GBS strains, suggesting it could make a strong vaccine candidate (43). BibA, and its ST-17 specific homologue HvgA, are both adhesins (44, 45). Other invasins include Spb1, a ST-17 specific PI-2b backbone protein (46), and SfbA (47).

Although a number of virulence genes have been identified in GBS, their regulation is poorly understood. Genome sequencing has shown that GBS has 17-20 two-component systems to regulate genes, but only five of them have been characterized. These are CovR/S, DltR/S, RgfA/C, and CiaR/H. GBS also has at least six stand-alone transcriptional regulators, but only four have been examined: MtaR, RogB, RovS and Rga (39).

The Neonatal Immune System

The severity of neonatal disease is attributed to susceptibility of the newborn and the ability of GBS to avoid immunologic clearance and adapt to the changing environments throughout disease progression. Infants generally become infected by GBS during the first three months of life (39), suggesting that the immaturity of the immune system likely plays a role in susceptibility to infection. A greater understanding of the interaction between GBS and the neonatal immune system will aid in the development of novel therapies or preventative measures for invasive disease.

Deficiencies in neonatal immunity

Deficiencies in the newborn immune system give them a heightened susceptibility to infectious diseases as the newborn immune system is relatively underdeveloped, which leads to a reduced number of available immune cells. Moreover, neonatal immune cells can be present in different proportions in different sites relative to adult immune cell populations (48). The neonatal immune system is also relatively naïve resulting in a lack of pre-existing memory immune cells, which leads to a dependency on maternal transfer of antibodies. The newborn immune system is also biased towards the production of anti-inflammatory cytokines (49). A thorough understanding of these deficiencies is an important step to help protect neonates from invading pathogens, such as GBS.

Innate immunity deficiencies. Since the adaptive immune system has had limited exposures to antigens *in utero* resulting in a deficient adaptive immune response, neonates mainly rely on their innate immune response to pathogens. Neutrophils are one of the main phagocytes found in the blood and act as a first line of defense against infection. However, the neutrophil storage pool is much lower than that of adults and neonatal rats challenged with GBS developed neutropenia and neutrophil storage pools rapidly become depleted (50). In addition to the small pool of stored neutrophils, neonatal neutrophils show impaired rolling adhesion, transmigration, and chemotaxis, resulting in poor recruitment to infection sites (51). Moreover, neutrophils from both preterm and term neonates have reduced phagocytic ability compared to that of adult neutrophils, but become comparable to adult neutrophils by three days after birth (52).

Neonatal neutrophils are capable of producing functional neutrophil extracellular traps, but the response is delayed and requires extended stimulation, making them less able to aid in clearing pathogens (53, 54). These findings show that not only is there decreased neutrophil recruitment to sites of infection, but also the neutrophils that do make it there are deficient in their ability to clear infection, making neonates particularly susceptible to infection within the first few days of life.

In contrast to neutrophils, the number of monocytes are much higher in neonates compared to adults and even higher in preterm neonates compared to term neonates (55, 56); the phagocytic ability of neonatal monocytes is the same as adults (52). Monocyte chemotaxis and recruitment to the site of infection, however, is attenuated (57) and cytokine concentrations are lower in neonatal monocytes resulting in a reduction in inflammatory responses (58). Neonatal monocytes have reduced levels of major histocompatibility complex (MHC) class II expression on their surface, resulting in diminished capacity for antigen presentation (59). Toll-like receptor (TLR) mediated signal transduction pathways are also impaired in neonatal monocytes resulting in reduced activation of NF-κB, an important transcription factor involved in immune response regulation (60).

Once monocytes travel to tissues, they differentiate into macrophages. Alveolar macrophage populations are much lower in newborns relative to adults, however the number rises to adult levels 24-48 hours after birth (61). Since inhalation of GBS during birth is the main predisposing mechanism for pneumonia, the initial reduced number of macrophages in the lungs makes them unable to rapidly clear the infection resulting in EOD. In mice, neonatal murine macrophages have reduced expression of genes

associated with antigen processing and presentation, including reduced expression of MHC class II, CD11b, CD14, CD80, and CD86 as well as TLR2, TLR4, and TLR9 compared to adult macrophages; their ability to induce T-cell proliferation was also reduced (62). Moreover, neonatal macrophages have a delayed response in recruiting neutrophils and monocytes to the site of infection (61), yet migration, chemotaxis, and production of reactive oxygen intermediates are normal in neonatal macrophages relative to adult macrophages (63). Upon stimulation through TLRs 1, 2, and 4, neonatal macrophages have enhanced production of IL-6 (64), demonstrating that neonatal macrophages are capable of secreting proinflammatory cytokines despite their other deficits.

Many different subsets of dendritic cells (DCs) can be found in the body and vary in phenotype, function, and tissue location. Although DCs are highly specialized, potent antigen presenting cells (65), cord blood DCs have been reported to be very immature. Cord blood DCs are unable to stimulate either adult or cord blood mononuclear or T cells; however, cord blood mononuclear and T cells were responsive to adult DCs indicating a deficit in the cord blood DCs (66). In addition, cord blood DCs have decreased expression of MHC I and II, ICAM-1/CD54, CD40, CD80, CD 83 and CD86, which is indicative of immaturity (66, 67). DCs stimulated through TLR7/9 have a reduced ability to produce IFN α/β, which are important immune regulators. This deficiency is as a result of reduced translocation of the transcription factor interferon regulatory factor (IRF) 7 into the nucleus (68). However, stimulated cord blood monocyte derived DCs have similar levels of NF-κB signaling and secretion of the proinflammatory cytokines TNF-α, IL-6, and IL-8 compared to adult DCs (69).

In addition to the reduced numbers and function of neonatal innate immune cells, the complement system is also underdeveloped. Depending on the activation stimuli, the complement system can be activated either through the classical, alternative, or lectin pathway through a cascade of enzymatic reactions. All three pathways result in the formation of the membrane attack complex (MAC) that forms a channel in cell membranes resulting in cell lysis. Additionally, throughout the cascade, a number of enzymatic intermediates and cleavage products are formed that play a role in the immune response, such as immune cell activation or bacterial cell opsonization (70). Complement proteins cannot be transferred from mother to fetus across the placenta and the number of neonatal complement proteins are only 10-80% of those found in adults (71). More specifically, classical pathway components C1q, C3, and C4 as well as the alternative pathway components properdin and factor B are deficient in neonates (71–73). These deficiencies in the neonatal complement system result in reduced ability to activate the complement cascade, thereby leading to reduced phagocytosis, ability to lyse pathogens, and recruitment of immune cells to sites of infection (71).

Adaptive immunity deficiencies. Since the innate immune system is responsible for activating the adaptive immune system, deficiencies in the innate immune system can lead to a reduced adaptive immune response. Additionally, there are a number of deficiencies and differences in neonatal adaptive immune cells relative to adults. Although neonates are capable of mounting an adaptive immune response, the response can range from no response to a similar response to that of adults (74). Although neonates mainly rely on their innate immune response to pathogens within the

timeline of GBS transmission, understanding how neonates differ in their adaptive immune response compared to adults can greatly influence vaccine development efforts.

T-cells can be classified into different subclasses that play specific roles in the immune response. $CD4^+$ T-cells, also known as T helper (Th) cells, play an important role in activating or stimulating maturation of other immune cells and can be further differentiated into other subtypes, with the two major subtypes being Th1 and Th2. Th1 cells play an important role in producing an inflammatory response to microbial pathogens, whereas Th2 cells secrete cytokines that play a role in response to parasites and allergens. Interestingly, the neonatal immune system has a much larger population of Th2 cells and diminished numbers of Th1 cells (75). In addition to the reduced number of Th1 cells, neonates also have reduced numbers or even a complete lack of Th17 cells, which play a role in developing immunity to both bacterial and fungal infections at mucosal surfaces (76).

Neonates have been shown to have a defective B cell response resulting in deficient humoral immunity as well. This defective B cell response could be due to an immature development of the surface immunoglobulins (Ig) and the lack of exposure to antigens. Additionally, follicular Th (TFH) cells play an important role in developing an antibody response by aiding in the proliferation and maturation of B cells. Neonates have reduced frequency of TFH cells, which are regulated by IL-4 production by Th2 cells (77). Moreover, B cell signaling through the B cell receptor (BCR) is deficient in neonatal B cells that can be caused by the higher expression of CD22, a negative regulator of BCR signaling in neonatal B cells (78).

Despite these deficiencies in adaptive immunity, neonates have protective antibodies that are passed on from mother to neonate either transplacentally or through breast milk. These maternal antibodies play an important role in protecting the neonate from infection, but can also impact the neonatal immune response to infection as well as vaccination (79). A study that examined specific antibody concentrations at birth and after immunization found an inverse correlation between birth concentrations and increases in antibody concentration after immunization. These data suggest that high antibody concentrations at birth could inhibit the neonatal immune response to vaccines. Nonetheless, most of the neonates in this study did develop antibodies and hence, there is not complete inhibition of neonatal antibody development in the presence of maternal antibodies (80).

Immune Response to GBS and Mechanisms of Immune Evasion

Recognition of GBS by the innate immune system

GBS is able to elicit a strong inflammatory response. Upon GBS infection, neonatal monocytes produce pro-inflammatory cytokines, including TNF and IL-6, but at reduced levels compared to adult monocytes (81). GBS activates phagocytes via interaction with TLR2 and TLR6, and this activation is dependent on the TLR adaptor protein myeloid differentiation factor 88 (MyD88) (82, 83). Additionally, transcriptional activation of inflammatory cytokines in response to GBS requires the c-Jun kinase pathway (84). Phagosomal GBS induces interferon in DCs via TLR7, MyD88 and the transcription factor IRF1 (85). Furthermore, GBS ssRNA is recognized by monocytes

and macrophages via a complex comprising of MyD88 and UNC-93B (86). Recognition of GBS ssRNA results in increased production of nitric oxide (NO) by host cells, which activates macrophages and aids phagosome acidification (87). The presence of GBS DNA has also been shown to induce release of IL-6, IL-12, and TNF-α via TLR9, but did not upregulate IFN-β or NO secretion (88). Elevated levels of TNF-α occurs during GBS sepsis, which is believed to play a role in clinical outcomes. GBS can induce TNF-α release from both monocytes and macrophages. Deposition of complement on GBS, more specifically C3 activation via the alternative pathway, triggers TNF-α production by monocytes (89). Additionally, monocytes are the most abundant innate immune cell in neonates, which could contribute to the abundance of monocyte derived TNF-α production (61).

GBS produces a surface associated β-hemolysin/cytolysin (β-h/c) toxin that is encoded by the *cyl* operon and is a major virulence factor (90). Not only does GBS β-h/c contribute to pathogenicity through its cytolytic properties and by promoting invasion across host cell barriers, it also stimulates a potent pro-inflammatory cytokine response by stimulating release of IL-1 and IL-6 as well as NO production in macrophages (91). Activation of the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome by GBS is dependent on expression of β-h/c. Inflammasomes are multiprotein complexes located inside innate immune cells that activate the immune system in response to pathogens through activation of caspase-1, which leads to an inflammatory response (92).

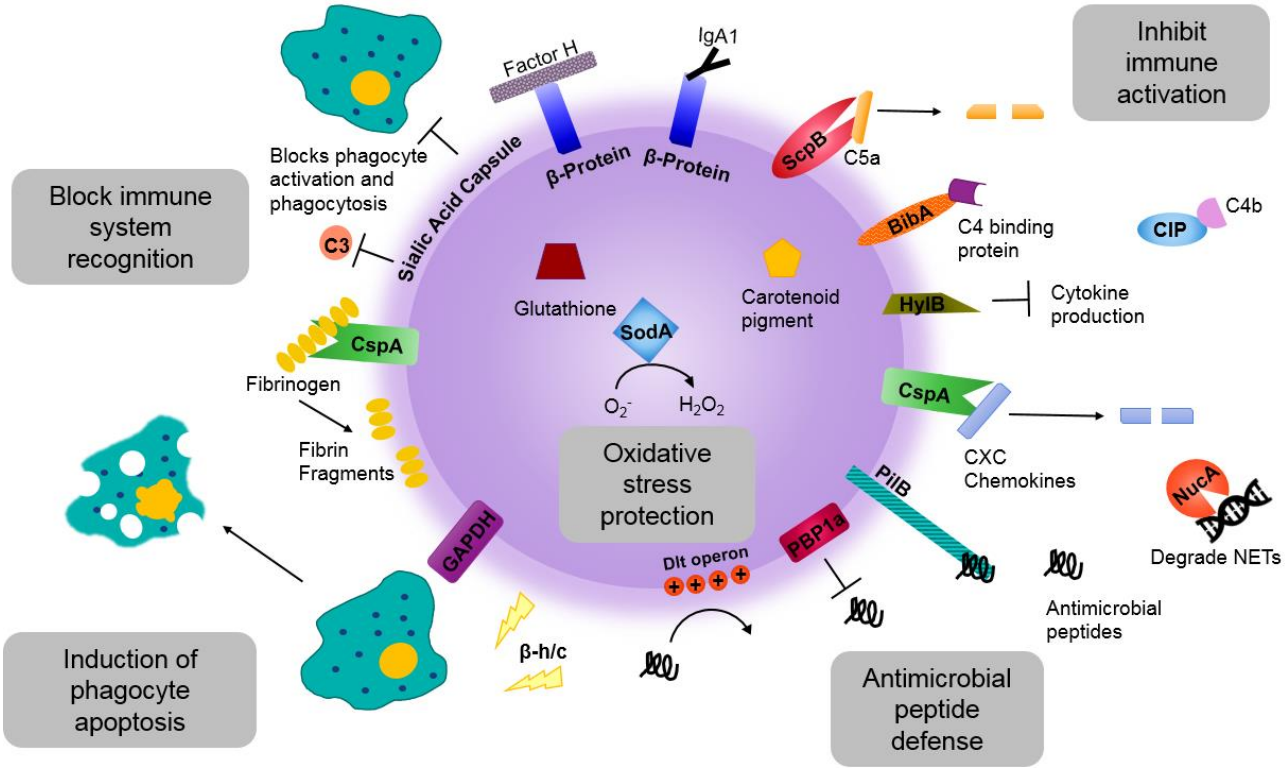
Antibody response to GBS

Because of the large number of deficits in the neonatal innate immune system, maternal antibody transfer is very important in passive immune protection of the newborn. Deficiency in low maternal antibodies targeting GBS has been considered to be important for neonatal infections (93). Moreover, CPS type III strains induce a lower antibody response compared to other CPS types (94). Human colostrum and milk contain very high concentrations of secretory IgA, suggesting an important role of IgA in protective immunity in newborns. The roles of IgA are to both recognize pathogens and trigger a response to eliminate them. Once IgA recognizes a pathogen, it interacts with CD89 on the surface of phagocytes to induce phagocytosis, reactive oxygen species (ROS) production, and production of inflammatory mediators (95). The GBS surface expressed β protein also plays a role in binding to the Fc region of IgA, which inhibits IgA binding to CD89 and blocks the proactive immunity from maternal IgA (96).

GBS immune system evasion

GBS employs several mechanisms to resist immune detection and phagocytosis, thereby increasing the chance of survival in the host. These mechanisms are summarized in Figure 1.1. One example is the expression of the capsule polysaccharide (CPS), which is considered a major virulence factor as unencapsulated GBS strains are less virulent in animal models (97). The GBS capsule contains a terminal sialic acid (Sia). Since Sia is also present on the surface of vertebrate cells, the Sia on the surface of GBS allows it to mimic host cells and avoid immune detection (98).

Figure 1.1. Mechanisms used by GBS to evade the immune system. GBS expresses many factors that help it evade the immune system and increase its survival in the host. The sialic acid capsule and fibrin fragments cleaved by CspA that coat the surface helps GBS present as "self" to the immune system. The capsule also blocks C3 deposition and recognition by phagocytes. Sialic acid in the capsule, β-protein, ScpB, BibA, and CIP inhibit the complement system by binding or cleaving complement components. The GBS β-protein also binds the Fc region of IgA1 to inhibit immune activation. HylB and CspA inhibit or cleave cytokines, while PilB, PBP1a, and proteins encoded by the Dlt operon assist in resisting antimicrobial peptides. NucA degrades the DNA matrix of neutrophil extracellular traps (NETs). Glutathione, carotenoid pigment and SodA all aid in defense against reactive oxygen species, and the β-hemolysin/cytolysin (β-h/c) and GAPDH both aid in inducing apoptosis in phagocytes.



Sia-binding immunoglobulin-like lectins (Siglecs) are located on the surface of leukocytes and are responsible for distinguishing between self and nonself to determine if an immune response should be activated. The Sia in the GBS capsule binds to Siglecs in order to reduce the activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling, thus inhibiting an immune response. Siglec-9 expressed on the surface of human neutrophils recognizes Sia on the surface of GBS and dampens the immune response (99). Additionally, the surface expressed β protein of GBS binds to hSiglec-5 in a Sia dependent manner (100). GBS binding to Siglecs results in impairment of phagocytosis, reduced oxidative burst, and poor formation of extracellular traps in leukocytes (99, 100). Macrophages lacking Siglecs have enhanced production of proinflammatory cytokines, phagocytosis, and bacterial killing of GBS (101).

Macrophages also express sialoadhesin on their surface, which is a unique type of Siglec with an elongated extracellular portion that is able to recognize Sia on the surface of pathogens and mount an inflammatory response. Sialoadhesin plays an important role in clearing GBS infection and block organ dissemination in mice (102). Another mechanism of host cell mimicry employed by GBS is by coating itself with the highly adhesive fibrin breakdown product of fibrinogen. GBS uses the cell surface protein CspA to cleave fibrinogen in a similar manner to thrombin resulting in exposure of the regions responsible for fibrinogen polymerization that leads to aggregation of GBS as well as coating of the GBS surface with fibrin. This fibrin coating allows GBS to appear as “self” to host immune cells and reduces the access of opsonins to the bacterial surface inhibiting opsonophagocytosis (103).

The CPS can also inhibit opsonophagocytosis by blocking C3b deposition on the bacterial surface. Both unencapsulated strains and encapsulated strains lacking sialic acid bind more C3 molecules compared to the wild type (WT) strain (104). GBS also expresses other surface components that prevent opsonophagocytosis as well as activation of the complement cascade. BibA, for example, resists opsonophagocytic killing by neutrophils via the specific binding of the C4-binding protein, a regulator of the complement pathway (44). The secreted complement interfering protein (CIP) binds to C4b inhibiting its interaction with C2 to reduce complement activation through the classical and lectin pathways, but not the alternative pathway (105). Similarly, the GBS β-protein binds the soluble complement inhibitor factor H to the bacterial surface in a way that inhibits C3b deposition and opsonophagocytosis (106); the Sia residues in the CPS can also bind factor H (107). Another important factor is a serine protease, ScpB, which is a C5a peptidase that proteolytically cleaves the complement-activated C5a, a powerful chemoattractant involved in the recruitment of inflammatory cells (35). In addition to its ability to cleave fibrinogen mentioned earlier, CspA also cleaves and inactivates CXC chemokines that recruit neutrophils to different infection sites (108).

Neutrophil extracellelar traps (NETs) are produced by neutrophils in response to invading bacteria and consist of DNA and antimicrobial peptides (AMPs). These NETs ensnare the bacteria and eliminate them to help clear infections (109). The GBS Nuclease A (NucA) degrades the DNA in the NETs to allow GBS to escape. Moreover, NucA was needed for GBS to persist in lung tissue and a *nucA* mutant was less virulent compared to the WT in a mouse model, suggesting that NucA is important for both initial infection as well as dissemination (110).

In response to tissue injury following pathogen invasion, for instance, hyaluronan (HA), a component of the extracellular matrix, is quickly degraded by host hyaluronidases and ROS (111). The small cleavage products are recognized by TLR2 and/or TLR4 to stimulate an inflammatory response to clear the pathogen as well as initiate wound healing (112, 113). GBS secretes hyaluronidase encoded by *hylB* to degrade HA to assist dissemination. Interestingly, HylB plays a role in enhancing survival inside macrophages, inhibiting proinflammatory cytokine expression, and utilizing HA as a carbon source in the host (114). The GBS hyaluronidase degrades HA into disaccharides instead of 4-16-mer fragments that produce a proinflammatory response. These HA disaccharides are capable of blocking TLR2/4 signaling resulting in reduced proinflammatory cytokine production (115).

Phagocytic uptake of GBS

Despite the mechanisms to avoid immune detection and phagocytosis described above, GBS is easily phagocytosed and killed by phagocytic cells in the presence of serotype specific antibodies via Fc receptors (116). Internalization of GBS can also occur through the complement receptor CR3 in the presence of other opsonins like lectins and L-ficolin (117). Since GBS elicits a poor antibody response and neonates have low levels of complement, opsonin-independent pathways of phagocytosis would be the more likely uptake mechanism of GBS. Additionally, GBS is rapidly taken up by macrophages in the absence of opsonins (116). Because CR3 is important for opsonin independent phagocytosis by macrophages, GBS was suggested to interact with CR3 in a C3-independent manner (118). Furthermore, uptake of GBS requires actin (116). In

addition to the complement binding domain, CR3 also contains a lectin domain that has been shown to interact with the type III CPS to initiate phagocytosis in neutrophils (119).

GBS induction of apoptosis in macrophages

One strategy to avoid immune activation after a pathogen is taken up by a phagocyte as well as persist at the site of infection is to induce apoptosis that kills the immune cell before it can become activated (120). Apoptosis is a process of programmed cell death that is less likely to induce a strong inflammatory response, such as that seen with necrosis or pyroptosis. However, there are certain cases in which apoptosis can be inflammatory (121). Because apoptosis plays a role in maintenance of cell populations in tissues as well as during development and aging, it is a tightly regulated process. This process involves Protein Kinase C (PKC) activity and modulation of cytoplasmic calcium levels, and is regulated by the caspase family of cysteine-directed proteases (caspase-dependent pathway) or calpains (caspase-independent pathway) as well as Bcl-2 family regulators (122).

GBS is one of the pathogens capable of inducing apoptosis in macrophages. This induction requires internalization of GBS and is bacterial dose dependent (123). During GBS induction of apoptosis, GBS stimulates persistent and sustained activation of c-Jun NH2-terminal kinase (JNK) and p38, but inhibits extracellular signal-regulated kinase (ERK), all three of which are members of the MAPK family (124). Moreover, GBS infection of macrophages also induces expression of TNF-α, IL-1, and inducible nitric oxide synthase (iNOS) leading to apoptosis. Inhibiting iNOS expression inhibited GBS induced apoptosis, but inhibiting TNF-α and IL-1 did not. Also, adding NO alone without

infection induced apoptosis indicating a direct effect of the GBS induced NO production in apoptosis (125).

The role of caspases in GBS induced apoptosis is not clear. One study showed GBS induced apoptosis was independent of caspase-1 and -3 (123), whereas another study showed caspase-3 and -9 were important for the process (125). These contradictory results could be due to the studies using different GBS strains; both used serotype III strains, but different strains of the same serotype have been shown to have varying host-pathogen interactions (126). Therefore, it is possible that different GBS strains use different mechanisms for inducing apoptosis.

Through β-h/c induced plasma membrane permeability, GBS is able to cause a massive increase in calcium levels inside macrophages leading to activation of the calcium sensitive calpains, which leads to degradation of structural and regulatory cytoskeletal proteins as well as induction of apoptosis (127, 128). GBS induced calcium influx also results in PKC activation (123) as well as activation of gelsolin, an important regulator of actin cytoskeleton and apoptosis (129). Glyceraldehyde 3-phosphate dehydrogenases (GAPDH) are enzymes that are capable of binding to host cell components and have immunomodulatory effects. Interestingly, GAPDH from GBS and other pathogens including *S. pyogenes* and *Staphylococcus aureus*, can induce apoptosis in macrophages, indicating yet another role of bacterial GAPDH in pathogenesis (130).

GBS survival inside phagocytes

Once a bacterium is taken up by a phagocytic cell, it gets trapped within a vacuole that goes through phagosomal maturation involving a series of fusion and fission events with compartments in the endocytic pathway resulting in a fully mature phagolysosome. The phagolysosome is a harsh, highly acidic and nutrient limiting environment where antimicrobial peptides, ROS, and reactive nitrogen species (RNS) are generated to kill the bacterium (131). Although most bacteria are efficiently killed by this process, many pathogens have developed ways to overcome these defense mechanisms. For instance, some pathogens can disrupt cellular signaling to prevent or slow down the phagosome maturation process and live inside the phagosome. Other pathogens can escape from the phagosome by lysing the membrane to replicate in the cytosol, while others can remain inside the phagolysosome by defending against the many stressors (132).

GBS is capable of persisting within macrophages and remains inside the phagosome, which recruits late endosomal markers. This recruitment indicates that GBS does not inhibit phagosome maturation as a survival strategy and likely uses a phagosomal stress defense mechanism (116, 133). This ability to survive inside innate immune cells allows GBS to avoid immune detection, protect against antibiotics, and facilitate dissemination to other sites of the body, making it a particularly important topic of study (134, 135). Interestingly, opsonization of GBS significantly reduces the ability of GBS to survive intracellularly (116). Although the CPS helps GBS avoid phagocytosis, it does not aid in intracellular survival. Indeed, unencapsulated mutants were found to get

internalized at a higher rate in a prior study, though the time surviving intracellularly was no different than the encapsulated WT (136).

GBS has several strategies to help it survive the antimicrobial conditions of the phagosome. Upon infection, macrophages undergo a number of changes in protein expression that result in decreased expression of enzymes impacting ROS production and NO synthesis, both of which are important for antimicrobial responses. Since these changes were not observed in macrophages infected with heat inactivated GBS, it is likely that GBS actively induces these changes (137). In addition to its ability to inhibit ROS production, GBS also has the ability to inactivate ROS through the use of superoxide dismutase (SodA), which converts superoxide into oxygen and hydrogen peroxide (138). Although GBS is catalase negative, genome sequencing shows GBS could potentially detoxify hydrogen peroxide using NADH peroxidase, a thiol peroxidase, and an alkylhydroperoxide reductase; however these have yet to be examined in GBS (9). Moreover, GBS has been shown to produce glutathione (139), which protects the bacterial cell from oxidative stress and low pH as well as other stresses (140). The cyl operon that encodes for β-h/c production also produces an orange carotenoid pigment, which has also been shown to protect GBS from oxidative damage (141).

In addition to ROS and RNS production, a number of AMPs and hydrolases are present in the phagosome to kill bacteria (142). The penicillin binding protein (PBP1a), for example, is important for resisting host AMPs (143). One mechanism to avoid the effect of cationic AMPs is to reduce the electronegativity of the cell membrane to decrease the affinity of the cationic AMPs. GBS is capable of doing this via the *dlt*

operon by increasing the number of D-alanine residues in the cell wall lipoteichoic acids (144). Additionally, GBS pili have been shown to mediate resistance to AMPs in addition to aiding in host cell attachment. PilB plays a role in intracellular survival by conferring resistance to cathelicidin and defensin families of AMPs and facilitates bloodstream survival in a mouse model. Moreover, expressing GBS PilB in *Lactococcus lactis*, which is susceptible to AMPs, conferred resistance to AMPs (145). The pilus backbone protein specific to ST-17 lineages, Spb1, was also shown to enhance both phagocytosis and intracellular survival of GBS. Additionally, the presence of *spb1* in GBS strains did not alter NO or TNF-α responses in the macrophages (146). Although having a protein that would enhance phagocytic uptake of the pathogen seems counterintuitive, that same protein can also be used to enhance survival inside macrophages while promoting dissemination. Spb1 along with several other ST-17 specific virulence factors may partly explain the enhanced virulence of this particular lineage and its association with neonatal infections (147).

As a lactic acid-producing bacterium, GBS has mechanisms to withstand low pH and should be expected to withstand the low pH of the phagosome. Indeed, a prior study demonstrated that ~18% of the genes in the GBS genome were differentially expressed at pH 5.5 relative to pH 7.0, and most of these genes are regulated by CovR/S (148). In addition to regulating many virulence factors, this CovR/S acid response regulator was found to be required for GBS to survive inside macrophages (133). Some of the genes upregulated in low pH encode for transporters, which may allow GBS to increase its scavenging ability to facilitate survival in the nutrient limiting conditions of the phagosome.

Summary

GBS is a highly versatile organism that causes invasive disease in neonates as well as elderly and immunocompromised adults. Since GBS is a leading cause of neonatal sepsis and meningitis, many studies have focused on these infections. The steady rate of EOD in neonates despite current preventative measures as well as high frequencies of antibiotic resistance emphasizes the need to find additional or alternative therapeutics and preventatives. Additionally, the current preventative practice of IAP has not had an effect on the rate of LOD. In order to better tailor the efforts in developing new therapeutic and preventive measures, a thorough understanding of the interactions between GBS and the immune system is required. The neonatal immune system has a number of deficiencies and limitations that render neonates more susceptible to infection. Furthermore, GBS has an arsenal of immune evasion strategies and virulence factors that make it an extremely successful pathogen in the neonate. A thorough understanding of these factors will be important in effective vaccine prevention strategies. Moreover, further identification and characterization of virulence factors will help identify targets for new therapeutic measures.

In an effort to better understand GBS pathogenesis and why certain GBS strains are more virulent than others, the primary objective of my dissertation work is to examine variation in host-pathogen interactions at various stages of neonatal disease progression among diverse GBS strains, with a primary focus on comparing ST-17 strains to strains of other lineages. If we have a better understanding of what makes these hypervirulent strains better able to cause infections, we can better tailor preventative and therapeutic measures to combat these highly infectious strains. With

this in mind, I first determined the variation in the ability of diverse GBS genotypes to associate with decidual cells and lung epithelial cells using strains of STs 17, 19, and 23. Second, I examined mechanisms of persistent colonization of mothers after receiving IAP by comparing a ST-17 strains, which persisted and a ST-17 strain that was eradicated by IAP. This project focused on two possible mechanisms: antibiotic tolerance and survival inside macrophages. Finally, I used RNAseq to identify key factors important for survival inside macrophages and begun characterizing two of the genes identified in this analysis.

CHAPTER 2

ASSOCIATION AND VIRULENCE GENE EXPRESSION VARY AMONG SEROTYPE III GROUP B *STREPTOCOCCUS* FOLLOWING EXPOSURE TO DECIDUAL AND LUNG EPITHELIAL CELLS

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Abstract

Group B *Streptococcus* (GBS) causes severe disease in neonates, the elderly and immunocompromised individuals. GBS is highly diverse and can be classified by serotype and multilocus sequence typing. Sequence type (ST)-17 strains more frequently cause invasive neonatal disease. Attachment and invasion of host cells is a key step in GBS pathogenesis. We investigated whether four serotype III strains representing STs 17, 19, and 23 vary in their ability to attach to and invade both decidual cells and lung epithelial cells. Virulence gene expression following host cell association and exposure to amnion cells was also tested. The ST-17 strains varied in their ability to attach to and invade decidual cells whereas there were no differences with lung epithelial cells. The ST-19 and ST-23 strains, however, attached to and invaded decidual cells less than both ST-17 strains. Although the ST-23 strain attached to lung epithelial cells better than ST-17 and -19 strains, none effectively invaded the lung epithelial cells. Notably, the association with host cells resulted in the differential expression of several virulence genes relative to basal expression levels. Similar expression patterns of some genes were observed regardless of cell type used. Collectively, these results show that GBS strains vary in their ability to attach to distinct host cell types and express key virulence genes that are relevant to the disease process. Enhancing our understanding of pathogenic mechanisms could aid in the identification of novel therapeutic targets or vaccine candidates that could potentially decrease morbidity and mortality associated with neonatal infections.

Introduction

Group B *Streptococcus* (GBS) is a leading cause of neonatal sepsis and meningitis, and is transferred from mothers to babies *in utero* or during childbirth (149). Approximately 30% of women are asymptomatically colonized with GBS and roughly 50-70% of babies born to those women will become colonized. Neonatal GBS infections are divided into two classes of disease: early-onset (EOD) and late-onset disease (LOD). EOD occurs within the first few days of life and LOD occurs between one week and three months of age (4). Current prevention practices rely on antibiotic prophylaxis administered to colonized mothers prior to childbirth. Although these efforts have been successful in preventing EOD, the prevalence of LOD remains the same. In addition, screen-and-treat approaches do not provide a safeguard against premature birth due to invasive GBS infections. Therefore, the identification and development of alternative preventative measures, such as vaccines and drug targets are greatly needed (2).

GBS can be classified into ten distinct serotypes based on capsular polysaccharide (CPS), with types Ia, III and V more often associated with disease (2, 8). GBS can be further classified using multilocus sequence typing, which examines the allelic profile of seven conserved genes and groups the strains into sequence types (STs) providing a classification based on the genetic backbone (11). Serotype III ST-17 GBS have been shown to cause a higher frequency of neonatal disease than other STs (12–15).

GBS, like many other pathogens, needs to cross physical barriers within the host to cause disease. Progression of GBS disease involves initial maternal colonization of vaginal epithelial cells, dissemination across extraplacental membranes, causing

chorioamnionitis, and across neonatal lung epithelial cells, bloodstream survival, and, in cases of meningitis, penetration of the blood-brain barrier (142). Infection of the newborn is a result of either invasive GBS that ascends the genital tract to infect through the extraplacental membranes to cause infection *in utero* or aspiration of infected vaginal fluid as the baby passes through the birth canal (4). In order to cross these anatomical barriers to infection, GBS must be able to adhere to and invade the host cells that comprise these barriers. Previous studies have shown that GBS effectively adheres to and invades epithelial and endothelial cells. Additionally, GBS of different serotypes vary in their ability to associate with host cells (25–27, 29, 37, 150); however, these studies have selected strains based on CPS type rather than ST. Because CPS is horizontally transferred between strains and there is evidence of capsule switching (10, 151), selecting strains based on ST, or genetic backbone, is warranted. Comparing the hypervirulent lineage, ST-17, with other lineages in their ability to attach to and invade host cells will facilitate the identification of factors that play an important role in GBS disease development.

In this study, the level of GBS attachment and invasion of two barriers that are typically encountered during the early stages of an infection was determined. These barriers include: decidual cells, which make up the outer layer of the extraplacental membranes, and lung epithelial cells, one site of inoculation in neonates during passage through the birth canal or during aspiration of contaminated amniotic fluid *in utero*. Four Serotype III GBS strains representing STs 17, 19 and 23 were compared to quantify differences in association with decidual and lung epithelial cells across and within phylogenetically distinct lineages. Additionally, the expression of known virulence genes

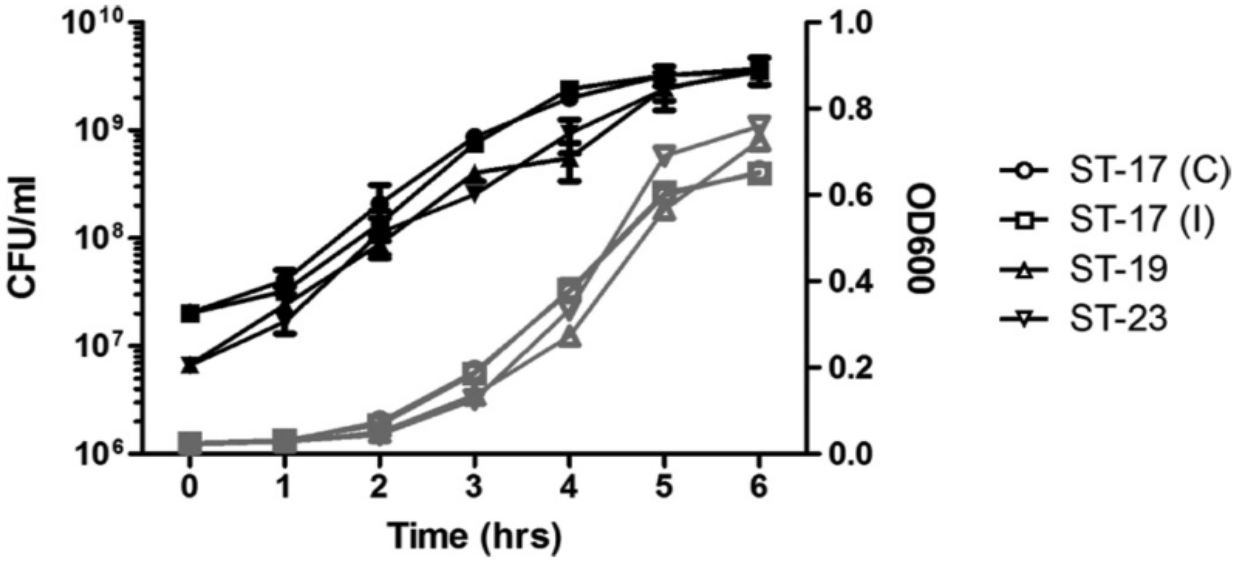
was examined in each strain upon association with host cells to better understand the role these factors play in GBS pathogenesis and identify gene targets useful for guiding disease prevention strategies.

Results

Bacterial growth rates did not differ among GBS isolates

Bacterial growth rate has previously been shown to influence the invasiveness of GBS in respiratory epithelial cells (152). Therefore, it was important that the strains used in this study all exhibited similar growth rates to reduce the effect of this parameter on the experiments. To determine the growth characteristics of the four strains used in this study, each strain was grown in the infection medium used for the association assays over a six hr period and the OD_{600} and CFU/ml were determined every hr (Figure 2.1). No significant differences in growth rate were observed for all four strains. Similar results were observed when grown in THB (data not shown).

Figure 2.1. **Bacterial growth curve to compare growth rates across strains.** Bacterial cultures of the four strains used in this study were incubated at 37°C for a 6hr period during which the cultures were sampled every hr to determine CFU/ml (black lines) and OD_{600} (gray lines). C=Colonizing, I=Invasive.

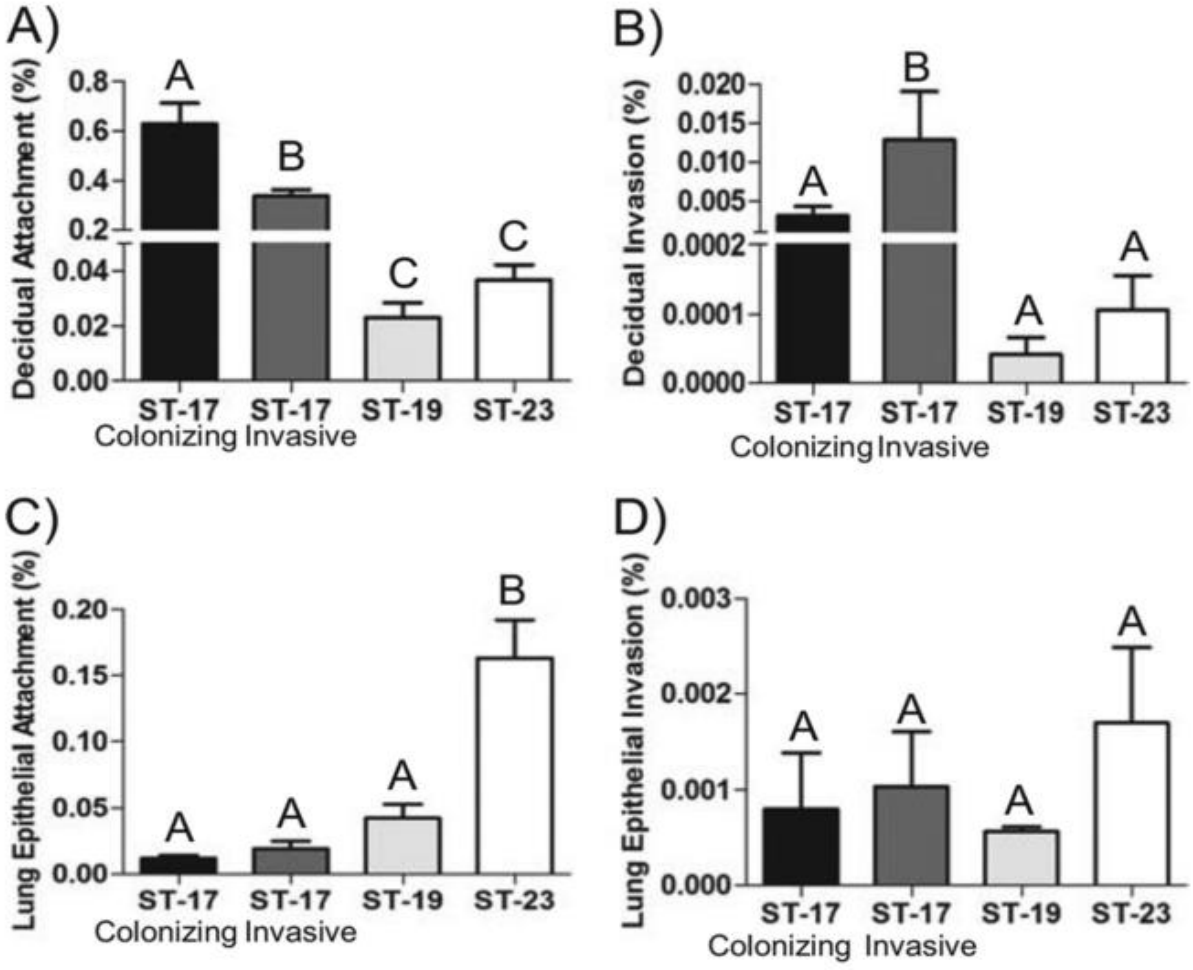


Association with host cells varies among serotype III GBS strains

Previous studies have shown that GBS of different serotypes vary in attachment and invasion of host cells, but few have compared GBS strains of the same serotype. Association assays were performed to determine if serotype III strains varied in their ability to attach to and invade host cells. Decidualized human endometrial stromal cells were used as an extraplacental membrane invasion model, and A549 lung epithelial cells were used as a model for infection through the lungs.

The hypervirulent lineage, ST-17, is most often associated with neonatal sepsis and meningitis. It is known that ST-17 strains have a greater propensity to cause severe disease than other lineages (12–15); therefore, we first compared strains within this lineage. Two ST-17 GBS isolates were selected: one colonizing strain isolated from a vaginal rectal swab of a healthy pregnant woman, and one invasive strain isolated from a newborn baby with septicemia. Interestingly, significantly more bacteria of the colonizing strain attached to decidual cells compared with the invasive strain ($P<0.01$), whereas the invasive strain invaded the decidual cells significantly more than the colonizing strain ($P<0.05$; Figures 2.2A and B). No difference was seen in the ability of either strain to attach to lung epithelial cells (Figure 2.2C) and neither strain effectively invaded the lung epithelial cells (Figure 2.2D). Overall, attachment and invasion of decidual cells was much higher than lung epithelial cells.

Figure 2.2. GBS association with decidual and lung epithelial cells. Host cell lines were infected with GBS at a multiplicity of infection (MOI)=1 for 2hr. The number of bacteria is expressed relative to the total number of bacteria in the well after the infection. A) Attachment to and B) invasion of decidual cells. C) Attachment to and D) invasion of lung epithelial cells. Bars represent mean ± SD. Bars labeled with different letters are significantly different from each other. Experiments were repeated in triplicate at least three times.



In a previous study, it was reported that both ST-17 and ST-19 strains were more often associated with invasive disease, but ST-23 strains were linked to asymptomatic colonization (15). To determine if variation exists in the ability to associate with host cells across these diverse GBS genotypes of the same serotype, the same association

assays were used to compare two strains representing STs 19 and 23 to the colonizing and invasive ST-17 strains. The ST-23 strain attached to lung epithelial cells significantly more than the ST-19 strain ($P<0.001$). However, there was no difference in attachment to decidual cells and neither strain effectively invaded either cell type (Figure 2.2). Both strains had an enhanced ability to attach to the lung epithelial cells compared to the decidual cells. Additionally, both ST-17 strains attached to decidual cells significantly more than either the ST-23 or ST-19 strains ($P<0.01$) (Figure 2.2A), whereas the ST-23 strain attached to the lung epithelial cells more than strains of STs 19 and 17 ($P<0.001$) (Figure 2.2C).

Virulence gene expression did not vary among ST-17 strains

Next, the expression of several known virulence genes was tested upon either attachment to or invasion of host cells in order to examine the effect host cells have on bacterial virulence gene expression and to determine if differential expression of these genes could help explain some of the differences seen in host cell association. The complete list of genes tested and their functions is shown in table 2.1. Although no significant differences in gene expression were detected between the two ST-17 strains, eight of the 12 genes tested were differentially expressed upon associating with host cells relative to basal gene expression (Figure 2.3). Of note, *fbsb* was highly upregulated under all conditions tested and both *hvgA* and *lrrG* were highly downregulated upon invasion of both cell types.

Table 2.1. Virulence factors examined during host cell association and invasion.

Category	Gene	Product	Function/Characteristics	Reference
Attachment	*srr*	Serine-rich repeat protein	Surface adhesins; two genetically distinct variants: *srr1* and *srr2. srr2* is specific to ST-17 strains.	(41, 42)
	bibA	Immunogenic bacterial adhesin	Binds human C4-binding protein to resist opsonophagocytosis; promotes adherence to epithelial cells.	(44)
	hvgA	Hypervirulent GBS adhesin	Homologous to BibA; occurs on the same genetic locus; specific to ST-17 strains	(45)
	lrrG	Leucine-rich repeat protein	Binds epithelial cells and elicits protective immunity.	(43)
	lmb	Laminin-binding protein	Promotes GBS colonization and translocation into bloodstream.	(39, 40)
	scpB	C5a peptidase	Dual function: cleaves and inactivates complement; promotes binding to epithelial cells and fibronectin.	(35)
Invasion	*spb1*	Surface protein of GBS	Pilus backbone of Pilus Island (PI)-2b; promotes invasion of epithelial cells; specific to ST-17 strains.	(46, 146)
	fbsB	Fibrinogen-binding protein B	Promotes invasion into epithelial cells.	(38)
	cylE	CylE protein	Predicted to function as a N-acyltransferase in the biosynthesis of the GBS pigment granadaene required for hemolytic/cytolytic activity of GBS.	(153)
	iagA	Invasion associated gene	Anchors lipoteichoic acid to the cell membrane. Involved in invasion of brain microvascular endothelial cells	(154)

Table 2.1. (cont'd)

Category	Gene	Product	Function/Characteristics	Reference
	sip	Surface immunogenic protein	Surface protein that elicits cross-protective immunity.	(155)
Other	*ponA*	Penicillin-binding protein 1a	Promotes resistance to antimicrobial peptides.	(39)
	cylX	CylX protein	Homologous to component of acetyl CoA carboxylase. Predicted to function in biosynthesis of GBS pigment granadaene.	(153)

To determine whether the ST-17 strains behave similarly in a more physiologically relevant cell type than immortalized cell lines, both strains were exposed to amnion cells isolated from the extraplacental membranes of three women after childbirth. Following bacterial RNA extraction and gene expression profiling, we observed upregulation of four genes (*srr2, lmb, fbsB* and *cylX*) that were also upregulated in response to decidual and lung epithelial cell exposure in one or both of the strains (Figure 2.3A and C). Expression of each gene was then compared across host cell exposures. No significant differences were observed in gene expression for the invasive ST-17 strain across all three cell types (Figure 2.4A); however, two genes differed for the colonizing ST-17 strain (Figure 2.4B). *fbsB* was upregulated by at least 2-fold when exposed to all cell types, but the magnitude of the fold change was significantly lower with amnion cells compared to decidual cells. Additionally, *cylX* was significantly downregulated with amnion cells where expression was unchanged with decidual and lung epithelial cell attachment.

Figure 2.3. Virulence gene expression in ST-17 strains. The expression of 12 virulence genes was determined upon attachment to and invasion of host cells in a ST-17 colonizing strain and ST-17 invasive strain. The genes were categorized as attachment, invasion or other according to their function (see table 2.1). Only the genes with at least a 2-fold change in expression relative to basal expression are shown here. A) Gene expression upon attachment to and B) invasion of decidual cells. C) Gene expression upon attachment to and D) invasion of lung epithelial cells. Bars represent mean ± SD of three independent experiments.

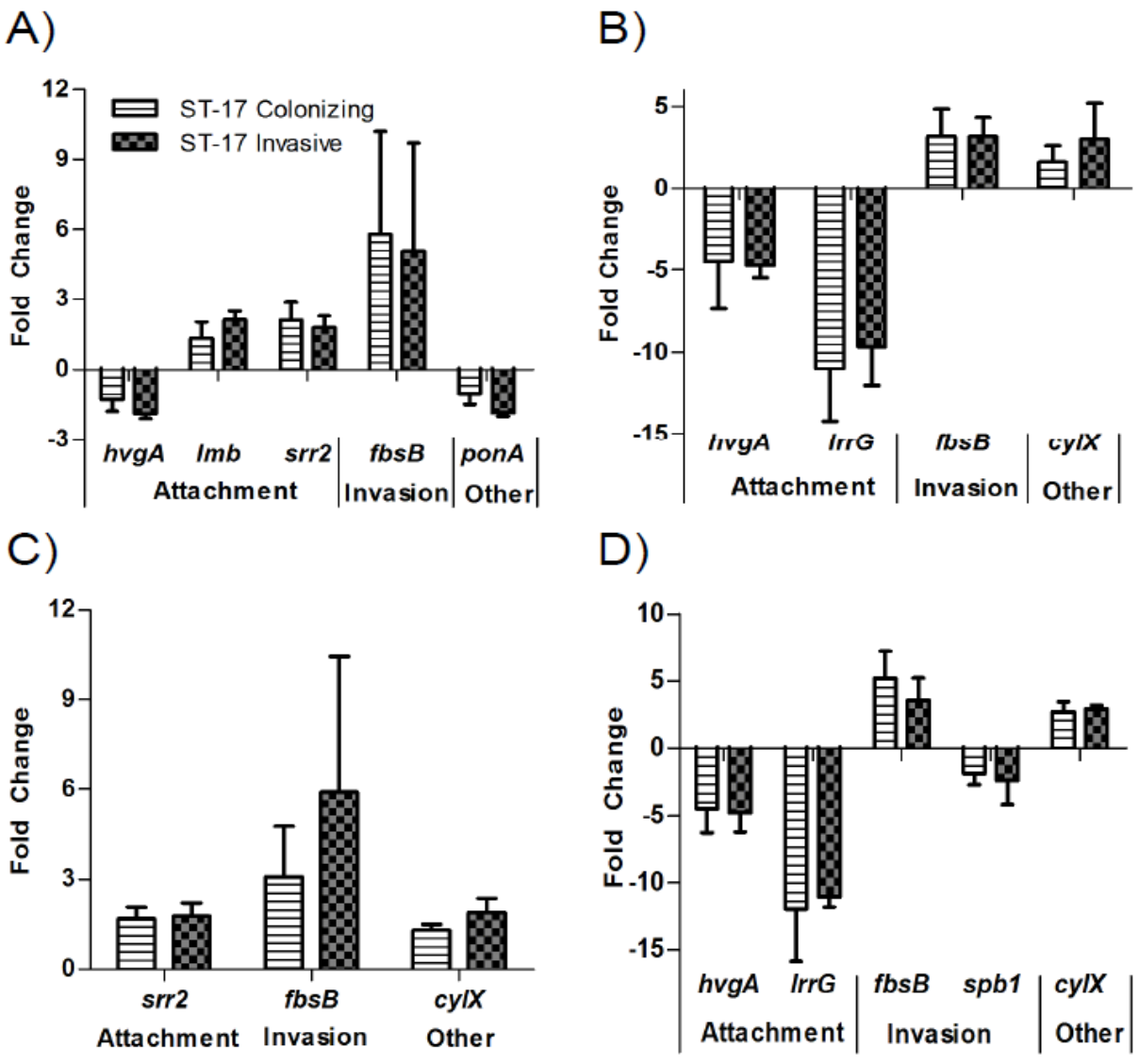
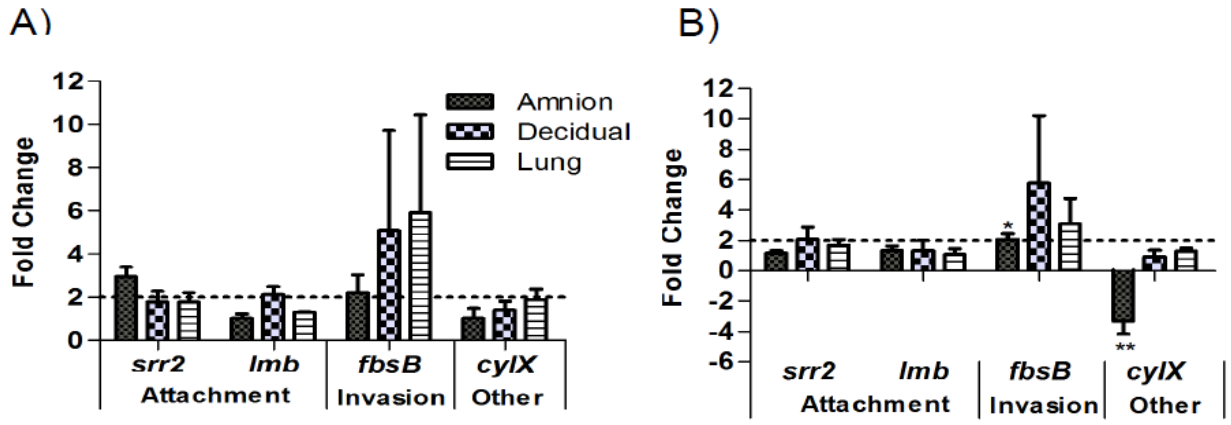


Figure 2.4. GBS ST-17 virulence gene expression compared across cell exposure types. The expression of four virulence genes upregulated upon host cell attachment (Figure 2.3) were examined after exposure to amnion cells isolated from human placental tissues and compared to expression after decidual and lung epithelial cell exposure. The genes were categorized as attachment, invasion or other according to their function (see table 2.1). A) Virulence gene expression of the invasive ST-17 strain. B) Virulence gene expression of the colonizing ST-17 strain. Dashed lines mark 2-fold change in expression. Bars represent mean ± SD of three independent experiments. Asterisks indicate significant difference in gene expression between exposures for each gene (* P<0.05 ** P<0.01).



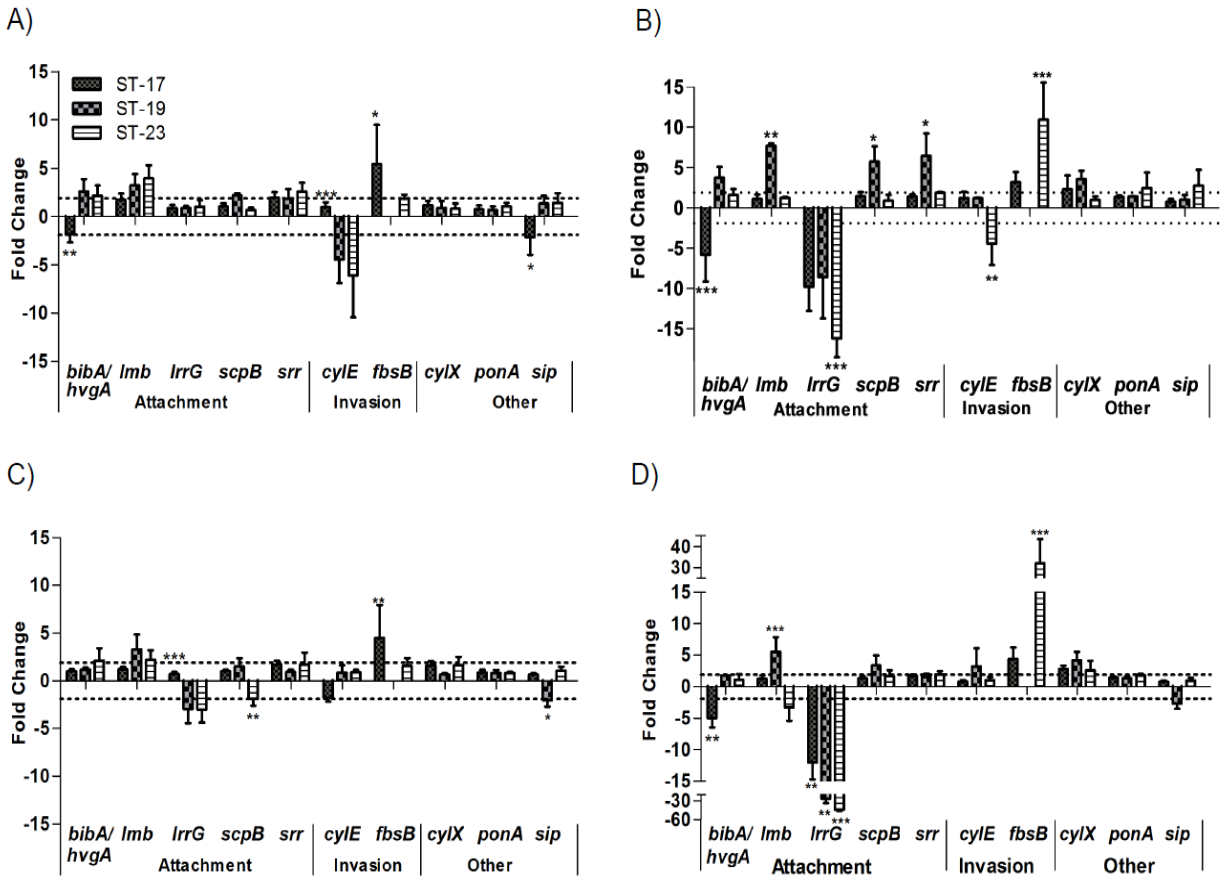
Differential expression of virulence genes across GBS STs

To determine if gene expression varies across diverse STs, virulence gene expression was compared between strains of STs 17, 19 and 23. Since no significant differences in gene expression were observed between the two ST-17 strains, the data were combined for this comparison. Upon attachment to decidual cells, seven of the genes tested were differentially expressed by at least 2-fold in one or more of the STs (Figure 2.5A). No significant differences in gene expression were detected between the ST-19 and ST-23 strains for all genes, whereas expression of four genes significantly

differed in the ST-17 strains compared to STs 19 and 23. Although the ST-17 strains attached to decidual cells more than the ST-19 and -23 strains in the association assays, none of the attachment genes examined were upregulated in the ST-17 strains relative to the other strains. Additionally, the ST-19 and ST-23 strains upregulated expression of *bibA* while both ST-17 strains downregulated *hvgA*, the homologue of *bibA* unique to ST-17 (45). Upon invasion of decidual cells, 10 of the virulence genes tested were differentially expressed (Figure 2.5B). No significant difference in expression was detected between the three STs for *cylX, ponA,* and *sip,* whereas one strain had significantly different expression levels for the remaining genes.

Attachment to lung epithelial cells resulted in differential regulation of seven virulence genes tested in this study (Figure 2.5C); of these, no significant difference in expression was detected among the strains for three genes. Additionally, the expression patterns for *lmb, srr*, and *fbsB* were the same upon attachment to both cell types. Although there was no difference in lung epithelial cell invasion among the strains in the association assays (Figure 2.2D), invasion resulted in differential regulation of the same 10 genes that were differentially regulated following decidual cell invasion (Figure 2.5D). Specifically, expression of *bibA/hvgA, lrrG, fbsB, cylX,* and *ponA* in response to lung epithelial cells showed a similar pattern to that observed upon decidual cell invasion. Of the 10 genes differentially regulated, six of them showed no significant difference in expression among the strains.

Figure 2.5. Virulence gene expression across GBS STs. The expression of known virulence genes was determined upon attachment to and invasion of host cells using ST-17, ST-19 and ST-23 strains. The genes were categorized as attachment, invasion or other according to their function (see table 2.1). A) Gene expression upon attachment to and B) invasion of decidual cells. C) Gene expression upon attachment to and D) invasion of lung epithelial cells. Dashed lines mark 2-fold change in expression. Expression of *iagA* was examined but not included because it was not differentially expressed under any condition. Bars represent mean ± SD of the results of three independent experiments. Asterisks indicate significant difference in gene expression levels between the STs for each gene (* P<0.05 ** P<0.01 *** P<0.001).



Discussion

Several studies have previously reported that unique GBS strains vary in their ability to attach to and invade host cells (25–27, 29, 37, 150). However, the strains tested in the prior studies were chosen based on serotype, a phenotypic characterization dictated by capsular polysaccharide genes, rather than a genotypic characterization, such as ST. The present study newly examined the ability of diverse GBS STs of the same serotype (serotype III) to associate with host cell types representing different anatomical barriers GBS would encounter early during neonatal infection. The three STs used in this study were ST-17, ST-19, and ST-23, which represent the most common genotypes worldwide (11). ST-17 was suggested to be a hypervirulent lineage because of its association with neonatal meningitis when compared to other lineages. Because ST-19 strains are also more often associated with disease than asymptomatic colonization, comparing between two invasive and distinct lineages is important. ST-23 strains are more often associated with asymptomatic colonization and therefore provide a useful comparison between invasive and colonizing STs.

The two ST-17 strains varied in their ability to associate with decidual cells. The colonizing strain attached more than the invasive strain while the invasive strain invaded more than the colonizing. This shows that two strains of the same ST can vary in their ability to associate with host cells. However, attachment and invasion of lung epithelial cells was the same for both strains and much lower than that of decidual cells. Additionally, the ST-17 strains attached to decidual cells more than the ST-19 and ST-23 strains, but the ST-23 strain attached to lung epithelial cells more than the ST-17 and

ST-19 strains. Overall, few differences in invasive ability were detected across the three STs. Collectively, these data show that there is variation in association with host cells within the same serotype and suggest that strains of the same phylogenetic lineage also vary in their ability to associate with host cells. Moreover, association ability of GBS appears to be specific to the host cell type. Further studies using larger numbers of strains of each ST are needed to further characterize the ability to associate with host cells of each ST. In addition, it is difficult to draw conclusions about invasive vs. colonizing strains because all invasive strains begin as asymptomatic colonizers.

One limitation of this study is that the invasion assay used in this study only accounts for the bacteria that pass through the cell layer using the transcellular route even though there are other routes that could be used by GBS to cross anatomical barriers (156). In a previous study using electron microscopy, Soriani, *et al*. found that GBS uses a paracellular route to cross cervical epithelial cells. The majority of GBS were found in the spaces between the cells with very few passing through the cells themselves (157). Therefore, variation in invasive ability among GBS strains needs to be further tested using other assays that account for the paracellular route of invasion as well. Additionally, the cells used in this study are not polarized and thus may not accurately represent the barriers *in vivo*.

Differential regulation of virulence genes in response to host cell association was also examined in this study. Interestingly, even though differences in association with decidual cells were detected between the colonizing and invasive ST-17 strains, there were no differences in expression of the virulence genes tested, showing that GBS strains belonging to the same phylogenetic lineage have similar transcriptional

responses upon attachment to and invasion of decidual and lung epithelial cells. This suggests that the differences in association could be either due to genetic variation in the genes themselves rather than differences in gene regulation or as a result of other unknown virulence factors. Additionally, we examined the expression of four genes in the ST-17 strains following exposure to primary amnion cells and compared them to lung epithelial and decidual cell exposure. Overall, gene expression was similar across all cell types showing that using immortalized cell lines to assess GBS gene expression in response to host cells accurately represents the response to primary cells from human tissues.

Comparing gene expression across all strains tested in this study showed that expression of several virulence genes upon association with distinct host cells differs among strains of the same serotype. Three of the five attachment genes examined in this study, *lmb, scpB, lrrG,* along with one other gene, *sip*, have been considered to be potential vaccine targets (2). These four genes are highly conserved and therefore have the potential to elicit protective immunity across GBS serotypes.

The laminin-binding protein, encoded by *lmb*, assists colonization by adhering to the extracellular matrix protein, laminin, and has been shown to play a role in adherence to brain microvascular endothelial cells (39, 40). The present study shows that expression of *lmb* is induced in response to host cell attachment and that there is no significant difference in this response across the three STs tested suggesting that *lmb* could be a potential vaccine candidate. *scpB* encodes a dual function serine protease that promotes adherence to epithelial cells and helps evade the host immune system by cleaving the human complement component C5a (35). Interestingly, even though ScpB

appears to be a good vaccine candidate, gene expression of *scpB* is upregulated in response to host cell association in only the ST-19 strain and is actually significantly downregulated in the ST-23 strain in lung epithelial cell attachment. *lrrG* encodes a highly conserved leucine-rich-repeat surface protein that has been shown to elicit protective immunity in mice and recombinant LrrG protein adheres to epithelial cells *in vitro*, thus making it a strong vaccine candidate (43). However, in the present study expression of *lrrG* is highly downregulated upon host cell invasion suggesting that *lrrG* is not expressed early on during GBS infections. LrrG is likely recognized by host cells in order to avoid immune system detection; therefore, GBS downregulates the expression of *lrrG*. The surface immunogenic protein (Sip), encoded by *sip*, is a protein of unknown function, but is conserved across GBS serotypes and elicits protective immunity in mice (155). In the present study, expression of *sip* upon host cell attachment was either not significantly changed or downregulated by 2-fold suggesting that it does not play an important role in host cell attachment. Further studies need to be done to better understand its role in pathogenesis. Upon invasion of decidual cells, expression of *lmb, scpB,* and *srr* increased in the ST-19 strain though expression either decreased or remained the same in the ST-17 and 23 strains relative to expression upon attachment to decidual cells. This suggests that the ST-17 and 23 strains no longer need these genes for invasion and turn off their expression while the ST-19 strain keeps their expression high.

Interestingly, decidual cell attachment resulted in upregulation of *bibA* in the ST-19 and 23 strains although *hvgA,* the *bibA* homologue, was downregulated in both ST-17 strains. This expression pattern was also observed upon invasion of both decidual

and lung epithelial cells. *bibA* and *hvgA* are located at the same genomic locus and the regulatory regions are highly conserved between the two genes with >90% sequence identity (45). Therefore, the difference in expression observed between these two genes may be more likely due to differences in the gene regulators between the different STs rather than differences in the genes themselves.

Expression of *fbsB* was upregulated upon attachment to host cells and even higher upon invasion of host cells for both ST-17 strains and the ST-23 strain. The high upregulation of *fbsB* upon invasion of host cells is consistent with a previous study that showed FbsB is important in invasion of epithelial cells, but not attachment (38). The ST-19 strain showed no expression of *fbsB* for any of the samples tested in this study. Upon further investigation of the *fbsB* gene sequence in this strain, we found that the *fbsB* gene contains an inversion of one segment of the gene and an insertion compared to the NEM316 sequence. Further investigation is required to determine if a functional protein is produced and if this sequence is conserved among other ST-19 strains.

The presence of host cells induced differential gene expression in all strains of GBS tested in this study. Although a number of genes were differentially expressed among the three STs, there was no clear connection between difference in ability to adhere to and invade host cells with expression of virulence genes. Future studies using whole transcriptome analysis would be helpful in explaining the differences in the ability to associate with host cells and identifying other pathways that are important for adherence to and invasion of host cells among GBS isolates.

One limitation of examining differential gene expression in response to host cells is that gene expression is very dynamic and the time at which the RNA is sampled could

affect which genes we see expressed. In this study, we sampled bacterial RNA at the same time point (after a two hr infection) for each experiment; therefore it is possible that some of the genes we did not see differentially expressed could have been expressed at a different time during the infection. For a more complete examination of the transcriptional response to host cells, a full time course may be more appropriate. In addition, there could be differences in protein expression that are not shown by mRNA expression due to post-translation regulation, which could help explain some of the differences shown in the association assays. Future studies assessing the patterns of protein expression could, therefore, be beneficial.

The current study provides a comparison of GBS strains based on genetic backbone, rather than serotype, a phenotypic characteristic. The results show that genetically distinct GBS strains of the same serotype vary in their ability to attach to and invade host cells and differentially express key virulence genes during host cell association. In addition, two strains of the same genotype vary in their ability to attach to and invade host cells, but do not differentially express key virulence genes, suggesting that other, unknown virulence genes are involved in this process. Because these strains were classified by MLST, it is quite possible that additional genetic characteristics are partly responsible for differences observed. Indeed, Tettelin et al. demonstrated that 20% from each sequenced strain is only partially shared or strain specific (158). Further studies with larger numbers of strains for a full representation of genotypes and whole genome transcriptome analysis will aid in identifying additional candidate genes important for attachment to and invasion of host cells.

Materials and Methods

Bacterial strains and growth conditions

Four GBS strains were used in this study, all of them serotype III: GB112, a ST-17 strain isolated from a follow-up vaginal rectal screen of a woman who recently gave birth (159); GB411, a ST-17 strain isolated from a newborn with septicemia (160); GB590, a ST-19 strain isolated from a vaginal rectal screen of a pregnant woman (159); and the genome strain NEM316 (ATCC 12403), a ST-23 strain isolated from a newborn with septicemia. Strains were cultured in Todd-Hewitt broth (THB) or agar (THA) or on sheep's blood agar plates (BD) at 37^{o}C with 5% CO_2.

Cell culture

The cell line A549 (ATCC CCL-185), a human alveolar epithelial carcinoma cell line was maintained by incubating at 37^{o}C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Hyclone) and 2% penicillin/streptomycin (pen/strep; Gibco). The human endometrial stromal cell line, T HESC (ATCC CRL-4003), was cultured in DMEM/ Nutrient Mixture F-12 Ham with L-glutamine (Sigma) supplemented with 1.5 g/L sodium bicarbonate, 1% BD ITS+ Universal Culture Supplement Premix, 10% charcoal treated FBS (Hyclone) and 2% pen/strep (referred to as HESC medium).

Association assays

T HESCs were first decidualized as previously described (161). Briefly, cells were grown to approximately 50-70% confluence then seeded into a 6 well plate and treated with 0.5mM 8-bromo-cAMP (Sigma) for three to six days. Decidualization was confirmed by examining the expression of prolactin and IGF binding protein 1, which are upregulated following decidualiation. A549 cells were seeded into a 6 well plate in DMEM containing 2% FBS the day before the experiment. Assays were not performed until the cells reached 100% confluency so that no part of the bottom of the well was exposed since the bacteria attached effectively to the plates. Bacterial strains were grown in THB to mid log phase, washed once with phosphate buffered saline (PBS) and resuspended in infection medium (HESC medium with 2% charcoal treated FBS, no ITS+, and no antibiotics). The same infection medium was used for both cell lines for comparisons in association across cell lines without factoring in effect of the medium used. Prior to infection, host cells were washed three times with PBS. They were then infected with GBS strains in the infection medium at a MOI of one bacterial cell per host cell. After two hr incubation at 37^{o}C with 5% CO_2, wells were washed three times with PBS to remove non-adherent bacteria.

To determine the number of associated bacteria (attached and invaded), host cells were lysed with 0.1% Triton X-100 (Sigma) for 30 min at 37^{o}C. Lysates were gently vortexed to further disrupt the host cells and liberate intracellular bacteria. After serial dilution, lysates were then plated on THA, incubated overnight at 37^{o}C, and colony forming units (CUFs) were counted. To test invasion, once non-adherent bacteria were washed away as described above, infection medium containing 100µg/ml of gentamicin

(Gibco) and 5µg/ml of Penicillin G (Sigma) was added to each well and incubated at 37ºC for one hr to kill extracellular bacteria. Wells were then washed with PBS two times and intracellular bacteria were enumerated as described above for associated bacteria. The number of attached bacteria was calculated by subtracting the number invaded from the number associated. All data were expressed as percent of the total number of bacteria per well after the two hr infection. Assays were run in triplicate at least three times.

Amnion cell isolation

Human extraplacental membranes were collected from healthy, non-smoking, singleton pregnancies undergoing scheduled cesarean delivery prior to onset of labor at the University of Michigan Birth Center as previously described (162). The University of Michigan Institutional Review Board approved this research (IRBMED# HUM0037054). Immediately following delivery, the membranes were transported to the lab in Dulbecco's PBS (DPBS). Membranes were rinsed with DPBS and blood clots removed. Membranes were then blunt dissected to separate the choriodecidua from the amnion. Amnion tissue was used to isolate amnion cells using methods adapted from three protocols (163–165). Briefly, amnion was digested with 0.25% trypsin-EDTA (Gibco) at 37ºC for 30 min. Amnion tissue was transferred to fresh trypsin-EDTA and the above digestion was repeated. Following each digestion, the trypsin-EDTA was neutralized with medium (DMEM:F12 supplemented with 10% FBS and pen/strep). Cells were pelleted by centrifugation, washed in medium, pelleted again, and resuspended in medium containing epidermal growth factor (EGF; Peprotech) (DMEM:F12

supplemented with 10% FBS, pen/strep, and 10 ng/mL EGF). Amnion cells were seeded at 500,000 cells/ well (12-well plates) in 1 mL medium, and grown to 70-80% confluence and medium was changed on day two of culture (DMEM:F12 supplemented with 10% FBS, and 100 ng/mL EGF without antibiotics). Viability of amnion cells prior to plating was assessed using trypan blue. After two days of culture, cell morphology and growth were assessed. Cells were infected on day three after forming a monolayer.

RNA preparation and real-time PCR (qRT-PCR)

Bacterial RNA was isolated from samples in the association assay described above. The four types of samples collected were for basal activity (growth in cell culture media with no exposure to host cells), bacterial cells in suspension (in the media, not attached to host cells), bacteria associated with host cells, and bacteria that invaded host cells. Samples used for RNA extraction were prepared using RNAprotect Bacteria Reagent (Qiagen) and the RNeasy Mini Kit (Qiagen) as described in the Enzymatic Lysis, Proteinase K Digestion and Mechanical Disruption of Bacteria protocol in the RNAprotect handbook with the addition of incubation with mutanolysin during the Proteinase K and lysozyme treatment. RNA samples were then treated with the Turbo DNA-free Kit (Ambion) and checked for DNA contamination by PCR without the addition of reverse transcriptase. cDNA was synthesized from 1µg RNA with random primers using the iScript Select cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed in a 15µl reaction using iQ™ SYBR Supermix (Bio-Rad) and 10µM each of gene specific primers. The list of primers used can be found in Table S1 in the appendix. Amplification and detection of specific products were performed using the CFX384

Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 1 cycle of 3 min at 95^{o}C and 39 cycles of 95^{o}C for 10 s and 60^{o}C for 30sec. Relative gene expression was calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$ method) (166) with *gyrA* as the internal control gene and normalizing expression relative to basal levels of expression. A 2-fold change in gene expression was considered significant.

Statistical Analysis

Data are reported as means ± standard deviations and are either pooled from or representative of at least three independent experiments with triplicates. GraphPad Prism 5.0 was used for statistical analysis. For the GBS growth rates and association assays, statistical analysis was performed using one-way ANOVA and Tukey's posttest. For gene expression, statistical analysis was performed using two-way ANOVA and Bonferroni's posttest. Differences with P values <0.05 were considered statistically significant.

Acknowledgments

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CHAPTER 3

DIFFERING MECHANISMS OF SURVIVING PHAGOSOMAL STRESS AMONG GROUP B STREPTOCOCCUS STRAINS OF VARYING GENOTYPES

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Abstract

Group B *Streptococcus* (GBS), a leading cause of neonatal sepsis and meningitis, asymptomatically colonizes up to 30% of women and can persistently colonize even after antibiotic treatment. Previous studies have shown that GBS resides inside macrophages, but the mechanism by which it survives remains unknown. Here, we examined the ability of four GBS strains to survive inside macrophages and then focused on two of the strains, belonging to sequence type (ST)-17 and ST-12, to examine persistence in the presence of antibiotics. A multiple stress medium was also developed using several stressors found in the phagosome to assess the ability of 30 GBS strains to withstand phagosomal stress. The ST-17 strain was more readily phagocytosed and survived intracellularly longer than the ST-12 strain, but the ST-12 strain was tolerant to ampicillin unlike the ST-17 strain. Exposure to sub-inhibitory concentrations of ampicillin and erythromycin increased the level of phagocytosis of the ST-17 strain, but had no effect on the ST-12 strain. In addition, blocking acidification of the phagosome decreased the survival of the ST-17 strain indicating a pH-dependent survival mechanism for the ST-17 strain. Congruent with the macrophage experiments, the ST-17 strain had a higher survival rate in the multiple stress medium than the ST-12 strain, and overall, serotype III isolates survived significantly better than other serotypes. These results indicate that diverse GBS strains may use differing mechanisms to persist and that serotype III strains are better able to survive specific stressors inside the phagosome relative to other serotypes.

Introduction

Commonly found as a commensal in the gastrointestinal and genitourinary tracts of up to 30% of adults, group B *Streptococcus* (GBS) is also a leading cause of neonatal sepsis and meningitis (167). GBS can be classified into ten serotypes based on the capsular polysaccharide (CPS): Ia, Ib and II through IX. These ten antigenically distinct CPS types play a role in GBS virulence, with types Ia, Ib, II, III, and V most often causing disease (8, 10). GBS isolates can be further characterized using a multilocus sequence typing (MLST) system that groups strains into phylogenetically distinct lineages, or sequence types (STs). Several studies have shown that serotype III strains belonging to ST-17 are more often associated with neonatal disease, indicating that ST-17 strains may be inherently more virulent than strains of other GBS lineages (11–15). Additionally, a previous study that examined the GBS colonization status of pregnant women before and after delivery showed that serotype III ST-19 and ST-17 strains were more likely to persistently colonize women after receiving intrapartum antibiotic prophylaxis (IAP), whereas ST-12 strains were more frequently lost (7). This ability of certain GBS STs to persist suggests an enhanced ability to evade the effect of antibiotics, either through enhanced antibiotic tolerance or protection via biofilm production or uptake into host cells. In addition, the capability of GBS to both colonize individuals as a commensal and cause disease in susceptible hosts indicates a heightened ability to evade host immune responses.

Several pathogens have developed mechanisms to survive inside macrophages, the innate immune cells designed to eliminate pathogens, thus evading the immune response of the host (132). Pathogens are taken up by macrophages and other

phagocytes through a process called phagocytosis, which contains the pathogen in a vacuole inside the macrophage. The vacuole then goes through phagosome maturation and becomes highly acidic as antimicrobial peptides, reactive oxygen species (ROS), and reactive nitrogen species (RNS) are generated to kill the bacteria. A number of pathogens have developed a variety of ways to overcome these defense mechanisms, including disruption of cellular signaling to prevent or slow down phagosomal maturation, phagosomal escape by lysing the membrane to replicate in the more favorable cytosol, and the production of enzymes to protect against ROS and RNS (132). GBS was shown to survive inside macrophages within the mature phagosome (116), but little is known about the mechanism used for intracellular survival. Since many antibiotics poorly penetrate eukaryotic cells, the ability to survive inside macrophages may provide protection from antibiotics and allow the bacterium to recolonize the host following antibiotic cessation. Additionally, residing inside phagocytic cells has been suggested to facilitate the dissemination of bacterial cells to other sites of the body via the bloodstream or penetration of host tissue barriers (134, 135).

This study examines the ability of distinct lineages of GBS to tolerate antibiotics, survive inside macrophages, and express virulence genes important for survival in each condition. Survival in a synthetic multiple stress medium comprising common phagosomal stressors was also developed to compare strains of varying serotypes, genotypes, and sources. Identifying bacterial factors important for persistent colonization and dissemination could aid in the discovery of therapeutic targets aimed at eradicating the commensal GBS population, which is particularly critical for pregnant women with an increased risk of transmitting GBS to their newborns.

Results

GBS genotypes differ in level of phagocytosis by macrophages and intracellular survival

Since macrophages play an important role in controlling bacterial levels in the host, we examined phagocytic uptake and intracellular survival of four GBS strains: NEM316, GB00590, GB00112, and GB00653 (Table 3.1). Importantly, the GBS strains representing diverse lineages varied in their interactions with PMA treated THP-1 macrophages. NEM316 and GB112, which belong to ST-23 and ST-17, respectively, were phagocytosed the most followed by GB590 (ST-19) and GB653 (ST-12) (Figure 3.1A). At 24 hr, the ST-23 strain had the greatest ability to survive inside the macrophages followed by the strains belonging to STs 17, 19, and 12, which had the lowest ability to survive (Figure 3.1B). Together these data show that distinct lineages of GBS vary in their ability to be phagocytosed by macrophages and survive intracellularly for up to 24 hr.

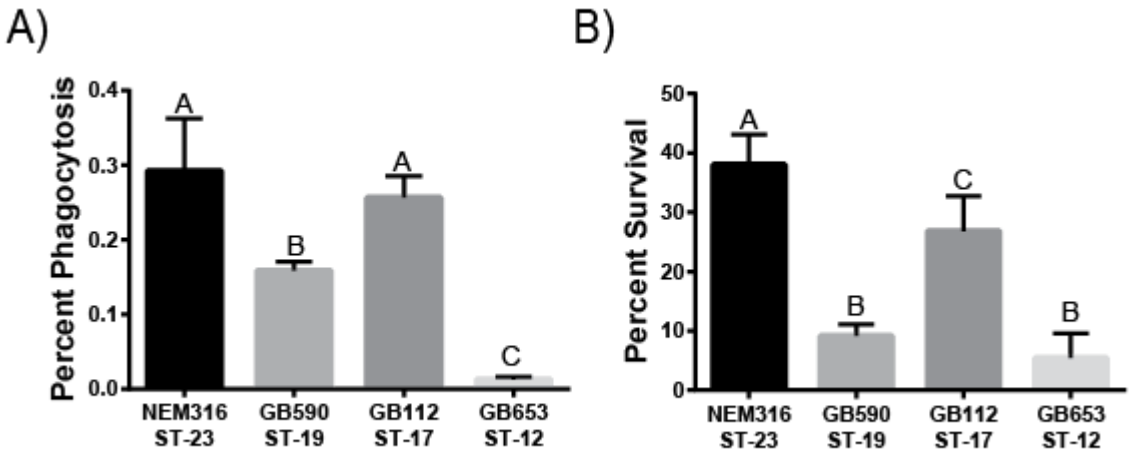
Since ST-17 strains were previously found to persistently colonize pregnant women after receiving IAP and ST-12 strains were more commonly lost (7), we selected the ST-17 (GB00112) and ST-12 (GB00653) strains to determine whether persistence is due to antibiotic tolerance or protection from antibiotic effects by surviving inside host cells. Both strains were collected from women during a routine vaginal/rectal screen before or after childbirth (7) and the administration of ampicillin for IAP. The ST-12 strain was recovered from a pregnant mother prior to delivery and IAP as the mother was GBS-negative at the postpartum visit. By contrast, the ST-17 strain was isolated

from a mother at her postpartum visit and was found to be highly similar to the strain recovered prior to childbirth and IAP, indicating persistent colonization (7).

Table 3.1. GBS strains used in this study by sequence type (ST).

ST	Strain	Serotype	Source
ST-1	GB00020	V	Colonizing-Persisted
	GB00305	Ia	Colonizing-Lost
	GB00620	Ia	Colonizing-Persisted
	GB00037	V	Invasive
	GB00310	V	Invasive
	GB00686	V	Invasive
ST-12	GB00285	II	Colonizing-Lost
	GB00555	Ib	Colonizing-Persisted
	GB00653	II	Colonizing-Lost
	GB00438	Ib	Invasive
	GB00910	II	Invasive
	GB01455	II	Invasive
ST-17	GB00097	III	Colonizing-Lost
	GB00112	III	Colonizing-Persisted
	GB00557	III	Colonizing-Lost
	COH1	III	Invasive
	GB00411	III	Invasive
	GB00418	III	Invasive
ST-19	GB00571	III	Colonizing-Persisted
	GB00590	III	Colonizing
	GB00651	Ib	Colonizing-Lost
	GB00036	III	Invasive
	GB00079	III	Invasive
	GB00377	III	Invasive
ST-23	GB00002	Ia	Colonizing-Persisted
	GB00279	II	Colonizing-Lost
	GB00644	Ia	Colonizing-Persisted
	NEM 316	III	Invasive
	GB00033	Ia	Invasive
	GB00397	III	Invasive

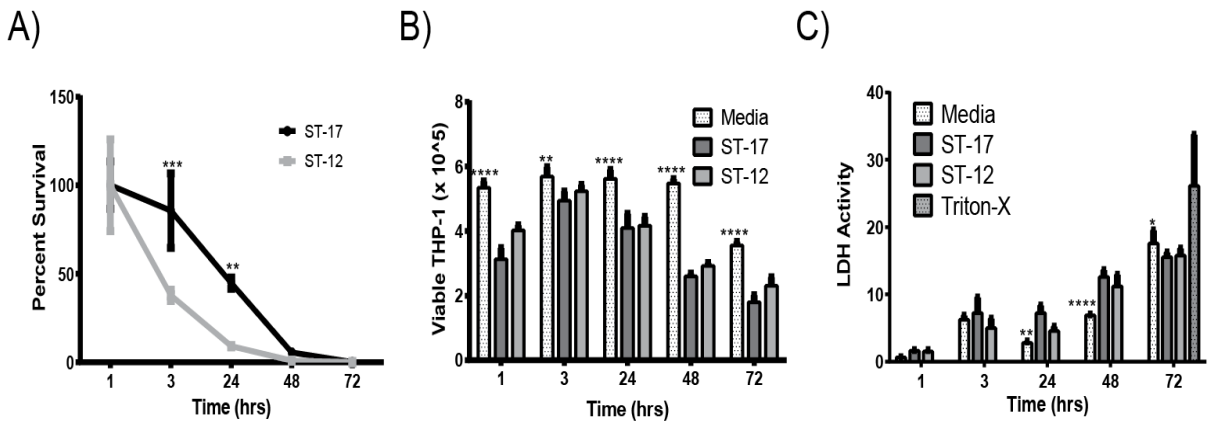
Figure 3.1. Phagocytosis and intracellular survival of diverse GBS strains in human macrophages. (A) Phagocytosis of GBS by macrophages after a 1 hr infection period. The number of phagocytosed bacteria was normalized to the total bacteria per well after the infection period to get percent phagocytosis. (B) Intracellular survival of GBS after 24 hr normalized to phagocytosis to account for differential uptake. Data represents the average of three separate experiments combined. Bars labeled with different letters are significantly different from each other.



We first compared the ability of these two strains to survive inside macrophages for up to 72 hr. Survival of the ST-17 strain was significantly higher than the ST-12 strain at 3 hr and 24 hr post infection. After 48 hr, the ST-17 strain was still detected inside the THP-1 cells, whereas the ST-12 strain was no longer detectable (Figure 3.2A). Since GBS has been shown to induce apoptosis in macrophages (123, 168), we wanted to confirm that the difference in survival between the two strains was not due to increased host cell death after infection by the ST-12 strain. Using trypan blue staining followed by viable cell counting, we found that GBS infection significantly decreases macrophage viability over time compared to the uninfected control (Figure 3.2B). It is important to note that overall viable cell counts per well did vary from approximately $3x10^5$ at 1 hr to $5x10^5$ at 3 hr. Variation was also observed across biological replicates and is likely indicative of variation in the recovery of cells from the well. Therefore, these

results were confirmed using a lactate dehydrogenase (LDH) assay, which measures cell lysis with triton-X as a positive control (Figure 3.2C). The LDH assay also showed reduced viability in the GBS treated macrophages versus the untreated; however, there was no difference in macrophage viability during infection between the two GBS strains for either method (Figures 3.2B and C). These data indicate that the difference in the ability to survive intracellularly was not due to differential killing of the macrophages.

Figure 3.2. Intracellular survival of the ST-17 (GB00112) and ST-12 (GB00653) strains in PMA treated THP-1 macrophages. (A) Long term intracellular survival of GBS. Data was normalized to the 1 hr time point to calculate percent survival. (B and C) Assessment of macrophage viability during intracellular survival using trypan blue staining and viable cell counting (B) and measurement of LDH activity in milliunits/mL in the supernatant to detect cell lysis (C). Cells incubated with media alone were used as a negative control to assess spontaneous cell death. Cells were incubated with triton-X for 30 min before collecting supernatant for LDH assay as a positive control for complete cell death. Data shown are representative experiments of three biological replicates performed in triplicate. (**P<0.01, ***P<0.001, ****P<0.0001)



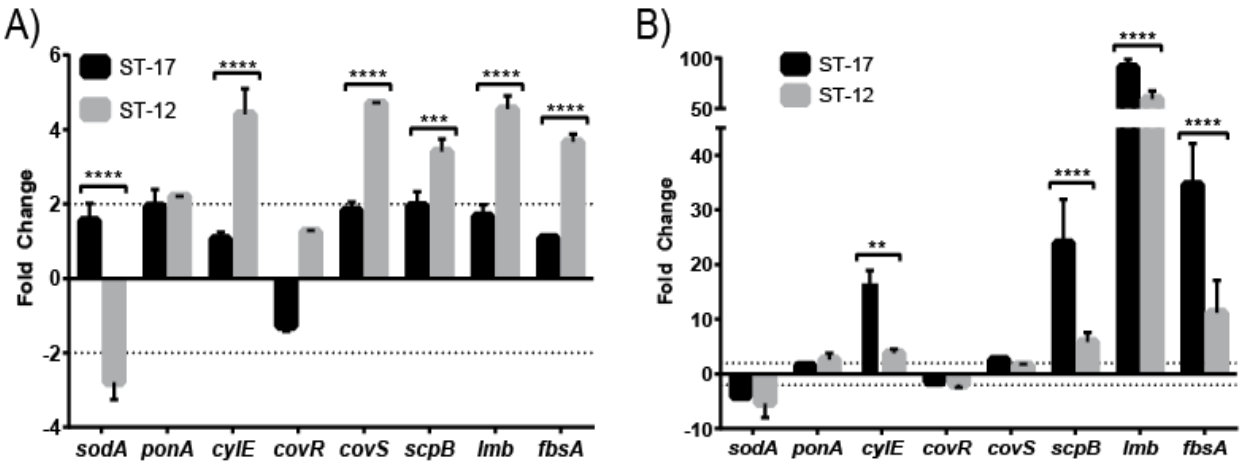
Virulence gene expression varies temporally between the ST-17 and ST-12 strains during intracellular survival

Next, virulence gene expression during intracellular survival at 1 hr and 24 hr post infection was examined to quantify both short and long term survival as well as compare gene expression profiles between the two strains. The genes examined were selected based on previous studies showing their importance in either intracellular survival or GBS virulence (Table 3.2). Interestingly, five of the eight genes (*cylE, covS, scpB, lmb,* and *fbsA*) were expressed significantly higher in the ST-12 strain compared to the ST-17 strain at 1 hr post infection (Figure 3.3A). By 24 hr post infection, however, *cylE, scpB, lmb,* and *fbsA* were significantly higher in the ST-17 strain versus the ST-12 strain (Figure 3.3B). Since the ST-17 strain was able to survive beyond 24 hr unlike the ST-12 strain, it is likely that the prolonged upregulation of these genes in the ST-17 strain could contribute to resisting phagosomal stress for the long term. Of note, *sodA*, which encodes superoxide dismutase, was downregulated during intracellular survival even though it has previously been shown to be important for survival inside macrophages (138).

Table 3.2. Virulence genes examined during intracellular survival in human macrophages.

Gene	Product	Function/Characteristics	Reference
sodA	Mn-cofactored superoxide dismutase	Converts superoxide anions to molecular oxygen and hydrogen peroxide	(138)
ponA	Penicillin-binding protein 1a	Promotes resistance to antimicrobial peptides.	(39)
cylE	CylE protein	Predicted to function as an N-acyltransferase in the biosynthesis of the GBS pigment granadaene required for hemolytic/cytolytic activity of GBS.	(153)
covR	DNA-binding response regulator CovR	Two component regulatory system response regulator involved in virulence	(169)
covS	Sensor histidine kinase covS	Two component regulatory histidine kinase involved in virulence	(169)
scpB	C5a peptidase	Dual function: cleaves and inactivates complement; promotes binding to epithelial cells and fibronectin.	(35)
lmb	Laminin-binding protein	Promotes GBS colonization and translocation into bloodstream.	(39, 40)
fbsA	Fibrinogen-binding protein A	Promotes adherence to epithelial cells	(37)

Figure 3.3. Virulence gene expression during intracellular survival. Differential expression of virulence genes (Table 3.2) relative to basal expression in culture medium was determined after 1 hr (A) and 24 hr (B) intracellular survival. Dashed lines mark 2 fold change in expression. Data shown are representative experiments of at least three biological replicates performed in triplicate. (**P<0.01, ***P<0.001, ****P<0.0001)



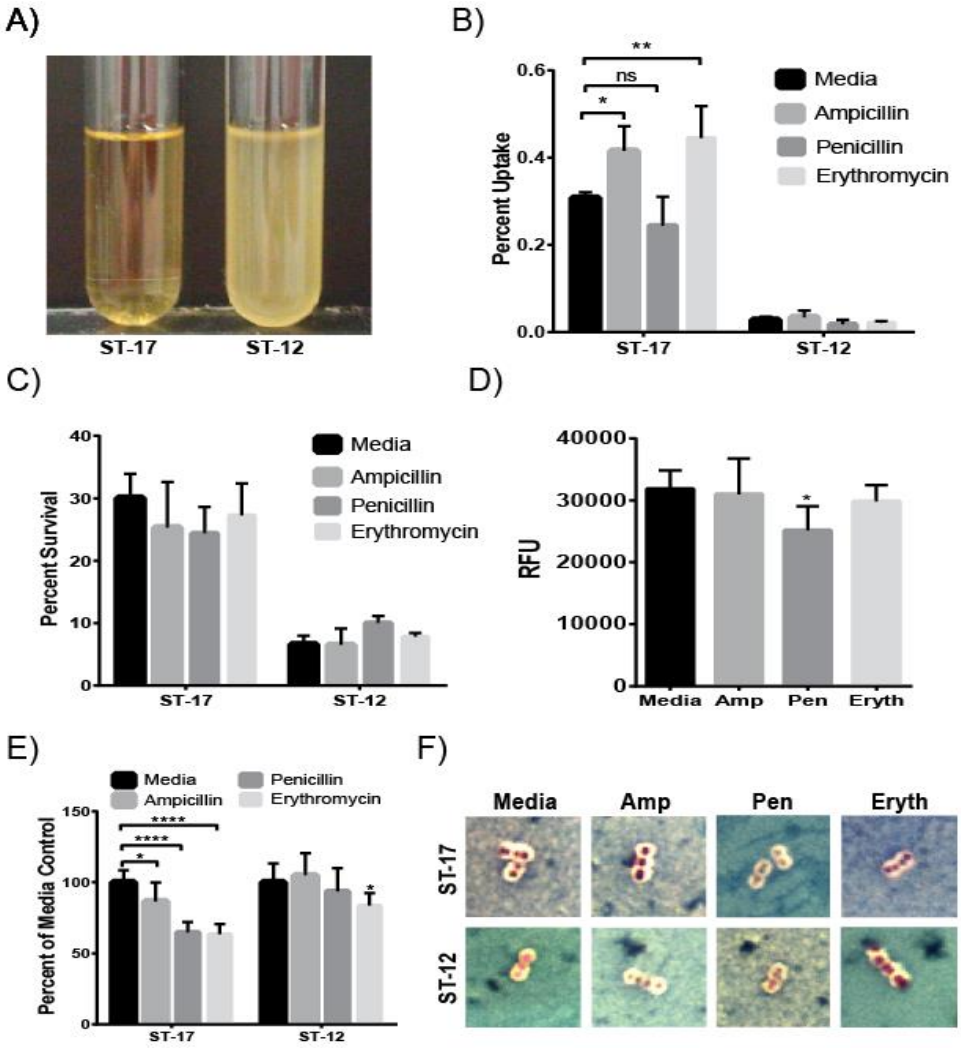
The ST-12 strain is tolerant to ampicillin but exposure to sub-inhibitory ampicillin and erythromycin enhances phagocytosis of the ST-17 strain

We next determined the ability of these two strains to tolerate antibiotic exposure by developing an ampicillin tolerance assay in which GBS was exposed to 5µg/ml of ampicillin for 48 hr followed by 24 hr incubation with 1µg/ml, after which the culture was resuspended in fresh medium to detect tolerant bacteria. Surprisingly, the ST-12 strain was tolerant to ampicillin exposure while the ST-17 strain was effectively killed by ampicillin (Figure 3.4A). It is important to note that the ST-12 strain was tolerant, not resistant, since no growth was detected during antibiotic incubation. Because the ST-17 strain is able to survive inside macrophages and cannot tolerate ampicillin exposure, we hypothesized that ampicillin exposure could induce the ST-17 strain to be more readily taken up by macrophages. Indeed, exposure to a sub-inhibitory concentration of

ampicillin during macrophage infection significantly increased phagocytosis of the ST-17 strain, but had no effect on the ST-12 strain (Figure 3.4B). Additionally, this ampicillin pretreatment had no effect on the ability of either strain to survive inside the macrophages over a 24 hr period (Figure 3.4C). Since penicillin and erythromycin are also commonly used during IAP, the effect of exposure to sub-inhibitory concentrations of both drugs on phagocytosis and intracellular survival was also examined. Although penicillin exposure had no effect on phagocytosis or intracellular survival for either strain, erythromycin exposure significantly increased phagocytosis of the ST-17 strain.

To determine if this increase in phagocytosis was due to a change in the bacteria or a change in the macrophages, we assessed the ability of antibiotic-treated macrophages to phagocytose FITC-labeled BioParticles. Ampicillin and erythromycin treatment did not alter phagocytosis of the BioParticles, but penicillin treatment significantly reduced the ability of the macrophages to take up the BioParticles (Figure 3.4D). This finding suggests that the enhanced uptake of the ST-17 strain after ampicillin and erythromycin exposure is due to a change in the GBS and not a change in the macrophages themselves. Moreover, since penicillin treatment reduced the phagocytic capacity of the macrophages, but phagocytosis of the ST-17 strain after penicillin exposure was the same as untreated, it is possible that penicillin impacts the bacterium resulting in enhanced uptake as well.

Figure 3.4. GBS exposure to antibiotics. (A) GBS cultures of the ST-17 strain (left) and ST-12 strain (right) were exposed to 5µg/ml ampicillin for 48 hr then 1µg/ml ampicillin for 24 hr. After ampicillin exposure, each culture was resuspended in fresh medium to detect any ampicillin tolerant bacteria. This figure shows representative results from four separate experiments (B and C) Phagocytosis (B) and 24 hr survival rate normalized to initial uptake (C) of GBS after cultures were exposed to a sub-inhibitory concentration of ampicillin (0.05µg/ml), penicillin (0.01µg/ml), or erythromycin (0.01µg/ml) during the macrophage infection period. (D) Phagocytosis of BioParticles after 1hr exposure to antibiotics as in B and C. B-D show all data combined from three separate experiments done in triplicate. (E) Relative capsule size of GBS after 1hr antibiotic exposure as described in B and C normalized to the media control. Data represents average relative capsule size from three separate experiments. (F) Representative images of capsule staining of GBS cultures used for relative capsule size shown in E. Capsule is represented by the clear zone around the cell. RFU: relative fluorescence units; Amp: Ampicillin, Pen: Penicillin; Eryth: Erythromycin (*P<0.05, **P<0.01, ****P<0.0001)



Since capsule is an important virulence factor in immune system evasion (170) and a previous study showed a correlation between capsule alteration after antibiotic exposure and enhanced phagocytosis in *E. coli* (171), we hypothesized that changes in capsule could contribute to enhanced phagocytosis of the ST-17 strain. To test this, we examined the GBS cultures after exposure to antibiotics using a capsule stain (Figures 3.4E and F). Interestingly, exposure to all three antibiotics significantly reduced capsule size relative to media treated for the ST-17 strain, with penicillin and erythromycin exposure showing the greatest reduction in capsule size. Ampicillin and penicillin treatment had no effect on capsule size, but erythromycin treatment caused a slight, but significant reduction in capsule size for the ST-12 strain.

The examined ST-17 and ST-12 strains differ in their ability to survive phagosomal stressors and alterations to phagosome acidification

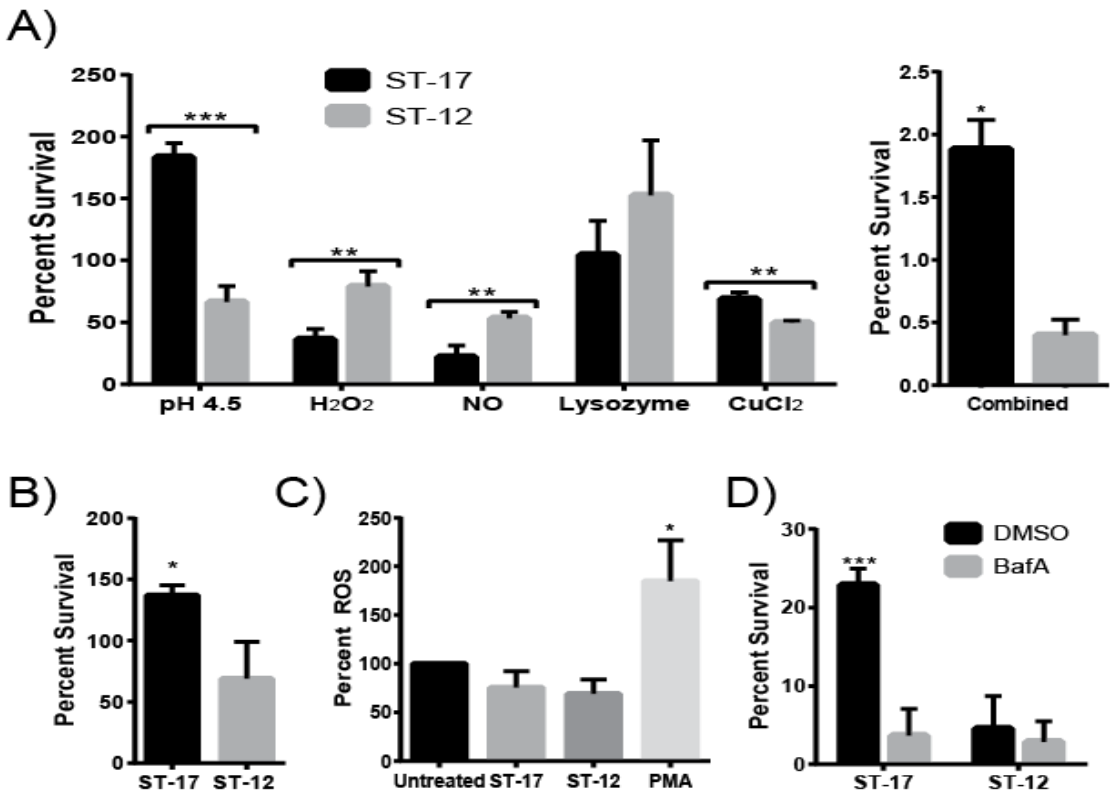
To better understand how GBS withstands phagosomal stress, a multiple stress medium was developed using stressors commonly found in the phagosome. The following stressors were first tested individually to determine how the two strains survived each stress and then tested in combination to assess survival in multiple stress conditions: pH 4.5, H_2O_2, NO, lysozyme, and $CuCl_2$. The ST-17 strain survived significantly better in acidic pH and $CuCl_2$ compared to the ST-12 strain, but the ST-12 strain survived significantly better in H_2O_2 and NO than the ST-17 strain (Figure 3.5A). Both strains were resistant to lysozyme at concentrations up to 10mg/ml. Congruent with the intracellular survival data, the ST-17 strain survived significantly better than the ST-12 strain in the combined multiple stress medium (Figure 3.5A). Although the ST-12

strain was better able to survive some of these stresses individually compared to the ST-17 strain, the combination of all stresses contributed to enhanced survival for only the ST-17 strain. To further examine this, we measured survival of both strains in pH 4.5 and H_2O_2 stress combined (Figure 3.5B). Interestingly, the survival rate in both pH 4.5 and H_2O_2 more closely resembled the survival rate in pH 4.5 alone. Moreover, the H_2O_2 was not breaking down more rapidly in the low pH (data not shown), ensuring that GBS was continuously encountering both stresses. Taken together, these data suggest that diverse strains of GBS use different mechanisms to survive phagosomal stress.

Since the ST-12 strain survived H_2O_2 stress significantly better than the ST-17 strain and GBS was previously shown to prevent the induction of ROS production in the macrophage during infection (133), we hypothesized that the ST-17 strain would have an enhanced ability to inhibit ROS production. To test this, we examined the production of ROS in placental macrophages every 30 min for 2 hr after GBS infection. Placental macrophages were selected because these cells are likely to be involved in host defense against bacterial infections of the placenta and associated membranes during ascending chorioamnionitis. Both strains equally inhibited ROS production to levels significantly less than the PMA-treated positive control and slightly less than the uninfected control at all of the time points tested with the highest ROS production at 2 hr. (Figure 3.5C). This ROS inhibition would not be due to overall cytotoxicity because we measured ROS activity up to 2 hr after infection and observed only a slight reduction in cell viability at 3 hr post infection (Figures 3.2B and C). Since both strains were capable of infecting placental macrophages without initiating a strong oxidative burst

compared to the positive control, this suggests ROS inhibition as a possible survival mechanism for GBS.

Figure 3.5. The GBS ST-17 strain shows an overall enhanced ability to survive a multiple stress environment and uses a pH dependent mechanism to survive the phagosome. (A) Survival of GBS after 1hr exposure to five stressors commonly found in the phagosome tested individually and combined in a single medium. Data is represented as percent of the untreated control. (B) Survival of GBS after exposure to both pH 4.5 and H_2O_2 combined. (C) Production of ROS by placental macrophages after exposure to medium alone (untreated), GBS, or PMA as a positive control. ROS production was measured every 30 min for 2hr. The 2hr time point is shown as representative data. A-C show all data combined from three separate experiments done in triplicate. (D) Representative data showing intracellular survival of GBS inside THP-1 macrophages pretreated with DMSO (control) or 100nM Bafilomycin A1 (BafA) to block acidification of the phagosome. (*P≤0.5, **P<0.01, ***P<0.001)



Since the ST-17 strain had higher CFUs/ml in the acid treated samples compared to the untreated control and the ST-12 strain was killed in acidic pH (Figure 3.5A), we hypothesized that survival in acid is critical for the ST-17 strain to withstand phagosomal stress for a longer period of time relative to the ST-12 strain. To test this, we examined the 24 hr survival rate of each strain inside human macrophages treated with bafilomycin A1 (BafA), which inhibits acidification of the phagosome. Interestingly, inhibiting phagosome acidification significantly reduced the ability of the ST-17 strain to survive intracellularly, but had no effect on the ST-12 strain (Figure 3.5D). Of note, the overall intracellular survival rate is less than that shown in Figures 3.1B and 3.2A. This is due to the inherent variation of this assay and therefore, data shown is representative from at least three independent experiments. Although the exact survival rate varies between experiments, the overall trends and differences between the strains were similar. Moreover, the range in survival rates for the two strains never overlapped across replicates and the differences in survival were significant in each experiment.

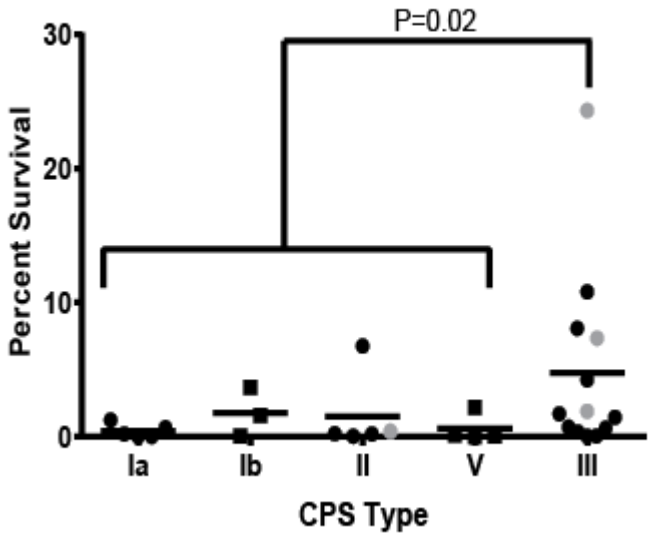
Survival in phagosome-like conditions is dependent on CPS type

Since intracellular survival assays using macrophages can be time consuming and labor intensive, we used the multiple stress medium to rapidly assess the ability of a large number of strains to survive phagosome-like conditions. To do this, 30 strains representing a range of CPS types, STs, and clinical types (Table 3.1) were selected. Clinical types included colonizing strains isolated from pregnant women that were either lost or persisted after IAP and invasive strains isolated from infected neonates. There was no significant difference in survival by clinical type or ST; however, CPS type III

GBS strains survived significantly better than any other CPS type combined (Figure 3.6A). Survival rates of 24.3%, 7.3%, 1.9%, and 0.4% were observed in the multiple stress medium for NEM316 (ST-23), GB00590 (ST-19), GB00112 (ST-17), and GB00653 (ST-12), respectively. Similar trends were observed using THP-1 cells with the exception of the ST-19 strain, which did not survive significantly better than the ST-17 strain after 24 hr.

Since low pH survival was an important factor for the examined ST-17 strain (Figure 3.5), we next determined if low pH survival played a role in the ability of other GBS strains to survive the multiple stress medium. To do this, we selected a subset of strains from each CPS type and ST for a total of 10 of the 30 strains tested in the multiple stress medium and examined their ability to withstand pH 4.5. These strains had a range of 0.01-6.75% survival in the multiple stress medium and a range of 70-190% survival in pH 4.5. Interestingly, we found no correlation between ability to survive multiple stress and low pH (R^2=0.0202, data not shown).

Figure 3.6. Survival of GBS clinical isolates in multiple stress medium. Thirty isolates representing a range of CPS types, STs, and clinical sources were assessed for their ability to survive a multiple stress medium. Data was represented as percent of the untreated control for each strain and stratified by CPS type. Data points in gray indicate the strains assessed in the macrophage survival assay in Figure 3.1. All data were combined from at least three independent experiments done in triplicate.



Discussion

In this study, we sought to better understand differences in the ability of diverse GBS strains to persist after antibiotic exposure by comparing a ST-17 strain that had persistently colonized a mother after IAP and a ST-12 strain that had been eradicated. We have shown that, in the case of the strains tested in this study, the ST-17 strain's ability to persist was more likely due to its ability to remain inside macrophages for an extended period of time rather than antibiotic tolerance. Although the ST-12 strain was tolerant to ampicillin, this strain was eradicated in the mother following IAP as it was not detectable at her postpartum visit. Since the strain was tolerant, but not resistant, it is possible that IAP limited the bacterial population enough for adequate clearance by the immune system. The differences in antibiotic tolerance and macrophage intracellular survival suggests varying mechanisms of persistence and warrants further investigation using a larger number of diverse strains to identify ST- or CPS type-specific mechanisms as well as other possible mechanisms not examined in this study.

Additionally, the ST-17 strain was more readily taken up by macrophages after ampicillin and erythromycin exposure. Similar to our findings, it has previously been reported that treatment with sub-inhibitory concentrations of antibiotics increased phagocytic uptake of *Listeria monocytogenes* (172)*, Staphylococcus aureus* (173)*,* and *E. coli* (171) by macrophages, but this enhanced phagocytic activity was not due to a change in the macrophages themselves (172). In addition, ampicillin or erythromycin exposure did not alter phagocytosis of the BioParticles; therefore, the enhanced phagocytosis of the ST-17 strain is likely due to a change in the bacteria rather than a change in the macrophages. Since penicillin treatment reduced the phagocytic capacity

of the macrophages, but phagocytosis of the ST-17 strain after penicillin exposure was the same as the untreated control, it is possible that penicillin exposure does result in enhanced phagocytic uptake.

Indeed, changes in the level of capsule production in response to antibiotics could increase the number of exposed surface proteins that are recognized by macrophages and result in increased phagocytosis. Similar findings of reduced encapsulation after antibiotic exposure were previously reported for *Bacteroides fragilis* after sub-inhibitory clindamycin (174). A link between encapsulation and enhanced phagocytic uptake after exposure to sub-inhibitory antibiotics in *E. coli* was also reported (171). Taken together, these data indicate a common response to sub-inhibitory concentrations of antibiotics across different bacterial species as well as antibiotics with different modes of action. However, ampicillin exposure resulted in a smaller reduction in capsule compared to penicillin and erythromycin. Since sub-inhibitory concentrations of antibiotics have also been shown to cause other morphological changes in bacteria (175), further investigation of the effect of antibiotics on GBS is warranted to have a full understanding of the molecular mechanisms behind the increased phagocytosis. In addition, the level of capsule production may vary across strains and could also influence differences in phagocytic uptake across strains.

Interestingly, antibiotic exposure had no effect on intracellular survival of either strain but enhanced phagocytic uptake, suggesting that antibiotics could increase the likelihood of persistent colonization, at least for the strain tested in this study. In a recent study, Lehar et. al. proposed a novel therapeutic using antibody-antibiotic conjugates to clear intracellular reservoirs of *Staphylococcus aureus* (176). Similar strategies could

also be used to eliminate persistent maternal GBS colonization and to potentially reduce the risk of neonatal infection. A previous study reported that serotype Ib GBS treated with sub-inhibitory concentrations of beta-lactam antibiotics, including penicillin, increased killing of GBS by phagocytes (177). However, our current study shows no difference in phagocytic killing of the two strains (serotypes II and III) examined here. The differing results suggest that the antibiotic effect on phagocytic killing of GBS could be dependent on serotype.

By examining gene expression during intracellular survival, we have shown that the ST-17 and ST-12 strains vary in their temporal response to macrophages. The finding that these genes are important for long term survival in macrophages is congruent with previous studies. Both strains, for example, had approximately 2-fold upregulation of *ponA*, which encodes the penicillin-binding protein 1a that was linked to phagosomal stress resistance in a prior study by protecting GBS against cationic antimicrobial peptides (143). The *cyl* operon, which includes *cylE*, produces a pore-forming β-hemolysin/cytolysin (β-h/c) and a carotenoid pigment that helps protect against reactive oxygen species. A previous study showed that a *cylE* deletion mutant does not produce either the β-h/c or pigment and is more susceptible to both oxidative and macrophage killing (141). Another study has found that the CovR/S two component regulatory system is required for survival inside macrophages. This system regulates the expression of many virulence genes including *cylE, scpB,* and *fbsA* (133) and could explain the upregulation of these genes in the ST-12 strain at 1hr since *covS* was also upregulated. However, the *covR/S* regulated genes are upregulated in the ST-17 strain during intracellular survival despite *covR/S* not being significantly differentially

expressed. The upregulation could be due to increased activity of CovR/S rather than upregulation of gene expression. Although *scpB, lmb,* and *fbsA* play important roles in GBS pathogenesis by aiding in attachment and invasion of host cells (35, 37, 40), their importance for survival inside macrophages has yet to be determined. Consequently, future studies should focus on constructing GBS mutants to better understand the role these genes play inside the macrophage.

Interestingly, *sodA* was found to be important for survival in oxidative stress and intracellular survival in mouse bone marrow-derived macrophages using a *sodA*-disrupted mutant (138). It is therefore likely that the downregulation of *sodA*, despite its importance in intracellular survival, results because the ROS burst occurs early on during phagosome maturation and the elevation in ROS is transient (178). Hence, by 1 and 24 hr, ROS concentration is reduced and expression of *sodA* would be turned off. Proper examination of *sodA* expression during intracellular survival would require sampling at earlier time points. Additionally, since GBS inhibits the ROS burst in macrophages, it is also possible that *sodA* upregulation is not necessary. Because mRNA expression does not always correlate with protein levels, future studies are necessary to examine protein levels for a more complete determination of their levels inside a macrophage for each of these strains.

In addition to enhanced intracellular survival, the ST-17 strain was phagocytosed significantly more than the ST-12 strain. Chattopadhyay et. al. demonstrated that GBS strains containing the pilus island 2b backbone protein, Spb1, had increased phagocytosis and intracellular survival compared to strains lacking Spb1 (146). Since Spb1 is specific to ST-17 strains (147), this finding suggests that the enhanced

phagocytosis and ability to survive intracellularly is ST-dependent. However, using our multiple stress medium, ST-17 strains did not survive significantly better than other STs. Since the mechanism of how Spb1 promotes intracellular survival is currently not known, it is possible that the factor Spb1 protects against was not included in our multiple stress medium.

Using the multiple stress medium we demonstrated that strains with CPS type III have an enhanced ability to survive multiple phagosomal stressors. Indeed, previous studies have reported that CPS type III GBS strains induce a lower maternal antibody response (94) and are more virulent. Since newborn infants rely on the transfer of maternal antibodies for defense against infections early in life, the low antibody response to type III strains could explain the high rate of type III infections (93). This low antibody response could also be due to the enhanced ability of type III strains to survive inside macrophages, which are common antigen presenting cells that alert the immune system of invading pathogens (179). Future work, however, requires the examination of a larger set of strains representing varying genotypes and CPS types using both THP-1 and placental macrophages for a more complete understanding of intracellular survival in GBS.

Because the exact trends of survival in macrophages for the four strains tested in this system did not completely match survival in the synthetic multiple stress medium, it is important to note that this multiple stress medium does not fully represent the phagosomal environment. This synthetic medium is missing a number of factors present in the macrophage as well as the timing of the addition of each stressor as the phagosome matures. Nonetheless, this medium would work as an excellent first step in

screening for novel factors important for surviving phagosomal stress, such as screening mutant strains, before proceeding to the more labor intensive assays with macrophages.

The ST-17 strain, which survived intracellularly longer than the ST-12 strain, had decreased survival when acidification of the phagosome was blocked with BafA treatment. BafA treatment possibly causes other changes in the cell in addition to inhibiting the vacuolar H^+-ATPase to block phagosome acidification (180). Nonetheless, the increased intracellular survival in pH 4.5, the survival rate in both pH 4.5 and H_2O_2 most closely resembling low pH alone, and the decrease in intracellular survival with BafA treatment indicates a pH-dependent mechanism of intracellular survival for the ST-17 strain. This pH requirement is congruent with a previous study that found the CovR/S acid response regulator was needed for intracellular survival (133). When comparing the individual effect of different phagosomal stressors on each of the strains, the ST-17 strain survived better in low pH and $CuCl_2$ but the ST-12 strain survived better under H_2O_2 and NO stress. Taken together, these results suggest that different GBS strains use different mechanisms to survive inside the phagosome.

Survival inside professional phagocytes can assist with the dissemination of a pathogen throughout the host to cause more severe disease. Pathogens can cross host cell barriers, such as the extraplacental membranes or the blood brain barrier, via phagocyte-facilitated invasion (181, 182). Therefore, a better understanding of the mechanism by which GBS survives inside macrophages could aid in preventing GBS infection. Additionally, GBS has the ability to use intracellular survival as a mechanism to evade antibiotics and persistently colonize women, providing a possible explanation

for the unchanged rate of late onset neonatal infections despite the implementation of IAP preventative measures (183).

Materials and Methods

Bacterial culture

GBS strains (Table 3.1) were cultured in Todd-Hewitt broth (THB) or on agar (THA) at 37^{o}C with 5% CO_2. With the exception of NEM316 (9) and COH1 (184), invasive strains were originally recovered from neonatal blood or cerebral spinal fluid in a prior study (160), and colonizing strains were recovered via vaginal/rectal swabs from women during and after pregnancy (159). The ST-17 strain (GB00112) was examined in our prior studies (126, 185), and all strains were previously characterized by capsular genotyping and MLST as described (11, 186).

Cell culture

The human monocyte cell line, THP-1 (ATCC TIB-202), was maintained by incubating at 37^{o}C with 5% CO_2 in Roswell Park Memorial Institute 1640 (RPMI) growth medium (Gibco) containing 10% fetal bovine serum (FBS; Hyclone) and 2% penicillin/streptomycin (Gibco), referred to as full RPMI for the rest of the paper. For experiments, 10^6 cells were seeded into each well of a 24 well plate in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) in RPMI 1640 with 2% FBS for 24 hr. The THP-1 cells were differentiated into adherent macrophages, which could be observed microscopically, after 24 hr (187).

Intracellular survival assay

Bacterial strains were grown in THB to mid-log phase, washed once with PBS and resuspended in RPMI. PMA treated THP-1 cells were washed twice with PBS then infected with GBS strains at a multiplicity of infection of 10:1 for 1 hr, after which the wells were washed three times with PBS. Remaining extracellular bacteria were killed using RPMI containing 2% FBS, 100 µg/ml gentamicin (Gibco) and 5 µg/ml penicillin G (Sigma). After 1 hr, the number of intracellular bacteria was determined by washing each well with PBS to remove antibiotics and adding 0.1% triton X-100 (Sigma) to lyse the cells for 30 min. Lysates were diluted and plated onto THA and incubated at 37^{o}C with 5% CO_2 overnight to count colony forming units (CFUs). This was repeated at several time points to determine survival rate over time. The number of intracellular bacteria was normalized to the total number of bacteria in the well after the 1 hr infection period. The survival rate was calculated as follows: survival rate= (intracellular bacteria at time *x* / intracellular bacteria 1 hr after adding antibiotics) * 100. Macrophage viability was assessed throughout the assay using two methods: 1) Cells were removed from the wells using trypsin-EDTA (Gibco) and stained with trypan blue (Gibco); viable cells per well were counted using a hemocytometer; and 2) Supernatants were collected from each well and the amount of LDH, an indicator of cell death, was assessed using the colorimetric LDH activity assay kit (Sigma) following manufacturer's instructions. Since LDH reduces NAD to NADH, LDH activity was calculated by determining the amount of NADH generated by the sample supernatant by comparing the absorbance from that sample to a NADH standard curve. LDH activity is reported as milliunit/mL where one unit is the amount of enzyme needed to generate 1µmol per minute. To

assess the effect of blocking acidification of the phagosome on intracellular survival, 100 nM BafA (Sigma) was added to the cells 1 hr prior to GBS infection and remained with the cells throughout the assay.

RNA isolation and RT-PCR

RNA preparation, cDNA synthesis, and RT-PCR were performed as previously described (126). RNA samples were collected from bacteria cultured in medium by adding samples to two volumes of RNAprotect Bacteria Reagent (Qiagen). RNA samples were collected from intracellular bacteria during the survival assay described above by washing the wells twice with PBS then adding 1ml RNAprotect Bacteria Reagent. RT-PCR analysis was performed using primers listed in Table S1 in the appendix and the relative fold change of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method using *gyrA* as the internal control gene (166).

Ampicillin tolerance

Overnight GBS cultures were exposed to 5 µg/ml ampicillin in THB for 48 hr at 37°C. The same population of bacteria was then re-suspended in THB containing 1 µg/ml ampicillin for an additional 24 hr exposure at 37°C. In an attempt to recover any live cells, the culture was resuspended in fresh THB and grown for 24 hr at 37°C; full growth represented ampicillin tolerance.

Phagocytosis after antibiotic exposure

Sub-inhibitory concentrations of antibiotic were determined by exposing GBS to antibiotic in RPMI for 1 hr to mimic the infection period of the intracellular survival assay described above. The highest concentration without a significant reduction in cell viability compared to growth in medium alone was used for the assay. To assess the effect of exposure to a sub-inhibitory concentration of antibiotics on phagocytosis and intracellular survival of GBS, cultures were exposed to 0.05 µg/ml ampicillin, 0.01 µg/ml penicillin, or 0.01 µg/ml erythromycin where indicated during the 1 hr infection period of the intracellular survival assay; phagocytosis and intracellular survival were determined as described above. Phagocytic activity of the macrophages after antibiotic exposure was determined by measuring the uptake of FITC-labeled *Staphylococcus aureus* BioParticles (Molecular Probes). BioParticles were opsonized using the *S. aureus* BioParticles opsonizing reagent (Molecular Probes) following the manufacturer's instructions. BioParticles were added to PMA treated THP-1 cells at 50 particles per cell in 96 well plates in RPMI and incubated for 1 hr. Wells were then washed three times with PBS. Extracellular fluorescence was quenched using 0.25mg/ml trypan blue. Intracellular fluorescence was read using the Cytation^{TM} 3 multi-mode microplate reader (Biotek Instruments, Inc.) at 480nm excitation and 520 nm emission.

Capsule staining

GBS capsules were stained using the Maneval method (188) after 1 hr exposure to media alone or sub-inhibitory concentrations of penicillin, ampicillin, or erythromycin as described above. One drop of culture was mixed with one drop of 1% Congo red

stain (Sigma). Once the smears were dry, they were counterstained with Maneval's stain (Carolina) for 2 min, drained and air dried. Antibiotic exposure and capsule staining were performed three separate times and representative images were taken from each. Capsule thickness was determined by measuring the diameter of the clear zone representing the capsule and subtracting the size of the cell to control for differential cell size. Three separate measurements were made for each cell examined and averaged to determine capsule thickness. Approximately 10-15 cells were analyzed for each treatment. Data was then normalized to the untreated media control and is reported as average percent of media control.

GBS survival in multiple stress medium

Survival of GBS in several phagosomal stressors was assessed as previously described (133). Briefly, stationary phase cultures were washed in PBS, then resuspended in 0.1M sodium phosphate buffer pH 7.5 alone or buffer with the indicated stressor at a final concentration of approximately $1x10^6$ cells/ml. Cultures were incubated for 1 hr at 37^{o}C then diluted and plated on THA to count viable bacteria. The number of viable bacteria in the treated samples was normalized to untreated samples to calculate percent survival. The ability of GBS to survive the following stressors at the indicated concentrations was tested individually: hydrogen peroxide (H_2O_2) at 5 mM, sodium nitrate ($NaNO_2$) at 10 mM in sodium phosphate buffer at pH 4.5 ($NaNO_2$ dissociates to yield nitric oxide (NO) at low pH), copper chloride ($CuCl_2$) at 0.25 mM, lysozyme at 100 µg/ml, and acidic sodium phosphate buffer at pH 4.5. For the combined multiple stress medium, the concentrations of the stressors were 1.5 mM H_2O_2, 3 mM

$NaNO_2$, 100 µM $CuCl_2$, and 100 µg/ml lysozyme in sodium phosphate buffer at pH 4.5. For the low pH and H_2O_2 survival, GBS cultures were exposed to 5mM H_2O_2 in sodium phosphate buffer at pH 4.5.

Isolation of placental macrophages

Placental macrophages were obtained from term, non-laboring placentas obtained at the time of Cesarean section as previously described (189). Samples were only obtained from healthy donors with no significant medical condition, aged 18-40 years. Briefly, the tissue was washed three times with PBS by centrifuging at 1,500 rpm for 10 min to remove circulating blood. The tissue was minced into small pieces and weighed to determine final grams collected. Tissue fragments were placed into 50 ml conical tubes with digestion solution containing 150 µg/ml deoxyribonuclease (Sigma), 1 mg/ml collagenase (Sigma) and 1 mg/ml hyaluronidase (Sigma) at 10 ml per gram of tissue. Cells were filtered through a 280 µm metal sieve, followed by 180 and 80 µm nylon screens (Millipore). Cells were centrifuged again and resuspended in 25% Percoll (Sigma) diluted in cold full RPMI and overlaid onto 50% Percoll, plus 2 ml of PBS on top of the density gradient. CD14+ macrophages were isolated by positive selection using the magnetic MACS® large cell separation column system according to the manufacturer's instructions (Miltenyi). Isolated CD14+ placental macrophages were rested overnight in full RPMI before experimentation.

Measurement of ROS production

The production of ROS following infection of placental macrophages by GBS was determined by seeding 2 x 10^5 human placental macrophages onto a 384-well cell culture-treated plate and rested overnight in full RPMI. The next day, placental macrophages were washed with PBS and labeled with 10 µM Carboxyl H2DCF-DA for 30 min following the manufacturer's instructions (Invitrogen). Placental macrophages were washed again with PBS before being inoculated with GBS at a MOI of 50:1 or PMA (300 nM) as a positive control. Cells were assessed for the generation of ROS every 30 min for 2 hr post infection, and ROS production was normalized to the percent of the untreated control.

Human subjects

These studies were approved by the Vanderbilt University Institutional Review Board (Protocol #131607). Tissue samples were provided by the Cooperative Human Tissue Network at Vanderbilt University, which is funded by the National Cancer Institute.

Statistical analysis

Data shown were either pooled from or representative of at least three independent experiments performed in triplicate. GraphPad Prism version 6 was used for statistical analysis. Significant differences between strains/treatments for two groups, multiple groups or two parameters comparisons was determined using Mann-Whitney U

test, one-way ANOVA and two-way ANOVA, respectively, along with Tukey's multiple comparison test where appropriate. Statistical significance was accepted at $P \leq 0.05$.

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CHAPTER 4

THE ROLES OF *cadD* AND *npx* IN CONTRIBUTING TO THE ABILITY OF GROUP B *STREPTOCOCCUS* TO SURVIVE IN HUMAN MACROPHAGES

Abstract

Macrophages play an important role in defending the host against infections by engulfing pathogens and containing them inside the phagosome which consists of a very harsh, microbicidal environment. However, many pathogens have developed mechanisms to survive inside macrophages. Group B *Streptococcus* (GBS), a leading cause of sepsis and meningitis in neonates, is one such pathogen that survives inside macrophages by withstanding phagosomal stress. Although a few key intracellular survival factors have been identified, the mechanism by which GBS survives is largely unknown. In this study, RNA sequencing was used to identify intracellular survival candidate genes by examining upregulated genes during intracellular survival. Of the genes significantly upregulated, two were selected based on their putative function related to stress response for further characterization through mutagenesis: NADH peroxidase (*npx)* and cadmium resistance protein (*cadD)*. The *npx* deletion mutant showed a slight decrease in ability to detoxify exogenously added H_2O_2 and was significantly more susceptible to H_2O_2. Moreover, Δ*npx* was more susceptible to killing by a multiple stress medium consisting of phagosomal stressors as well as killing by PMA treated THP-1 macrophages. The *cadD* deletion mutant was more susceptible to heavy metal toxicity by cobalt, copper, nickel, and zinc and showed elevated levels of divalent metal cation accumulation in the cytoplasm. Moreover, Δ*cadD* was more susceptible to killing by a multiple stress medium as well as macrophages and was less virulent in a mouse model. These data suggest that Npx aids in intracellular survival by detoxifying reactive oxygen species and CadD plays a role in protection against divalent metal cation toxicity by acting as an efflux pump.

Introduction

Preterm birth affects 15 million people worldwide each year and approximately one million deaths occur as a result of complications related to preterm birth (190). Moreover, preterm birth causes significant neonatal morbidity including neurological and respiratory disorders (191). One of the most common causes of preterm birth is inflammation or infection of the maternal (decidua) and fetal (chorion and amnion) extraplacental membranes, commonly referred to as chorioamnionitis, accounting for approximately 25% of preterm births (192, 193). Chorioamnionitis is often associated with polymicrobial ascending infections of the reproductive tract which leads to elevated cytokine levels in the amniotic fluid. Amniotic fluid cytokine levels mediate weakening of the fetal membranes as well as inflammation which can lead to premature rupture of the membranes and preterm delivery (194, 195).

Several bacterial species have been identified as contributing to pregnancy-related infections and disease outcomes. Of the organisms commonly identified in clinical cases of chorioamnionitis, *Streptococcus agalactiae*, or Group B *Streptococcus* (GBS) accounts for 8-11% of all cases (196). GBS can be transmitted vertically from mother to fetus *in utero*, a scenario that is often associated with chorioamnionitis. GBS colonizes the vagina and/or gastrointestinal tract of about 15-30% of healthy adults. Because antibiotic resistant strains of GBS are emerging (197), it is critical to gain a better understanding of this host-pathogen interaction to develop potential targets for novel treatment strategies.

As the predominating antigen presenting cell in the decidua (198), decidual macrophages play an important role in maintaining a balance between fetal-maternal

immune tolerance and protection against invading pathogens. Decidual macrophages effectively suppress immune responses during pregnancy by secreting high levels of the anti-inflammatory cytokine IL-10 to maintain a tolerant response (199). Additionally, decidual macrophages express high levels of pattern recognition receptors, indicating an important role in recognition and clearance of a pathogen (200). However, the exact role of these macrophages in defense against bacterial pathogens during pregnancy remains poorly defined.

GBS infection induces a strong inflammatory response which aids in clearance of the pathogen. Once GBS is recognized by phagocytic cells, it is readily engulfed and contained within the phagosome, which matures through a series of fusion events with endosomes and lysosomes resulting in a highly microbicidal environment which includes acidic pH, reactive oxygen species (ROS), reactive nitrogen species (RNS), antimicrobial peptides, and heavy metals (131). However, GBS is capable of surviving inside macrophages and remains inside the fully mature phagosome (116). Although some key factors involved in intracellular survival have been identified (133, 138, 141, 143, 145, 146, 201), the mechanism by which GBS survives inside the phagosome is largely unknown. A more thorough understanding of this mechanism is needed as survival inside macrophages is an important mechanism of crossing host cell barriers (181) and this could be one mechanism GBS uses to infect the extraplacental membranes resulting in chorioamnionitis and preterm birth.

This study explores how a particular GBS strain of sequence type (ST)-17 survives inside macrophages and withstands phagosomal stress. Using RNA sequencing (RNAseq), two putative factors for intracellular survival were identified and

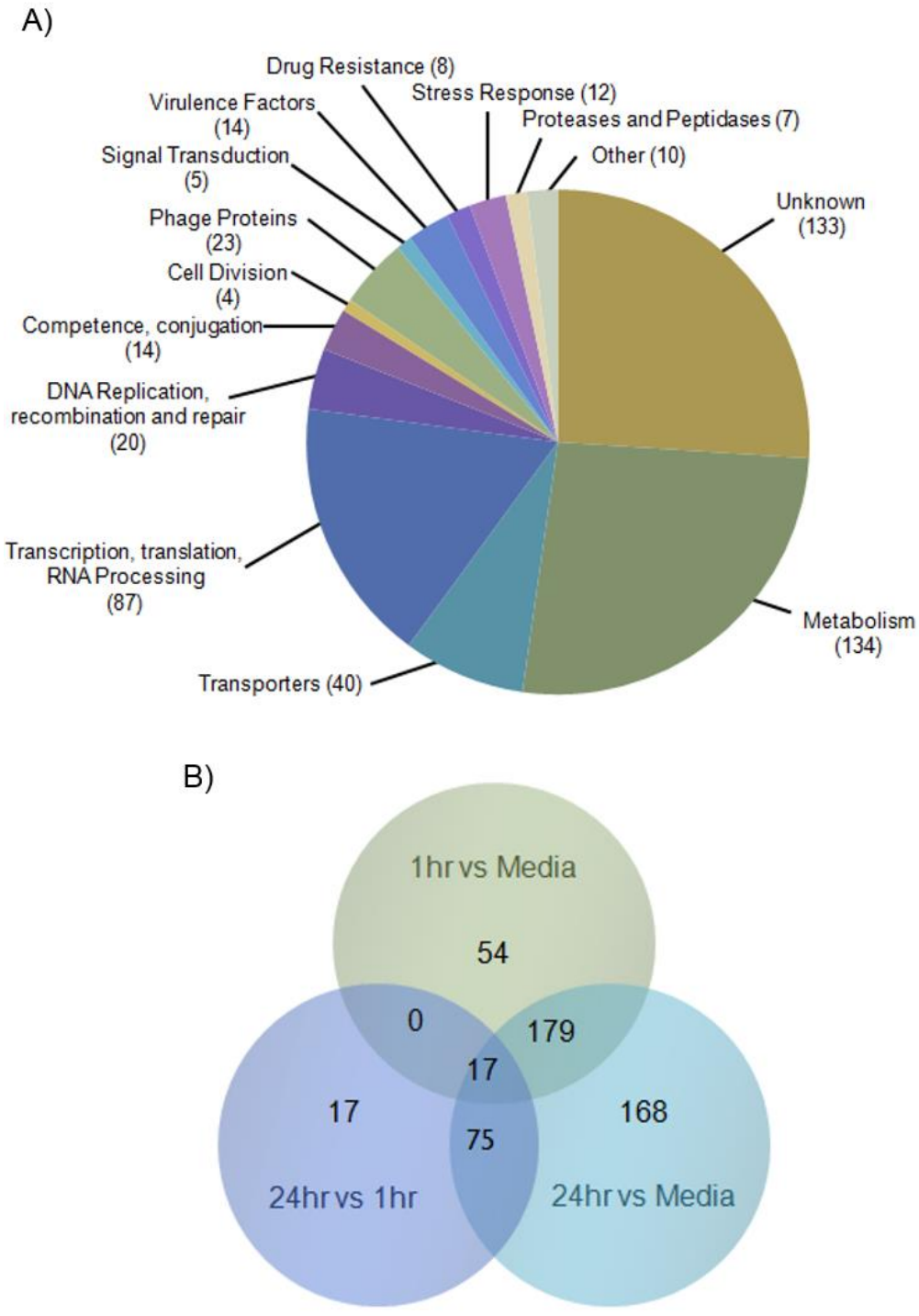
characterized for their role in promoting survival of GBS inside human macrophages: NADH Peroxidase and Cadmium Resistance Protein. Through mutagenesis studies, we show that these two factors promote intracellular survival by detoxifying hydrogen peroxide (H_2O_2) and pumping toxic heavy metals from the cytosol, respectively. A better understanding of what factors are important in promoting intracellular survival of GBS will aid in identifying targets for alternative therapies for treating and preventing GBS infections.

Results

Transcriptome remodeling occurs in GBS during survival in human macrophages

In a previous study, we examined the ability of the ST-17 strain, GB00112, to survive inside human macrophages and found it survived significantly better than an ST-12 strain over a prolonged period of time (202). In order to identify possible genes involved in the ability of GBS to survive inside macrophages, RNAseq was used to examine the transcriptome of this strain after 1hr and 24hr survival inside human macrophages compared to growth in cell culture media alone. By comparing gene expression at 1hr survival vs media alone, 24hr survival vs media alone, and 1hr vs 24hr survival, a total of 511 genes were found to be significantly upregulated during intracellular survival (Figure 4.1A). Among these genes was the *cyl* operon, which produces the β-hemolysin/cytolysin toxin as well as caratenoid pigment and was previously shown to be important for intracellular survival (141, 203), validating our findings. Using these comparisons, we were also able to determine there was a temporal transcriptome remodeling (Figure 4.1B). For example, 54 genes were upregulated only during the 1hr vs media comparison indicating these genes were turned on initially during intracellular survival, but were then turned off by 24hr, whereas 168 genes were turned on at 24hr, but not 1hr. Moreover, 179 genes were initially turned on at 1hr survival and then their expression remained on at a similar level through 24hr survival, whereas 17 genes were turned on at 1hr and were still on by 24hr, but at a higher level compared to expression at 1hr.

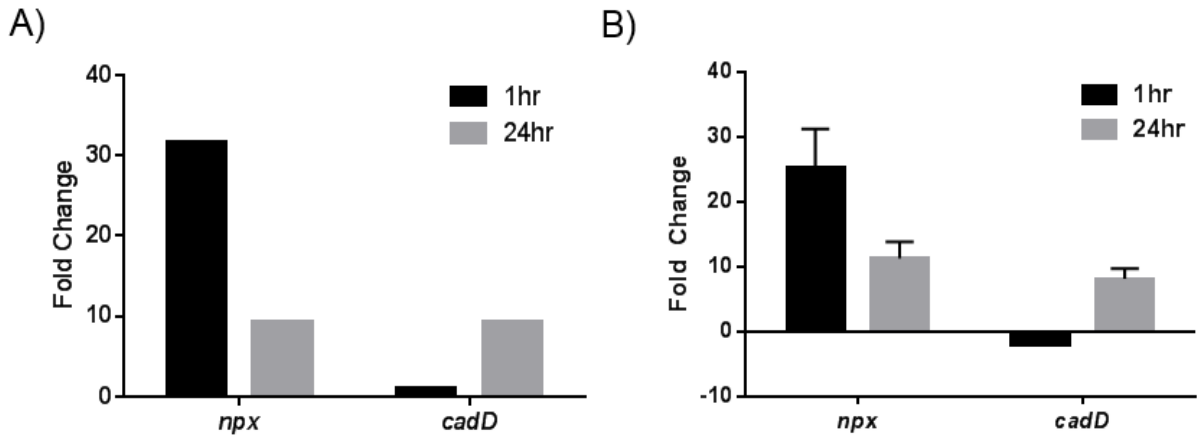
Figure 4.1. Genes significantly upregulated in GBS after 1hr and 24hr survival in human macrophages. (A) The total 511 genes that significantly upregulated during intracellular survival grouped by function. (B) Venn diagram showing the number of significantly upregulated genes found from each of the indicated comparisons.



The cadD and npx genes are highly upregulated during intracellular survival

Since the goal of this analysis was to identify factors that play a role in helping GBS withstand phagosomal stress, we focused on upregulated stress response genes (Figure 4.1A). Of the 12 significantly upregulated genes, two were selected for further characterization, GB112_04315 and GB112_06484, which encode a putative NADH peroxidase (Npx) and a putative cadmium resistance protein (CadD), respectively. Moreover, BLAST analysis shows that both genes are highly conserved among many other diverse GBS strains. qPCR analysis confirmed the expression of these two genes during intracellular survival and shows that *npx* is highly upregulated at 1hr and remains upregulated, but not as high at 24hr, whereas *cadD* is upregulated at 24hr but not at 1hr (Figure 4.2). In order to begin further characterizing the roles of these two genes in intracellular survival, deletion mutants of each gene were constructed in the GB00112 background and complementation was done *in trans* using the pLZ12 plasmid with the constitutive *rofA* promoter controlling the wild type (WT) allele of each gene (204).

Figure 4.2. Expression of *npx* and *cadD* after 1hr and 24hr survival inside human macrophages relative to expression in media alone. (A) Gene expression determined by RNAseq analysis. (B) Gene expression determined by qPCR.

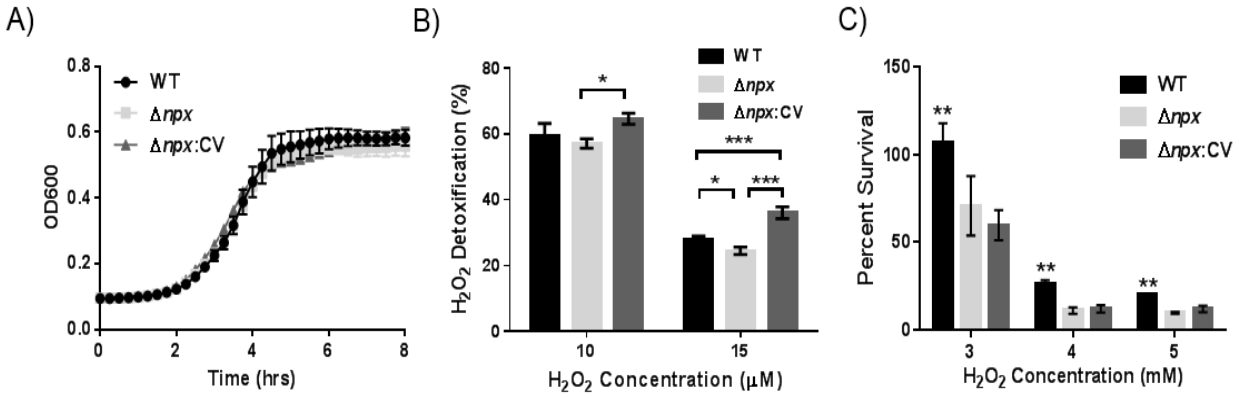


Npx promotes survival by detoxifying hydrogen peroxide

The presumed function of NADH peroxidases is to detoxify endogenously produced hydrogen peroxide (H_2O_2), such as that produced during aerobic growth, metabolism or through dismutation of superoxide; however, it may also detoxify exogenous H_2O_2 (205). To determine if the GBS Npx plays a role in endogenous H_2O_2 produced during aerobic growth, the growth of an *npx* deletion mutant was compared to that of the GB00112 WT strain and the complemented strain (Δ*npx*:CV) under aerobic conditions. All three strains exhibited the same level of growth over the examined 8hr growth period indicating that deleting *npx* has no effect on aerobic growth (Figure 4.3A). To examine the role of Npx in detoxifying exogenous H_2O_2, GBS cultures were incubated with H_2O_2 supplemented medium and the concentration of H_2O_2 remaining in the solution was determined using a fluorometric assay. Interestingly, the *npx* mutant showed a significant decreased ability to detoxify H_2O_2 and the complemented mutant was able to detoxify H_2O_2 significantly more than both WT and Δ*npx* when exposed to 15µM H_2O_2. However, when exposed to 10µM H_2O_2, the difference between WT and Δ*npx* was insignificant (Figure 4.3B). We next compared the ability of these strains to survive H_2O_2 stress of various concentrations over a 1hr period. Interestingly, Δ*npx* had a significantly reduced ability to survive compared to the WT, (Figure 4.3C); however, this phenotype was not complemented despite the ability of the complemented strain to detoxify H_2O_2 significantly more than the mutant (Figure 4.3B and C). One hypothesis for the lack of complementation in this assay was that the plasmid was not able to produce as much Npx as the WT under these concentrations of H_2O_2. To test this, we determined the level of expression of *npx* at 15, 30, and 60 min after H_2O_2 exposure in

both the WT and Δ*npx*:CV strains, but found that *npx* is not induced by H_2O_2 exposure in the WT under the conditions examined and that *npx* expression from the complementation plasmid is 3-4 fold higher than that of expression in the WT (data not shown).

Figure 4.3 Role of the *npx* locus in response to H_2O_2 in GBS. (A) Growth of GBS under aerobic conditions. (B) The level of H_2O_2 detoxification by GBS at two different H_2O_2 concentrations. Percent detoxification was calculated by normalizing the amount of H_2O_2 remaining after incubation with GBS to the amount of H_2O_2 in the culture free control. (C) Survival of GBS following a 1hr exposure to H2O2. Survival is expressed as the CFUs after H_2O_2 exposure normalized to the untreated control. (* P<0.05 ** P<0.01 *** P<0.001).

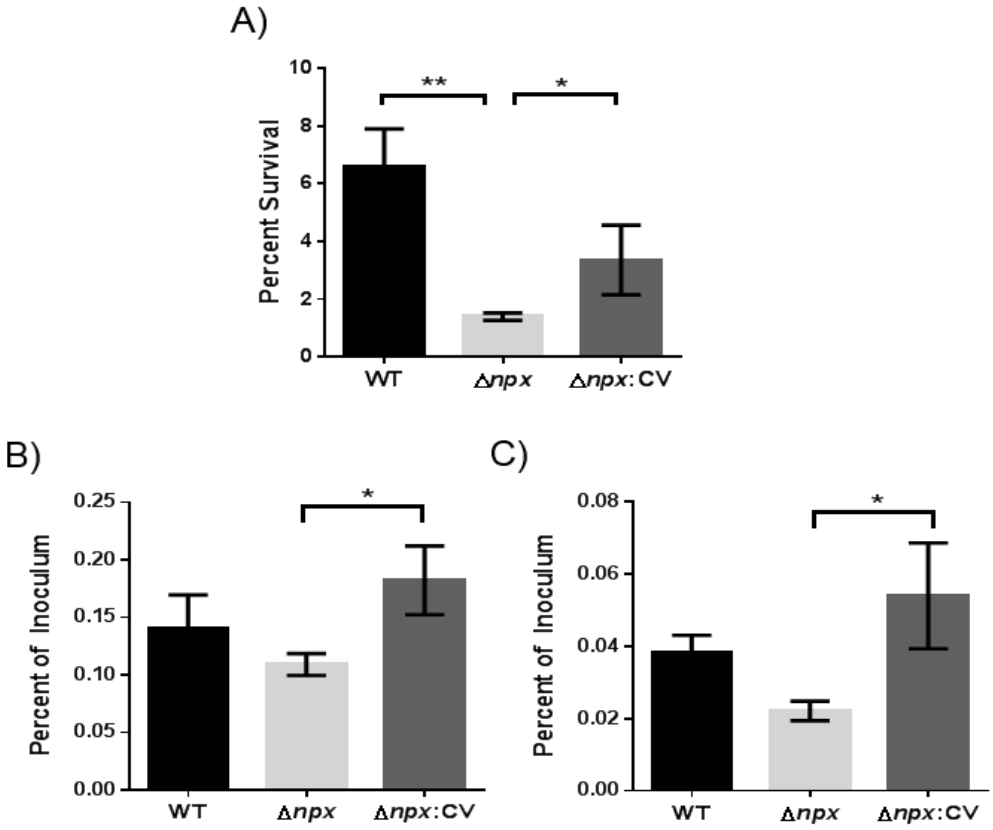


Role of Npx in phagosome survival

Since *npx* was highly expressed in the macrophage, we next examined the role of *npx* in the ability of GBS to survive the phagosome. We first examined the survival of these strains in a previously developed multiple stress medium that consists of common stresses found in the phagosome: acidic pH (pH 4.5), H_2O_2, NO, lysozyme, and Cu^{2+} (202). The *npx* mutant was more susceptible to killing by this medium, which was

partially rescued by complementation (Figure 4.4A). Next, the ability of these strains to survive inside human macrophages was determined after 1hr and 24hr. At both time points, the *npx* mutant showed a slight, but insignificant decrease in ability to survive and the complemented mutant survived similarly to the WT, but was significantly better able to survive compared to Δ*npx* (Figure 4.4B and C).

Figure 4.4. The role of the *npx* locus in surviving phagosomal stress. (A) Survival of GBS in a multiple stress medium consisting of acidic pH (pH 4.5), H_2O_2, NO, lysozyme, and Cu^{2+}. Survival was calculated by normalizing the number of CFUs after treatment to the untreated control. (B and C) Survival of GBS inside human macrophages after 1hr (B) and 24hr (C). The amount of intracellular bacteria was normalized to the total amount of bacteria in the well after the initial infection period. (* P<0.05 ** P<0.01).



CadD promotes survival through efflux of certain divalent metal cations to protect against heavy metal toxicity

In collaboration with Dr. Jennifer Gaddy at Vanderbilt University, we began to characterize the role of *cadD*. Previous studies have shown that heavy metal efflux, such as copper and zinc, plays an important role in survival inside the phagosome (206, 207). Therefore, we hypothesized that *cadD* contributes to intracellular survival through efflux of heavy metals to prevent divalent cation intoxication. To test this, we examined the growth of the WT, *cadD* mutant and complemented mutant (Δ*cadD*:CV) strains in the presence of calcium, cobalt, copper, iron, magnesium, manganese, nickel, and zinc at increasing concentrations from 0-7.5mM, concentrations at which bacteria may encounter within the phagosome (208). Of the metal cations examined, the *cadD* mutant was inhibited by cobalt, copper, zinc, and nickel and this sensitivity was rescued by complementation (Figure 4.5).

In order to demonstrate that CadD is working as an efflux pump of these divalent metal cations, we performed an elemental analysis using inductively-coupled plasma mass spectrometry (ICP-MS) on bacterial cultures after incubation in either media alone or media supplemented with cobalt, copper, nickel or zinc (Figure 4.6). Copper, nickel, and zinc accumulate to higher levels within the cell in the *npx* mutant compared to both the WT and Δ*npx*:CV strains after exposure to the heavy metals. However, cobalt accumulates within the cell at similar levels for all three strains.

Figure 4.5. The *cadD* locus confers resistance to certain divalent metal cations. GBS culture were grown in either medium alone or medium supplement with either (A) cobalt, (B) copper, (C) nickel, or (D) zinc. Other metals examined were not included as there was no significant difference in growth between the three GBS strains. (* P<0.05)

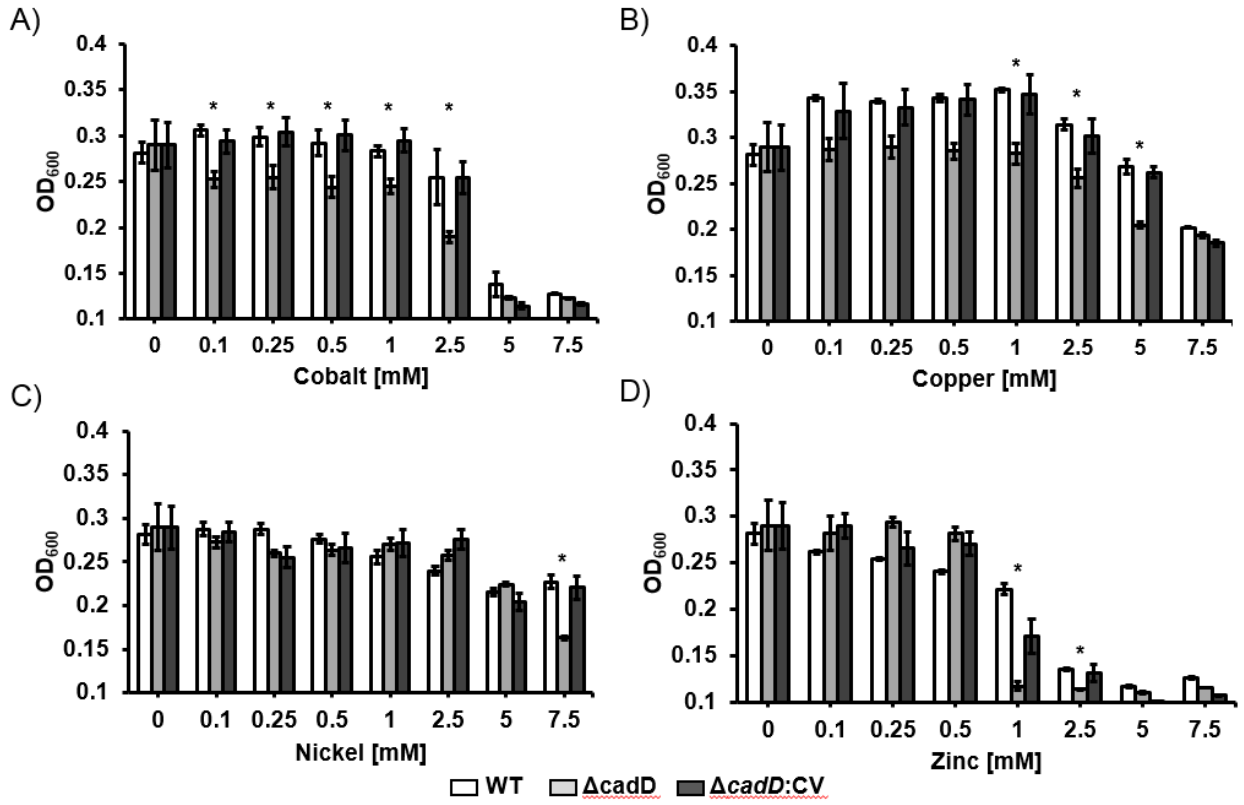
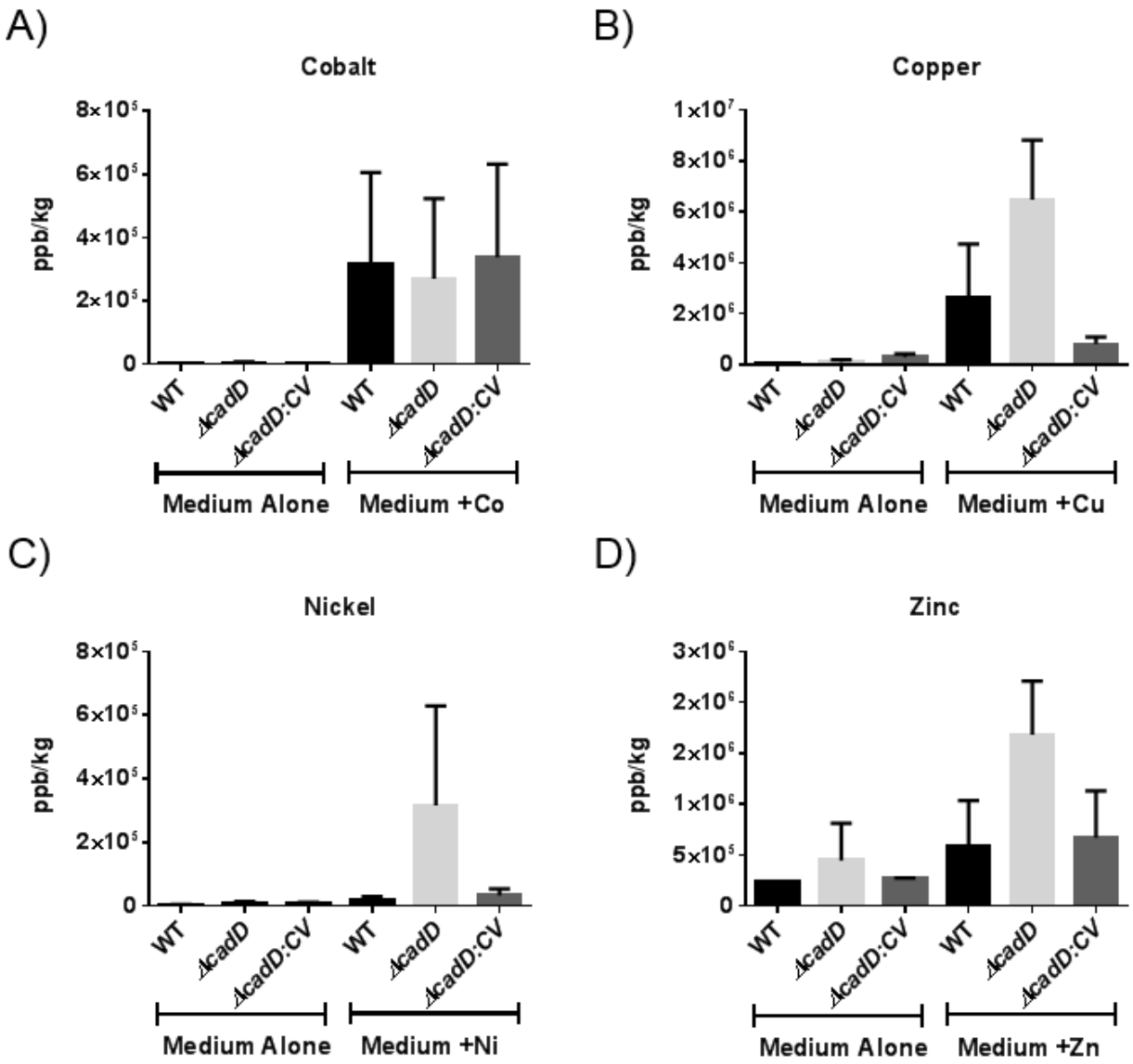


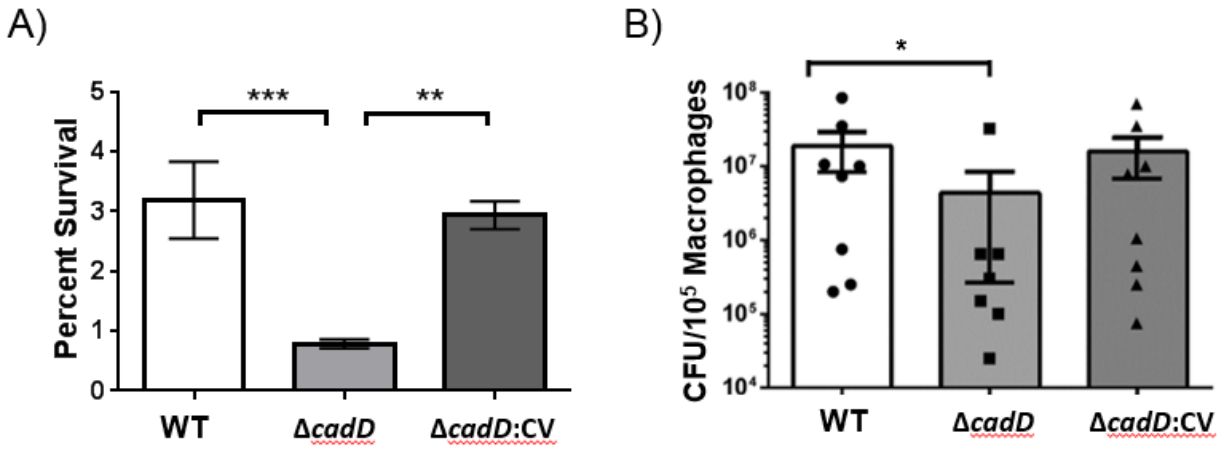
Figure 4.6. Certain divalent metal cations accumulate within the *cadD* mutant. GBS cultures were incubated in medium alone or medium supplemented with either (A) cobalt, (B) copper, (C) nickel, or (D) zinc for 24hr. After incubation, the amount of intracellular metal was determined using ICP-MS.



CadD plays an important role in phagosome survival and ascending infection in a pregnant mouse model

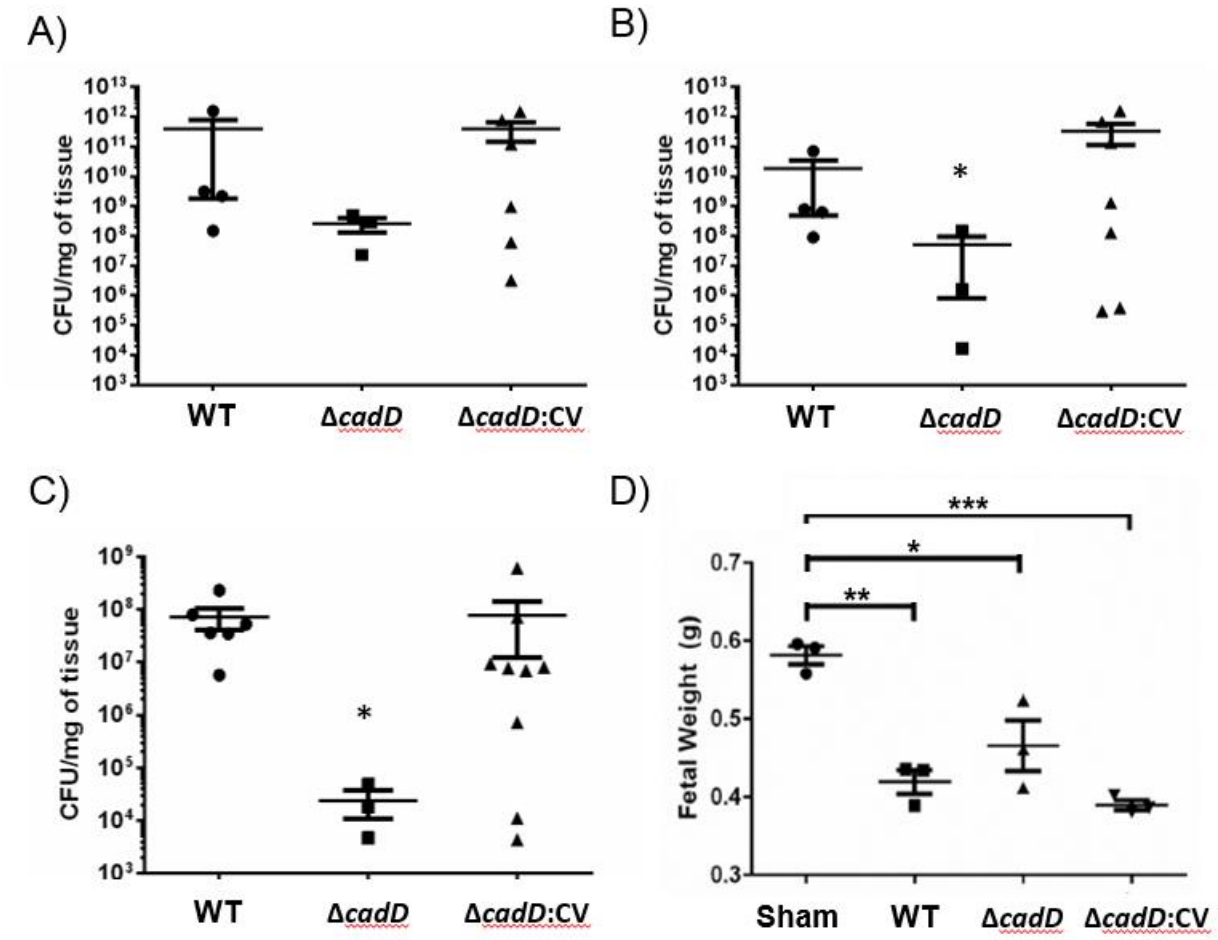
Since macrophages use heavy metals as a way to kill intracellular bacteria and *cadD* was found to be highly expressed in the macrophage, we sought to determine how CadD contributes to survival within the phagosome. We first used the multiple stress medium described above for the *npx* mutant. The *cadD* mutant was significantly more susceptible to this medium compared to the WT and this reduced survival was rescued through complementation (Figure 4.7A). Next, we examined the survival of these strains in human macrophages after 24hr. As with the multiple stress medium, the *cadD* mutant was more susceptible to macrophage mediated killing relative to the WT (Figure 4.7B).

Figure 4.7. The role of the *cadD* locus in surviving phagosomal stress. (A) Survival of GBS in a multiple stress medium consisting of acidic pH (pH 4.5), H_2O_2, NO, lysozyme, and Cu^{2+}. Survival was calculated by normalizing the number of CFUs after treatment to the untreated control. (B)The number of intracellular bacteria after 24hr survival in human macrophages. (* P<0.05 ** P<0.01 *** P<0.001).



We next hypothesized that the ability of GBS to survive inside macrophages through defense against metal toxicity was an important factor for ascending infection during pregnancy. To test this, we used an *in vivo* model of ascending vaginal infection. Pregnant mice were vaginally infected with GBS or negative controls (sham) on embryonic day 13.5 and reproductive tissues were collected after necropsy on embryonic day 15.5 to determine bacterial burden as well as fetal growth (determined by weight). The *cadD* mutant showed reduced burden of the decidua, placenta, and the fetus compared to both WT and complemented mutant (Figure 4.8A-C). GBS infected animals exhibited poor fetal development as determined by fetal weight. Animals infected with the WT and complemented strains showed an average weight deficiency of 28% and 33%, respectively, relative to sham-treated animals. However, this deficiency was attenuated when infected with the *cadD* mutant, which exhibited only a 19% deficiency in weight compared to sham-treated animals (Figure 4.8D).

Figure 4.8. The *cadD* locus is important for ascending infection and stunts fetal growth in a pregnant mouse model. Pregnant C57BL6/J mice were vaginally inoculated with 10^3 CFU GBS or medium alone (sham control). Mice were sacrificed on embryonic day 15.5 and bacterial burden of the (A) decidua, (B) placenta, and (C) fetus was determined as CFU per mg of tissue as well as (D) level of fetal growth. (* P<0.05 ** P<0.01 *** P<0.001).



Discussion

Macrophages play an important role in establishing immune tolerance during pregnancy as well as immune defense against invading pathogens. However, pathogens with the ability to survive inside these macrophages can evade immune system clearing and cross host cell barriers undetected inside these important immune cells. Therefore, it is essential to have a better understanding of how these pathogens remain inside macrophages in order to develop new therapeutics to eliminate this intracellular threat. Here, we have identified two novel factors that promote GBS survival inside human macrophages and have begun further characterizing them.

RNAseq analysis shows a high level of transcriptome remodeling in response to being inside a macrophage. It also indicates that certain genes are important only early on during survival, some are only important during long term survival, and others are needed throughout survival over the 24hr period examined here. Transcriptome remodeling also appears to continue to occur throughout intracellular survival. Approximately one quarter of the upregulated genes in this analysis are involved in metabolism. This is not surprising since the bacteria must adapt to the drastically different environment of the phagosome, which requires using alternative metabolic pathways (131). Another large portion of genes identified in the RNAseq were annotated as hypothetical proteins with no sequence homology to other known genes. This large number of genes further emphasizes our lack of knowledge of GBS pathogenesis and intracellular survival and requires further investigation.

Interestingly, 12 known virulence factors were upregulated during intracellular survival, including nine genes in the *cyl* operon, C5A peptidase (*scpB*), laminin binding

protein (*lmb*), and Fibrinogen-binding protein (*fbsA*). There were also two other genes homologous to virulence factors in other bacterial species, but have not been characterized in GBS. As stated previously, the *cyl* operon has been shown to be important in intracellular survival (141, 203). The genes *scpB*, *lmb,* and *fbsA* were found to contribute to immune evasion and host cell attachment (35, 37, 40), but their role in intracellular survival has not been determined and warrants further investigation. GBS also contains a Mn-cofactored superoxide dismutase that was previously shown to contribute to defense against ROS stress and killing by macrophages (138) and synthesizes glutathione (139), which may protect against both low pH and oxidative stress (140). However, neither of these genes were upregulated in the RNAseq analysis.

Here, as well as in previous studies (133, 138), GBS has been shown to survive high levels of H_2O_2. The ability of GBS to resist oxidative damage by the ROS burst is in part due to superoxide dismutase; however, GBS is catalase negative and therefore, requires other ways to detoxify H_2O_2. In this study we identified a putative NADH peroxidase to be highly upregulated during intracellular survival and demonstrated that *npx* could play a role in detoxifying H_2O_2. However, it is likely not the only factor contributing to H_2O_2 survival as the *npx* mutant was still able to detoxify H_2O_2 at levels slightly less that the WT and the *npx* mutant was not completely killed by H_2O_2 stress or macrophages *in vitro*. Genome sequencing shows that the GBS genome also encodes a thiol peroxidase and an alkylhydroperoxide reductase that could also contribute to H_2O_2 detoxification (9), but the role of these two genes in ROS defense requires further investigation. Although these genes were not found to be upregulated in the RNAseq

analysis, their basal levels of expression could be contributing to the ROS defense and survivability inside macrophages.

Although the complemented *npx* mutant survived inside macrophages significantly better than the *npx* mutant, there was no significant difference between WT and Δ*npx*. This could be, in part, due to the type of macrophage used in this study. Since THP-1 cells represent an immortalized cell line, they may not behave the same as primary cells *in vivo.* In order to further establish the importance of *npx* in macrophage survival, other macrophage types, such as decidual macrophages, should be examined. In addition, macrophages lacking a functional NADPH oxidase, and therefore do not produce a ROS burst should also be examined.

The *npr* gene of *Enterococcus faecalis*, which encodes a NADH peroxidase, has been well characterized and has 23% homology to the GBS *npx* examined here. Congruent with the current findings an *E. faecalis npr* mutant had similar growth to the WT under aerobic conditions, but was more susceptible to killing by H_2O_2 as well as macrophages (205). Since NADH peroxidases have been found in a number of other firmicutes (209), this may be a conserved mechanism to survive intracellular H_2O_2 and ROS stress. Although there was no effect on aerobic growth, the *E. faecalis npr* mutant was inhibited by endogenously formed H_2O_2 when intracellular oxidative stress was induced through growth in glycerol, which is oxidized by glycerophosphate oxidase resulting in H_2O_2 production (205). Future work will involve similar studies with the GBS *npx* mutant in order to further determine the role of Npx in detoxifying endogenous H_2O_2.

Metals are essential cofactors for many cellular processes for both pathogen and host, but can be toxic at high levels. Therefore, the host has developed a number of mechanisms to limit metal availability in some areas while generating microbicidal concentrations in other areas to limit pathogen growth during infection (210). Macrophages in particular have developed mechanisms to increase metal concentrations in the phagosome leading to metal poisoning of intraphagosomal bacteria. More specifically, proinflammatory cytokines induce the trafficking of the ATP7A copper transporter to the phagosome resulting in copper influx (207). Moreover, zinc accumulates in phagosomes containing *Mycobacterium tuberculosis* as well as *Escherichia coli* (207, 211). Therefore, successful intracellular pathogens must be able to detoxify heavy metals or pump them out of the cell to maintain nontoxic cellular concentrations to avoid metal poisoning by macrophages. Here, we have shown that one mechanism GBS uses to defend against certain heavy metals is efflux through CadD, which acts as a promiscuous efflux pump of nickel, cobalt, zinc, and copper, but not all metal cations.

Not only was *cadD* important for survival inside macrophages, it was also important for ascending infection in pregnant mice. Moreover, infection with the *cadD* mutant resulted in a reduced level of fetal development inhibition compared to WT and complemented mutant. This is likely due to the reduced level of bacterial burden in both the placenta and fetus. Taken together, these results indicate that *cadD* expression, resulting in the ability to survive inside macrophages, is important for crossing the extraplacental membranes and infect the fetus *in utero* in a mouse model. This suggests that *cadD* could be a potential target for alternative therapeutics to prevent

ascending infection, which results in neonatal infections and preterm birth. The role of *cadD* in establishing persistent colonization should also be examined in future work to determine the potential of eliminating this reservoir through *cadD* targeted alternative therapeutics.

In summary, we have identified two new factors involved in stress response that are highly expressed in GBS during survival inside macrophages: NADH Peroxidase, which defends against H_2O_2 stress, and Cadmium Resistance Protein, which protect against heavy metal toxicity by pumping certain divalent metal cations out of the bacterial cell. As both of these genes were found to be highly conserved among GBS, they should be considered as possible targets of novel therapeutics to help eradicate intracellular GBS that pose a threat both as a reservoir for persistent colonization and through dissemination across host cell barriers while hiding inside macrophages.

Materials and Methods

Bacterial strains and growth conditions

The GBS strain GB00112 (a ST-17 strain isolated from a vaginal rectal screen of a woman who had recently given birth (159, 185)), and isogenic mutants of this strain, including a *npx* deletion mutant (*Δnpx*) harboring the empty vector, a complemented *npx* mutant harboring a plasmid containing the *npx* locus (*Δnpx*:CV), *a cadD* deletion mutant (Δ*cadD*) harboring the empty vector, a complemented *cadD* deletion mutant harboring a plasmid containing the *cadD* locus (*ΔcadD*:CV), and the parental strain harboring the empty vector alone were used for these studies. Bacterial strains were grown on trypic soy agar plates supplemented with 5% sheep blood (blood agar plates), Todd-Hewitt agar (THA) plates or in Todd-Hewitt broth (THB) at 37ºC. Derivatives harboring the pLZ12 plasmid were growth in media supplemented with 3μg/mL of chloramphenicol.

Cell culture

THP-1 monocyte-like cells (ATCC TIB-202) were cultured in RPMI medium supplemented with 2mM L-glutamine (Gibco), 10% fetal bovine serum (FBS; Hyclone), and 2% penicillin/streptomycin (Gibco) at 37ºC in ambient air containing 5% carbon dioxide. THP-1 cells were differentiated into macrophages by incubation with 100nM phorbol 12-myristate 13-acetate (PMA; Sigma) in RPMI medium with 2% FBS for 24hr (187).

Macrophage survival assays

Intracellular survival assays were performed as previously described (202). Briefly, PMA treated THP-1 macrophages were infected with GBS strains at a multiplicity of infection of 10:1 in RPMI for 1hr. Extracellular bacteria were killed using the addition of RPMI supplemented with 2% FBS, 100µg/ml gentamicin (Gibco), and 5µg/ml penicillin G (Sigma). At the indicated time points, intracellular bacteria were enumerated by lysing the macrophages with 0.1% triton-X (Sigma) then serially diluting and plating lysates to determine CFUs. Survival was normalized to the total amount of bacteria present in the well after the 1hr infection period.

Generation of GBS competent cells and construction of isogenic bacterial mutant strains

GBS electrocompetent cells were generated by growing GBS culture in THB with 0.5M sucrose and a sublethal, but inhibitory concentration of glycine to early log phase (OD=0.25). The culture was then pelleted at 4°C, the supernatant removed, and the pellet washed with ice cold 0.625M sucrose solution. The culture was pelleted again and resuspended in 0.625M sucrose and stored at -80°C.

The genes of interest were deleted using the thermosensitive plasmid pG^{+}host5 as previously described (212). Primers used can be found in Table S2 in the appendix. The 5’ and 3’ flanking regions of *cadD* or *npx* were amplified using the primer sets P1/P2 for 5’ and P3/P4 for 3’ regions. The two amplified flanking regions were combined in a crossover PCR, resulting in a single PCR product of the deletion sequence. The PCR product and the pG^{+}host5 plasmid were digested with *BamHI* and *KpnI* then

ligated to create pG$^+$host5Δ*cadD* or pG$^+$host5Δ*npx*. The created plasmid was electroporated into GB112 competent cells and transformants were selected by growth on 2µg/ml erythromycin at 28°C. Cells in which the plasmid integrated into the chromosome were selected for by growth on erythromycin at 42°C. Colonies that grew at 42°C were then grown in broth with no antibiotic selection at 28°C for several passages to allow for excision of the plasmid. The cultures were then plated and single colonies were tested for erythromycin susceptibility and screened for gene deletion using PCR with primers P5 and P6 for the indicated genes. Deletion was confirmed using sequencing.

Complementation of both deletions was done using the pLZ12 plasmid with the *rofA* constitutive promoter (204). The coding sequence of *cadD* or *npx* was amplified using the pLZ12 primer sets in Table S2 in the appendix. The PCR product and the pLZ12-*rofA*pro plasmid were digested with *Bam*HI and *Pst*I then ligated to create *cadD*-pLZ12 or *npx*-pLZ12. The plasmid was electroporated into GBS competent cells and transformants were selected by growth on 3µg/ml chloramphenicol.

RNA isolation, RT-PCR and RNAseq

RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (126). Briefly, RNA samples were collected from bacteria culture in liquid medium by adding two volumes of RNAportect Bacteria Reagent (Qiagen) or from bacteria inside macrophages by washing the cells twice with PBS then adding 1ml RNAprotect Bacteria Reagent directly to the cells. RNA was then extracted using the RNeasy minikit (Qiagen) using the “Enzymatic Lysis, Proteinase K Digestions, and

Mechanical Disruption of Bacteria" protocol in the RNAprotect handbook. Residual genomic DNA was removed using the Turbo DNA-free kit (Ambion). The iScript Select cDNA synthesis kit was used to synthesize cDNA using random primers. As a control, samples were processed without reverse transcriptase. RT-PCR analysis was performed using the iQ SYBR Supermix (Bio-Rad) and gene specific primers. Relative fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *gyrA* as the internal control (166). Primer sequences can be found in Table S1 in the appendix.

The same RNA isolated above was also used for RNAseq. RNA samples collected from bacteria inside host cells were first depleted of host cell RNA using the MICROB*Enrich* Kit (Ambion) following the manufacturer's instructions. Ribosomal RNA was removed from all samples prior to sequencing using the Ribo-Zero rRNA Removal Kit for Gram-Positive Bacteria (Epicentre) according to the manufacturer's directions. Samples were then submitted for NGS library prep and sequencing to RTSF Genomics Core (Michigan State University). Libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep Kit; the protocol was modified to skip the oligo-dT bead step and proceeded directly to fragmentation and first strand synthesis. The libraries were pooled and loaded on one lane of an Illumina HiSeq 2500 Rapid Run flow cell (v2). Paired end 2x100bp (PE100) sequencing was carried out using Rapid SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.64 and output of RTA was demultiplexed and converted to FastQ files with Illumina Bcl2fastq, v1.8.4. Analysis was carried out on the CLC Genomics workbench using the RNAseq analysis tool by mapping the reads to the GB00112 genome. Differential expression analysis was performed using EdgeR using the following comparisons: 1hr vs media

alone, 24hr vs media alone, and 1hr vs 24hr. A significant difference in expression was accepted for genes with at least a 2 fold change in expression and $P < 0.05$.

H_2O_2 quantification

Bacterial cultures were incubated at 37^0C with the indicated concentrations of H_2O_2 in sodium phosphate buffer for 1hr in a 96 well plate. Buffer with bacteria alone (no H_2O_2 added) and buffer with H_2O_2, but without cultures were included as controls. After 1hr, the bacteria were pelleted and supernatants were transferred to a new 96 well plate. The amount of H_2O_2 remaining in the supernatants was determined using the Fluorimetric Hydrogen Peroxide Assay Kit (Sigma) using the manufacturer's directions. The concentration of H_2O_2 was calculated by comparing fluorescence readings from the samples to a standard curve. Percent detoxification was calculated by normalizing data to the H_2O_2 controls without bacterial cultures.

Survival in H_2O_2 and multiple stress medium

The ability of GBS to survive phagosomal stress was done as previously described (202). Briefly, GBS were exposed to either the indicated concentration of H_2O_2 alone or a multiple stress medium, which consist of 1.5mM H_2O_2, 3mM $NaNO_2$, 100 µM $CuCl_2$, and 100µg/ml lysozyme in acidic sodium phosphate buffer (pH 4.5) for 1hr then diluted and plated to enumerate viable bacteria. Percent survival was calculated by normalizing viable bacteria in the treated sample to the untreated samples.

GBS growth in heavy metals and inductively-coupled plasma mass spectrometry (ICP-MS) analyses

Overnight GBS cultures were diluted 1:100 into THB alone or supplemented with increasing concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 7.5mM) of calcium, manganese, magnesium, iron, nickel, cobalt, zinc, and copper. Cultures were grown for 24hr and the bacterial density was measured using OD_{600} throughout the growth period.

Elemental analyses of cell-associated metals were performed with ICP-MS as previously described (213). Briefly, bacterial cells were grown in THB alone (medium alone), or supplemented with the indicated metal salt for 24hr, with shaking, at 37°C in aerobic conditions. Cells were pelleted at 4,000 × g for 15 min before washing once with 0.5 M EDTA and three times with 5 mL of distilled water. Samples were weighed and digested in 1 mL of 50% nitric acid overnight at 50°C and elements were quantified using a Thermo-Element 2 HR-ICP-MS apparatus (Thermo, Fisher Scientific, Bremen, Germany), as previously described (214).

Mouse model of GBS ascending infection during pregnancy

GBS infection of pregnant mice and subsequent analyses were performed as previously described (215). Briefly, C57BL6/J mice were purchased from Jackson Laboratories and mated in harem breeding strategies overnight. Pregnancy was confirmed the following day by the presence of a mucus plug to establish the embryonic date (E0.5). On embryonic day 13.5, dams were anesthetized and 50µL of inocula containing 10^3 CFU in THB medium containing 10% gelatin was introduced into the vagina. Sham controls were inoculated with 50µL of THB medium containing 10%

gelatin. Upon recovery from anesthesia, animals were housed singly in cages and monitored for weight gain, general health, and preterm delivery. Animals were sacrificed on embryonic day 15.5 and necropsy was performed to isolate reproductive tissues including vagina, uterus, placenta, decidua, amnion, fetus, amniotic fluid, and maternal blood. Tissues were analyzed for bacterial burden by enumerating CFUs per mg of tissue from homogenized tissues.

Statistical analysis

With the exception of the RNA analyses (see above), statistical analyses were performed using GraphPad Prism version 6 and statistical significance was accepted at $P<0.05$. Bacterial growth assays, H_2O_2 detoxification, bacterial survival in H_2O_2, survival in multiple stress medium, macrophage intracellular survival, and the log transformation of bacterial burden in host tissues were analyzed using One-way ANOVA.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Despite increased prevention efforts, Group B *Streptococcus* (GBS) remains a leading cause of neonatal infections and current case rates have remained the same in the last several years (216). The current preventative measure is intrapartum antibiotic prophylaxis (IAP) for GBS positive or high risk pregnant mothers. Although this has been effective, the use of antibiotics during pregnancy can have adverse effects on the infant, such as altering the gut microbiota, increasing the risk of sepsis due to antibiotic resistant strains, an increased risk of asthma, and others (217–219). Moreover, antibiotics are the only treatment option, which is becoming increasingly problematic due to the rise in antibiotic resistance (220). Despite the use of antibiotics, mothers also can remain persistently colonized by GBS, providing a reservoir for future infections (7). No vaccine is currently available for GBS and efforts to develop vaccines have been complicated by the high level of genetic diversity of GBS strains (2). Therefore, there is a great need to better understand how strains differ at various steps of disease progression and identify factors that can be targets for alternative therapeutics and vaccine development.

In an effort to better understand GBS pathogenesis, the focus of this dissertation was to gain a better understanding of how diverse strains of GBS vary in their interactions with host cells. The overall goal of the first study was to determine if strains of the same serotype differed in their ability to associate with host cells as well as their gene expression in response to this association. This study found that strains of the same serotype, and even the same genotype, can vary in their ability to associate with host cells and this association ability is host cell type specific. Moreover, gene expression profiles showed that certain key virulence factors are similarly expressed

across host cell types within a single strain. Also, virulence factors were differentially expressed in the strains of different STs examined in the study. The second study examined two mechanisms of persistent colonization: antibiotic tolerance and survival inside macrophages. This study showed that CPS type III strains are better at surviving phagosomal stress and diverse strains use different mechanisms to withstand phagosomal stress. More specifically, the GBS ST-17 strain examined in the study uses a pH dependent mechanism to survive, whereas pH was less important for the ST-12 strain. Moreover, antibiotic exposure increased the phagocytic uptake of an ST-17 GBS strain in a capsule dependent manner for certain antibiotics. Finally, the third study revealed temporal transcriptome remodeling during survival in macrophages and identified key factors to be important for intracellular survival. More specifically, NADH peroxidase was shown to promote intracellular survival via H_2O_2 detoxification and the cadmium resistance protein protects against heavy metal intoxication through efflux of certain divalent metal cations. Taken together, the findings in this dissertation demonstrate a high level of pathogenic diversity across GBS strains at multiple steps of disease progression suggesting multiple mechanisms employed by GBS to cause disease. Future projects described here are directed at examining additional strains for more ST specific characterizations as well as identification and characterization of additional virulence factors.

With the level of variation observed during host cell interactions and the enhanced ability of the hypervirulent lineage, ST-17, to attach to, invade and survive inside host cells, further characterization of how and why these strains differ will lead to identification of targets that will allow for the treatment and prevention of infections of

highly pathogenic strains. To this end, the examination of a larger set of strains of different CPS types and STs will allow for phenotypic characterizations of high or low level invasiveness. Genomic comparisons of highly invasive strains to those with little or low invasive ability will identify factors that are unique to the invasive phenotype. Furthermore, whole transcriptome analyses during association with host cell barriers, such as cells of the extraplacental membranes, lung epithelium, and the blood-brain barrier, will uncover factors important in breaching these barriers that are both unique to invasive strains and conserved across strains. Additionally, there is a need for new models to study GBS pathogenesis and work shown here will aid the development of a microfluidics model of GBS infections through helping direct strain selection and serving as a basis for understanding how GBS interacts with the different host cells examined here to guide host cell selection.

Antibiotic tolerance (not resistance) and the effect of exposure to subinhibitory concentrations of antibiotics is not well studied in GBS. However, the work shown here demonstrated that subinhibitory antibiotics have the potential to increase virulence by causing increased uptake by macrophages where GBS can survive and be protected by antibiotics and the immune system as well as disseminate to other areas of the body. Future work should involve further exploration of the effect of subinhibitory antibiotics as well as antibiotic tolerance in a diverse set of GBS strains with large set of antibiotics in order to determine if this is a characteristic associated with more invasive strains. Additionally, other stages of pathogenesis should be examined, such as invasion of host cell barriers. Factors involved in tolerance and the changes in response to subinhibitory antibiotics can be identified through transcriptome analyses as well as genomic

comparisons as described above. Therapeutics targeting these factors could then be combined with IAP in order to make the treatment more effective and reduce the likelihood of persistent colonization.

The GBS transcriptome analysis following survival inside macrophage provides the greatest source for future work. This analysis provided a large list of both known and unknown factors that specific GBS genotypes use while persisting in the phagosome that can be further explored and characterized. These factors can be examined through mutagenesis experiments to determine their function as well as gene expression analyses to better understand their regulation. Although unexplored in this dissertation, the analysis also provided a list of genes turned off during intracellular survival and could help identify factors that may be detrimental to the ability of GBS to survive. In this work, the cadmium resistance protein, CadD, was determined to be an efflux pump for certain divalent metal cations to promote resistance to metal toxicity. Within the RNAseq analysis, a large set of other putative metal transporters were also highly upregulated during survival. The large number of transporters indicates a high level of involvement of metals in the phagosome and provides an intriguing area of further exploration in GBS research. These transporters may provide redundant function to CadD or work to protect against the other metals that CadD did not transport. Furthermore, some of these transporters may function in nutrient acquisition.

Lastly, the work provided here provides new insights for drug discovery and therefore the development of a reporter strain to make drug discovery more efficient is greatly needed. Sastalla et. al. have codon-optimized a number of fluorescent proteins that can be expressed in gram positive bacteria with low GC content (221), which will be

very useful for creating GBS reporter strains to identify drugs that specifically target the virulence factors identified in this dissertation as well as in future work. Furthermore, GBS reporter strains will also be useful in visualizing GBS interactions with host cells using microscopy. This will further aid in our understanding of the importance of GBS diversity in host-pathogen interactions.

APPENDIX

Table S1. Oligonucleotide primers used for qRT-PCR in this dissertation.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
gyrA	CGGGACACGTACAGGCTACT	CGATACGAGAAGCTCCCACA
srr1	GTCACTTCCGTTTGTTCTGCC	CTGGTAGGTGGAGCGAGTTT
srr2	GCTGTAGTTGGAGGGACGAC	TTACTTCTGGCGCAACCACT
bibA	CTAGCGGAAACTTGGTGGCT	GGCTTCACCCGTTGATGGTA
hvgA	ATACAAATTCTGCTGACTACCG	TTAAATCCTTCCTGACCATTCC
lrrG	TACGCCAGATTCCTTTGGCA	CTTCTGCCGCTGCATTTCG
lmb	GATCCCTTGCCCAAGCTTCT	TCCAATCAGGTGCAGGCATT
scpB	GTAACTACGCTCAAGCTATC	CCCAAAGCTACTATCATTAC
spb1	TCGCTGTTAGTGGCGAGTTT	TGTCTCAGCGGCAAAAGCTA
fbsB	GCGATTGTGAATAGAATGAGTG	ACAGAAGCGGCGATTTCATT
cylE	TGGAAATTGCTAAGTTAGATAACG	AGCCCTCGTTAAGTTTGCCA
iagA	CCCCCAAGTTTCGGGAGTTT	ACGTTTGACATTACGGTCGGT
sip	CATCGACAATGGCAGCTTCG	GCTGTCCACGTCGTATCTGT
ponA	AGGAAGTTTGGCTTGGGCTT	AGCGAGCAAAGCAAGTTGTG
cylX	TGGCTTGTATAAAACGCGGCT	CAACGACACTGCCATCAGCA
sodA	TGGGAATTGATGTCACCAGA	CCTGAACCAAAACGTCCTGT
covR	TGCTCCACGATCAAGACCAG	ACGGTCGTGAAGGATTGGAC
covS	AGCTTCCTTGGACGCGAATC	TTAAGAACGCCTGTCGCTGT
fbsA	GCGGTTTGAGACGCAATGAA	AAAAGTCACCCTAACCAACCT
cadD	GCACAAGTCCCTTCTGTTGG	GCCTAACACAGCCCATAGCA
npx	GACCGCCTTCCCTGATTCAT	TAGCAGTTGTTGGGGCAGG

Table S2. Oligonucleotide primers used to generate GBS mutagenesis and complementation vectors.

Primer Name	Sequence (5' to 3')
cadD-P1 [a]	CCGC**GGATCC**CAGTACCTGCACGTCACACT
cadD-P2 [b]	CCCATCCACTAAACTTAACAAAGCCCTGCCATCGAAATGA
cadD-P3 [b]	TGTTTAAGTTTAGTGGATGGGTGCTATGGGCTGTGTTAGGC
cadD-P4 [c]	GGG**GGTACC**CCTGCATTGCCTAGTTCGCT
cadD-P5	CAGTACCTGCACGTCACACT
cadD-P6	TACGTATAGCTCGAAGCGGT
npx-P1 [a]	CCGC**GGATCC**CCAAAGCCCCAGATTTTGCTG
npx-P2 [b]	CCCATCCACTAAACTTAACAAAGAGGATTGGGCTATGCGA
npx-P3 [b]	TGTTTAAGTTTAGTGGATGGGACCATAAAGTCGGTCTCAGCA
npx-P4 [c]	GGG**GGTACC**TTAGACCCCATTATGAGGCTGC
npx-P5	CTAAGGCTGCGTCTAACCGT
npx-P6	TGCTGTAGCTATTGCAGCGT
cadD-pLZ12-F2 [a]	CGC**GGATCC**AGGAGGACAGCTATGATTCAAAATGTTGTTAC
cadD-pLZ12-R2 [d]	AAAA**CTGCAG**CTAGCCTAACACAGCCCATA
npx-pLZ12-F2 [a]	CGC**GGAT**CCAGGAGGACAGCTATGACAGAAAAATATGTA
npx-pLZ12-R2 [d]	AAAA**CTGCAG**CTATTCGCATAGCCCAATCC

[a]BamHI restriction site is shown in bold
[b]Complementary sequences for P2 and P3 sets are underlined
[c]KpnI restriction site is shown in bold
[d]PstI restriction site is shown in bold

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