CHARACTERIZATION OF INTRACELLULAR TRAFFICKING OF

STREPTOCOCCUS AGALACTIAE

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Characterization of Intracellular Trafficking of *Streptococcus agalactiae*

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ABSTRACT OF THE THESIS

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Streptococcus agalactiae (Group B Streptococcus, GBS) is an important human pathogen because of its ability to cross the blood brain barrier (BBB) to cause meningitis in neonates. Previous studies have demonstrated that GBS can invade and transcytose through the cells that constitute the BBB to enter the brain. However, the exact mechanisms of intracellular trafficking have not been characterized. Previous electron micrographs have shown that when GBS invades brain endothelial cells, they reside in membrane-bound vesicles. Therefore, I hypothesized that GBS may be trafficked through the endocytic pathway, which can serve as an important host defense mechanism against intracellular pathogens. In order to examine if GBS is shuttled through this pathway, I infected human brain microvascular endothelial cells (hBMEC) with GFP-expressing GBS and stained for early and late endosomal markers, Rab5 and Rab7 respectively. GFP-GBS was shown to co-localize with early and late endosomes, as well as acidic vesicles. I have also shown that an important GBS response regulator CiaR, which promotes bacterial intracellular survival, acts to limit bacterial trafficking to acidic compartments and that one of the CiaR-regulated genes, SAN_2180, contributed to intracellular survival. Since approximately 50% of GBS was co-localized with acidic compartments, I hypothesized that selective autophagy may be induced in hBMEC to respond to GBS infection. An important indicator of autophagy is the microtubule-assocate protein light chain 3 (LC3). LC3 exists in 2 forms: LC3-I, an inactive cytosolic form, and LC3-II, the active lipidated form that associates with the formation of the autophagosome; therefore, an increase in LC3-II conversion is indicative of autophagy activation. During GBS infection, I have observed an increase in LC3-II levels, indicating induction of autophagy. I also demonstrated that autophagy is a critical host defense mechanism against intracellular GBS, and that specific bacterial factors such as the β-hemolysin/cytolysin may modulate this response. Further studies will focus on examining other host trafficking pathways that are not destined to fuse with lysosomes and further elucidate the various mechanisms associated with autophagy induction.
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CHAPTER 1

INTRODUCTION

1.1 SIGNIFICANCE OF GBS

*Streptococcus agalactiae* (Group B *Streptococcus*, GBS) is a Gram-positive bacterium that can colonize healthy individuals in the gastrointestinal tract and female reproductive tract [1]. It is the leading cause of neonatal meningitis and can cause invasive diseases in adults [2]. In order to cause meningitis, GBS must survive and persist in the bloodstream, interact with, and penetrate the blood-brain barrier (BBB), a single layer of highly selective microvascular endothelial cells, to gain entry to the central nervous system (CNS). However, it is still unclear how GBS trafficks through the brain endothelial cells to cross into the brain.

There are 9 different GBS serotypes, which are based on the differences of the polysaccharide capsule [2]. Serotypes Ia, Ib, II, III, and V are responsible for causing the majority of invasive disease and are isolated in 96% of neonatal cases [3].

1.2 IMPORTANT GBS VIRULENCE FACTORS

GBS encodes for several virulence factors that contribute to disease progression and some have been shown to aid in intracellular survival [1]. Since certain factors have been shown to help the bacteria persist inside a host cell, I hypothesized that they may also have an important role in how GBS is trafficked intracellularly.

1.2.1 CiaH/R Two-Component Regulatory System

Two-component regulatory systems (TCS) involve a transmembrane sensor kinase and a cytosolic response regulator. External stimuli can activate the sensor kinase, which in turn will phosphorylate and activate the response regulator that will bind to a specific part of the bacterial genome to allow for the transcription, or repression, of certain proteins [4]. In the case of CiaH/R, CiaH is the sensor kinase and CiaR is the response regulator. Previous studies have shown that CiaR is an important factor that aids in GBS intracellular survival.
within phagocytic cells, as well as hBMEC [5]. Therefore, I hypothesize that CiaR is another protein that can also be involved in intracellular trafficking. Furthermore, if CiaR does contribute to intracellular trafficking, then it may regulate a gene important for trafficking.

According to microarray data from the ciaR isogenic mutant, CiaR regulates variety of genes that encode for metallopeptidases and some genes of unknown function, one being SAN_2180, one of the most highly down-regulated gene [5]. To determine if SAN_2180 contributes to GBS intracellular survival, a mutant was previously created via plasmid insertion in the SAN_2180 gene. Since the function of SAN_2180 is still unknown, I investigated whether this gene contributes to GBS adherence, invasion, or intracellular survival in hBMEC.

1.2.2 Beta-Hemolysin/Cytolysin

The GBS β-hemolysin/cytolysin (β-h/c) is a pore-forming toxin that can directly damage host cell membranes, resulting in cell lysis. This toxin is encoded by the cyl operon and the cylE gene is important for hemolysin production [6, 7]. Not only does β-h/c contribute to virulence, but it was also found to contribute to GBS survival within phagocytic cells [8]. Since β-h/c is important for intracellular survival, it may also play an important role in intracellular trafficking.

1.2.3 CovR/S Two-Component Regulatory System

The CovR/S TCS is responsible for regulating the cylE gene and β-h/c production. Recently, the response regulator, CovR, was shown to play an important role in acid response, allowing GBS to survive in macrophages [9]. Again, because CovR is important for GBS survival in the phagosome, it may also contribute to trafficking in hBMEC.

1.2.4 Pili

Gram-positive bacteria have been shown to have pili, which are hair-like appendages on the cell surface. In GBS, pili are comprised of the PilA, PilB, and PilC proteins [10]. PilA is the tip-adhesion protein that aids in GBS attachment to and invasion of brain microvascular endothelial cells (hBMEC) [10, 11]; PilB comprises the pilus backbone and aids in GBS hBMEC invasion and survival in macrophages and neutrophils by mediating antimicrobial peptide resistance [10, 12]; PilC is the protein associated with the base of the
pilus [10]. Because PilB contributes to GBS survive in phagocytic cells, it could also have a role in how GBS is trafficked once inside a host cell.

1.2.5 Invasion-Associated Gene

Lipotechoic acid (LTA) is a major cell wall component in Gram-positive bacteria capable of eliciting a host immune response through Toll-like Receptor (TLR) recognition [13]. The cell wall-associated [Glucose(β1-6)Glucose (β1-3)(gentiobiosyl)diacyl-glycerol] (DGlcDAG) is an important glycolipid that serves as an anchor for LTA. A GBS invasion-associated gene (iagA) encodes for a DGlcDAG synthase allowing for the production of DGlcDAG to anchor LTA to the cell wall [14]. When iagA is absent, the LTA anchor is not produced, which leads to significant impairment in the ability of GBS to invade hBMEC [14].

1.2.6 Polysaccharide Capsule

GBS are categorized into serotypes based on the arrangement of their polysaccharide capsule, which also contains sialic acid. This capsule aids in bloodstream survival by inhibiting opsonization, via the complement pathway, by phagocytic cells. Capsule-deficient mutants were found to have an increase invasion rate of hBMEC, when compared to WT, indicating that the polysaccharide capsule inhibits invasion of hBMEC possibly because the capsule hides important cell surface factors that promote attachment and invasion [15].

1.3 ENDOCYTIC PATHWAY

The endocytic pathway is a host defense mechanism that can degrade invading intracellular pathogens, like Group A Streptococcus (GAS) [16, 17]. After a pathogen facilitates its own uptake into a host cell, it can reside in an early endosome, a single membrane vesicle typically decorated with Rab5 GTPases. The early endosome can mature into a Rab7-labeled late endosome. These endosomes can fuse with a lysosome, which results in the degradation of the pathogen [16, 18]. Rab proteins are activated through the binding of GTP and are involved in the formation and motility of vesicles and fusion with endosomes [19]. Rab5 has also been shown to play a critical role in fusion of endosomes and Rab7 is involved in endosomal maturation and fusion with lysosomes [19-22]. Previous electron micrographs of hBMEC demonstrate intracellular GBS have shown that it stays
within a single membrane vesicle [15]. Therefore, I hypothesize that GBS may be shuttled through the endocytic pathway (Figure 1.1). To determine if GBS associates with endocytic markers, I utilized GFP-expressing GBS in intracellular survival assays and stained for the host proteins Rab5 and Rab7, as well as for acidic compartments.

![Diagram of the endocytic pathway within host cell.](image)

**Figure 1.1. Diagram of the endocytic pathway within host cell.**

### 1.4 SELECTIVE AUTOPHAGY

Autophagy has been typically associated with the degradation of the host’s own cellular components under starvation conditions [23]. However, antimicrobial selective autophagy (xenophagy) has been described as another host defense mechanism against invading bacterial pathogens, such as GAS [24]. Additionally, previous studies with GAS have shown that Rab5 and Rab7-labeled endosomes were associated with bacterial degradation through the autophagic pathway [17]. After bacterial invasion, intracellular bacteria can be sequestered into double-membrane structures known as autophagosomes, which are destined to fuse with the lysosome [25, 26]. One of the main proteins associated with the formation of the autophagosome is a cytosolic protein called microtubule-associated...
protein 1 light chain 3 (LC3). When autophagy is activated, the ATG5-12-16L1 complex lipidates LC3-I, by the addition of phosphatidylethanolamine, to its active form, LC3-II, allowing for the elongation of the autophagosome [26]. Targeting of intracellular pathogens with LC3-II is facilitated through adaptor proteins, such as sequestosome 1 (SQSTM1/p62), nuclear dot protein 52 kDa (NDP52), and optineurin (OPTN) [27, 28]. Previous work from the lab has shown that autophagy is induced in GBS-infected hBMEC (Figure 1.2). However, the bacterial factors that induce autophagy are unknown.

Figure 1.2. Diagram of autophagic pathway within host cell.
CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

*Streptococcus agalactiae* (GBS) wild-type (WT) clinical isolates COH1 (serotype III) [29] and NCTC10/84 (serotype V) [30], and their isogenic mutants, including COH1 Δ2180, COH1 ΔciaR [8], COH1 ΔcovR [31], COH1 ΔcylE [7], COH1 ΔiagA [13], HY106 [15], NCTC10/84 ΔcylE [7], NCTC10/84 ΔpilA [11], and NCTC10/84 ΔpilB [12] were used in this study. All GBS strains were grown in Todd-Hewitt Broth (Hardy Diagnostics) at 37°C. For antibiotic selection, 2μg/mL of chloramphenicol (Cm) or 5μg/mL of erythromycin (Em) were used.

2.2 CONSTRUCTION OF GREEN FLUORESCENT PROTEIN (GFP)-EXPRESSING GBS

MC1061 *E. coli* containing the pDESTerm GFP plasmid was obtained from Victor Nizet (UCSD). Plasmid purification was performed via the Zymogen Zyppy™ MiniPrep. Competent bacterial cells were created by growing GBS in THB with 0.6% glycine to early-log phase. Bacteria were then centrifuged at 4000RPM for 30 minutes at 4°C. The supernatant was removed and pellets were washed with ice-cold 0.625M sucrose buffer. Bacteria were centrifuged again as described above and again the supernatant was removed. 1μg/μL of purified plasmid was added to 75μL competent GBS in a 0.1cm electroporation cuvette and electroporated at 1500V for 2-4 milliseconds. All steps were performed on ice. Transformed cells were incubated in recovery media (THB supplemented with 0.25M sucrose) at 37°C for 1 hour. The culture was then plated on THB agar plates containing 5μg/mL Em and incubated at 37°C. Colonies obtained were assessed for plasmid uptake by fluorescent microscopy and fluorescent cell sorting (FACS). Procedure and results for fluorescence and growth curve studies can be found in Appendix A.
2.3 Cell Lines

Immortalized human brain microvascular endothelial cell lines (hBMEC) were obtained from Kwang Sik Kim (Johns Hopkins University) and were grown in RPMI 1640 with L-glutamine (Corning, CellGro) supplemented with 10% NuSerum, 10% fetal bovine serum (FBS), and 1% modified Eagle’s medium nonessential amino acids at 37°C with 5% CO₂. ATG5 -/- knockout and ATG5 +/- wild-type mouse embryonic fibroblasts (MEF) cell lines were obtained from Noboru Mizushima (University of Tokyo) and cultured in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO₂.

2.4 In Vitro Infection Assays

GBS adherence, invasion, and intracellular survival assays were performed as described previously for hBMEC [15]. Cells were grown to confluence (monolayer) in 24-well tissue plates containing 1% rat-tail collagen. Cells were infected with bacteria grown to mid-log phase at multiplicity of infection (MOI) ranging from 1~10.

2.4.1 Adherence Assay

To assess the number of adherent bacteria to the cell surface, cells were incubated with mid-log phase GBS (MOI of 1) for 30 minutes at 37°C with 5% CO₂. After co-incubation, cells were washed 5 times with phosphate buffer saline solution (PBS). Then, 0.25% Trypsin-EDTA was added to liberate cells and 0.025% Triton X-100 added to lyse the cells. Serial dilutions were performed on lysates and plated on THB agar (THA) to obtain bacterial colony forming units (CFU). The percentage of adherent bacteria was calculated as (recovered CFU/original inoculum X 100%).

2.4.2 Invasion Assay

The amount of intracellular bacteria was determined by incubating cells with GBS (MOI of 1) for 2 hours. After infection, cells were washed with PBS 3 times and then antibiotic media (RPMI containing 100μg/mL of gentamycin and 5μg/mL of penicillin) was added for an additional 2 hours to eliminate any extracellular bacteria. Cells were again washed 3 times with PBS and lysed as described above. Lysates were plated on THA to obtain CFU counts. Invasion rate was calculated using the same equation as mentioned above.
2.4.3 Intracellular Survival Assay

To quantify the number of intracellular bacteria at a given time point, GBS (MOI of 1) and cells were incubated together. After washing cells 3 times with PBS, antibiotic-containing media was added to cells for specific time points. After each time point, cells were washed 3 times and lysed as described above to liberate intracellular bacteria. Cell lysates were plated on THA to obtain viable GBS colony forming units (CFU). The percentage of survival was calculated using the equation mentioned earlier.

2.5 Immunofluorescent Staining and Microscopy

Cells were grown to 80-100% confluence on round-glass coverslips in 24-well tissue plates for co-localization studies by immunofluorescent staining. The primary antibodies bind to the target cell-associated antigen and the secondary antibody conjugated to a fluorophore was added to bind to the primary antibodies. The coverslips were then mounted onto glass slides using Vectashield with DAPI (Vector Labs) and fluorescence was observed utilizing a Zeiss Axiovert 200 inverted microscope (Carl Zeiss). For each condition, 200 cells containing intracellular GBS were examined.

2.5.1 Co-Localization with Endosomal Markers

To determine if intracellular GBS co-localized with Rab5 and Rab7, GFP-expressing GBS (MOI of 10) was incubated with hBMEC for 1 hour, then washed 3 times with PBS. Antibiotic containing media was added to cells for specific time points. Cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature. 4% FBS blocking reagent was added to the fixed cells for 1 hour at 4°C. The primary antibody (anti-Rab5 or anti-Rab7 antibody, 1:100 dilution in 2% FBS in PBS) was incubated with the cells at 4°C overnight. The secondary antibody with the Alexa Fluro 594 fluorophore (1:2000) was added to cells for 1 hour at room temperature. At each step, cells were washed 3 times with PBS.

2.5.2 Co-Localization with Acidic Compartments

To determine if GBS associated with acidic vesicles, or lysosomes, cells were incubated with GFP-GBS (MOI of 10) and antibiotic media was added for specified time points as described above. 30 minutes before each time point, 1μM of Lysotracker
(Invitrogen, in RPMI with antibiotics) was added to cells and the coverslips were fixed and mounted as described above. Cells were washed with PBS 3 times at each step.

**2.6 Western Blotting**

Cell lysates were obtained by using RIPA buffer (Thermo Scientific) containing 1μM NaF, 1mM PMSF, and protease inhibitor cocktail (Calbiochem). Protein concentration was determined by using the Bio-Rad DCTM Protein Assay Kit and 10μg of cell lysate was loaded into 10-20% Tris-Glycine protein gels (Invitrogen). PVDF membranes were probed with antibodies against human LC3 (1:1000, Cell Signaling Technologies), SQSTM1/p62 (1:2000, Santa Cruz Antibodies), and GAPDH (1:150,000; Millipore).

**2.7 Transmission Electron Microscopy**

To examine if GBS resides in autophagosomes, hBMEC was pre-treated with 1μM bafilomycin. As a control, uninfected hBMEC was pre-treated with 1μM bafilomycin and 50nM rapamycin. Cells were incubated with GBS (MOI 10) for 4 hours at 37°C with 5% CO₂, washed 3 times with PBS, and then supernatants were collected and centrifuged at 1000RPM for 5 minutes, washed once with PBS, then centrifuged again. Cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer for 90 minutes and rinsed 3 times in 0.1M cacodylate buffer for 10 minutes each rinse. Samples were post-fixed in 1% osmium tetroxide for 90 minutes, and then dehydrated at increasing concentrations of ethanol and acetone for 10 minutes each. Samples were embedded in Epon Acetone and baked at 60°C overnight. Thin sections were cut using a diamond knife on a Leica Microtome, stained with uranyl acetate and lead citrate, and viewed using a FEI Tecnai 12 transmission electron microscope. Images were taken at 9700X and 37000X magnifications.

**2.8 Statistical Analysis**

Densitometry of Western blots were measured with ImageJ version 1.46r. All data was analyzed with GraphPad Prism version 6.0b using the Student’s t test. Statistical significance was accepted at a $p < 0.05$. 
CHAPTER 3

RESULTS

3.1 Characterize Intracellular Trafficking of GBS in Brain Endothelial Cells

It has been established that GBS can invade and survive in human brain microvascular endothelial cells (hBMEC) [15]. However, the exact mechanism of GBS intracellular trafficking in hBMEC has not been described. My research goal was to investigate if GBS could possibly be shuttled through the endocytic pathway and what GBS factors may be important for its cellular trafficking within hBMEC.

3.1.1 Intracellular Lifestyle of GBS

The assessment of intracellular GBS in hBMEC over time has not been described. Samples from a time course experiment for thin-section transmission electron microscopy (TEM) was prepared by a previous lab member. In that particular experiment, hBMEC was infected with GBS (MOI of 1) for 1 hour. After infection, antibiotic-containing media was added to kill extracellular bacteria and samples were taken at various time points to visualize intracellular organisms. After each time point, the samples were prepared for embedding. I have examined more of those samples to determine the intracellular location of GBS and whether it is within a vesicle or in the cytoplasm. Upon examination of the later time point samples, GBS still resides in single-membrane vesicles at 24 hours post-infection (Figure 3.1). Furthermore, GBS was never seen inside the cytoplasm. These findings indicate that these single-membrane structures may be endosomes.

3.1.2 GBS Associates with Early and Late Endosomes

To determine whether the vesicles observed earlier could be endosomes, hBMEC was infected with GFP-GBS (MOI 10), and then antibiotic media was added with samples taken at various time points. The cells were stained with Rab5 antibodies to label early endosomes.
Figure 3.1. Transmission electron microscopy of GBS-infected hBMEC. Intracellular GBS are enclosed in single-membrane vesicles; scale bar=2μm. Insets were taken at 37000X magnification; scale bar= 500nm.
In Figure 3.2A, representative images show GFP-GBS co-localization with Rab5 along with the corresponding quantification (Figure 3.2B), which shows that at early time points, more than 50% of intracellular GBS associates with Rab5. Next, to examine if GBS can also associate with late endosomes, the same experiment was performed as outlined above, but the cells were stained for Rab7. GBS does co-localize with Rab7, but at a much lower rate (Figure 3.2C & D). These results show that a certain percentage of intracellular GBS is shuttled through the endocytic pathway by interacting with early and late endosomes.

Figure 3.2. Immunofluorescent staining of GBS co-localization with endosomal markers. (A) Representative images of GFP-GBS co-localization with Rab5. (B) Quantification of the percentage of intracellular GBS co-localized with Rab5. (C) Immunofluorescent staining of GFP-GBS co-localization with Rab7. (D) Quantification of the percentage of intracellular GBS that co-localized with Rab7. Nuclei were stained with DAPI (blue) and all images were examined under 630X magnification.
3.1.3 Examination of GBS Interaction with Acidic Vesicles

To determine if the endosomes fuse with the lysosomes, hBMEC was infected as described above. Lysotracker, a cell permeable stain that labels acidic compartments, like lysosomes, was added to the cells 30 minutes prior to the time point. The slides were examined for co-localization of intracellular GFP-GBS with Lysotracker. In 3.3A, representative images show GFP-GBS and Lysotracker co-localization. Approximately 50% of intracellular GBS associated with acidic compartments up to 24 hours post-infection (Figure 3.3B). These results indicate that intracellular GBS does get shuttled to the lysosome, but since only 50% do, there might be another pathway that GBS can be shuttled to that does not result in the fusion of the lysosome. There is also the possibility that intracellular GBS could be trafficked through another host defense mechanism.

3.1.4 Determination of Bacterial Factors Involved in Intracellular Trafficking

Certain bacterial factors have been shown to promote intracellular survival in phagocytic cells, such as PilB [12], CylE [8], and CiaR [5], and therefore may also play a role in intracellular trafficking. To determine if these factors are also important in intracellular trafficking, cells were infected with WT GBS and mutants deficient in PilB, CylE and CiaR and stained with Lysotracker as described above. There was no significant difference in the ability of a ΔpilB mutant to co-localize with lysosomes, when compared to WT (Figure 3.4A). However, for the cylE mutant, there was a significant increase in the percentage of intracellular GBS associating with the lysosome at the 2-hour time point (Figure 3.4B). When GBS lacks CiaR, there is also a significant increase in the percentage of intracellular GBS co-localized with lysosomes, especially at the late time points (Figure 3.4C). The CiaR data also correlates with the intracellular survival data previously published [5].

Since the ΔciaR mutant co-localized more readily with lysosomes than WT, I also investigated if CiaR would contribute to trafficking to early and late endosomes. Cells were infected with either WT or ΔciaR and then stained for Rab5 or Rab7. There was no significant difference in the percentage of intracellular GBS co-localized with Rab5
Figure 3.3. GBS co-localizes with lysosomes. (A) Immunofluorescence of GFP-GBS co-localization with Lysotracker. (B) Quantitative analysis of the percentage of intracellular GBS co-localized with Lysotracker. Nuclei stained with DAPI (blue) and all images taken at 630X magnification.
Figure 3.4. GBS mutants co-localized with lysosomes. (A) Percentage of Δ*p*il*B* co-localized with Lysotracker compared to WT. (B) Percentage of Δ*cylE* co-localized with Lysotracker compared to WT. (C) Percentage of Δ*ciaR* co-localized with Lysotracker compared to WT. *, *p* < 0.05.
(Figure 3.5A) or Rab7 (Figure 3.5B), indicating that CiaR does not contribute to GBS trafficking to early and late endosomes.

![Figure 3.5. ΔciaR co-localization with early and late endosomes. (A) Percentage of ΔciaR co-localized with Rab5 compared to WT. (B) Percentage of ΔciaR co-localized with Rab7 compared to WT.](image)

### 3.1.5 CiaR-Regulated Genes

CiaR has been shown to be shuttled more readily to lysosomes and also does not survive well in hBMEC [5], suggesting that GBS lacking CiaR regulation are more readily degraded. Therefore, CiaR may regulate a protein that promotes its survival in acidic environments. From the previous microarray data of the *ciaR* mutant, I hypothesized that the most highly down-regulated gene, *SAN_2180*, may be important in intracellular survival. The lab currently has a plasmid insertion mutant of *SAN_2180*. Using this mutant, I performed adherence, invasion, and intracellular survival assays with hBMEC. In Figure 3.6A, *SAN_2180* did not affect the ability of GBS to invade cells. However, the absence of *SAN_2180* caused a slight decrease in invasion, when compared to WT (Figure 3.6B). There was also a significant decrease in the percentage of Δ*SAN_2180* recovered at 12 and 24 hours post-infection, when compared to WT (Figure 3.6C). These results show that *SAN_2180* does aid in intracellular survival in hBMEC, especially at later time points.

### 3.2 EXAMINE AUTOPHAGY ACTIVATION DURING GBS INFECTION

Induction of autophagy in hBMEC, during GBS infection, has been recently demonstrated in the lab. When hBMEC is infected with WT GBS, there is an increase in the amount of LC3-II conversion compared to uninfected cells (manuscript submitted for
In order to determine if certain bacterial factors differentially activate autophagy, COH1 mutants ΔciaR, ΔcylE, ΔiagA, and HY106 (capsule mutant) and NCTC 10/84 mutants ΔcylE, ΔpilA, and ΔpilB were utilized for this study.

### 3.2.1 Determine if Intracellular GBS Resides in Autophagosomes

I first sought to use TEM to visualize GBS and autophagic structures. hBMEC were pre-treated with Bafilomycin A1, a known inhibitor of autophagy that blocks the ability of vesicles fusing with a lysosome, for 1 hour prior to GBS infection. By blocking lysosomal fusion, the cell will accumulate autophagosomes and potentially increase the chance of visualizing GBS inside one. When hBMEC was infected with GBS, most of the intracellular GBS visualized were contained in single-membrane vesicles (Figure 3.7A), which is consistent with what has been observed previously (Figure 3.1). When GBS-infected cells were treated with bafilomycin, there was a noticeable increase in the amount of vesicles inside the cell, when compared to untreated cells (Figure 3.7B). There also seemed to be an increase in the amount of intracellular GBS inside each vesicle, so it may be possible that GBS is replicating inside these vesicles when autophagy is inhibited. An uninfected control was pre-treated with bafilomycin and rapamycin, which induces autophagy by inhibiting mTORC1, an inhibitor of autophagy, to induce and accumulate autophagosomes.
Figure 3.7. Transmission electron micrographs of GBS-infected cells pre-treated with bafilomycin. (A) hBMEC was infected with GBS for 4 hours, fixed, then prepared for electron microscopy. (B) hBMEC infected with GBS and treated with bafilomycin. Scale bar for $= 2\mu$m; for insets, scale bar = 500nm.
There were also instances when GBS was in a vesicle with multiple membranes, which may be an autophagosome. In Figure 3.8B, there is a structure that resembles a phagophore that is surrounding a mitochondria and a GBS-containing vesicle. The micrographs obtained from this experiment suggest that some intracellular GBS can reside in autophagosomes. There is also evidence that GBS can damage the vesicular membrane, but it is not seen free in the cytoplasm; it seems to remain in the vesicle (Figure 3.8C). However, the classical double-membrane autophagosome structure has, so far, been elusive in the sections I have examined (Additional TEM micrographs are included in Appendix B). Therefore, more sections from these samples need to be cut and analyzed.

![Figure 3.8. Transmission electron micrographs of possible phagophore. (A) hBMEC infected with GBS for 4 hours. (B) Higher magnification image of (A). White arrowhead indicates possible phagophore enclosing around GBS-containing vesicle and mitochondria. Black arrowhead indicates region of GBS-containing vesicle with multiple membranes. (C) Intracellular GBS surrounded by damaged membrane.](image)

### 3.2.2 Determine Bacterial Factors that Induce Autophagy

To identify the bacterial factors that promote autophagy induction, hBMEC was infected for 4 and 1 hour with mutants from the COH1 and NCTC10/84 background, respectively. After infection, antibiotic-containing media was added for 1 hour and then cell lysates were collected. Western blots were performed on the cell lysates to examine LC3. Analysis of Western blots revealed that both WT strains of GBS show an increase in LC3-I conversion when compared to the uninfected control (Figure 3.9). Interestingly, when both GBS WT strains (representing serotypes III and V) lacked cylE, the rate of LC3-II
Figure 3.9. Western blots for LC3 in hBMEC infected with various GBS mutants. (A) Representative blot from mutants in COH1 (serotype III) background. Without hemolysin, autophagy is not activated. (B) Semi-quantification of 3 replicates. (C) Representative blot from mutants in NCTC 10/84 (serotype V) background. Autophagy not activated when infected with ΔcylE mutant. (D) Semi-quantification of 3 replicates. *, p<0.05; **, p<0.005.
conversion was about the same as uninfected. This finding indicates that the β-hemolysin/cytolysin may be the factor triggering the cell to undergo autophagy. The capsule-deficient strain of GBS shows very small levels of LC3-II and no levels of LC3-I (Figure 3.9A). HY106 is more invasive in hBMEC, so it is possible that there is a constant turnover of LC3-I and LC3-II because there is a high bacterial load in the cell.

Another factor that may influence the amount of autophagy activation is the presence of IagA because the mutant has the similar LC3-II conversion rate as uninfected (Figure 3.9A). Moreover, the Δ pilB mutant also has a lower LC3-II conversion rate (Figure 3.9C). This suggests that autophagy activation may be dependent on GBS invasion because both IagA and PilB are important GBS invasion factors.

I also examined the levels of p62, which is an adaptor protein that aids in LC3-II binding to intracellular pathogens [27, 28]. When autophagy is activated, p62 is degraded in the process. hBMEC was infected with NCTC10/84 WT and Δ cylE for 1 hour and then antibiotic-containing media for 1 hour. Cell lysates were collected and blotted for p62 and LC3. In WT infected cells, there is a significant decrease in p62 recovered when compared to uninfected (Figure 3.10). The increase in LC3-II conversion with WT infection also correlates with the decrease in p62 levels. When the β-h/c is absent, the amount of p62 recovered is similar to levels in uninfected cells. Therefore, p62 may be the adaptor protein that recruits LC3 during GBS infection.

### 3.2.3 Autophagy Contributes in Clearing GBS Infection

To determine if autophagy is important in GBS clearance, mouse embryonic fibroblasts (MEFs) deficient in ATG5 were utilized. Without ATG5, the ATG5-12-16L1 complex that converts LC3-I to LC3-II cannot form and autophagy will not be activated. ATG5+/+ WT and ATG5−/− KO MEFs were infected with COH1 WT GBS (MOI of 1) and intracellular survival was examined over time. As shown in Figure 3.11A, there is a significant increase in GBS survival in ATG5−/− KO MEFs compared to the low survival rate in WT MEFs. The results from this experiment indicate that autophagy is an effective defense mechanism against intracellular GBS. Since the β-h/c was determined to be one of the factors responsible for autophagy activation, I investigated whether or not the Δ cylE
Figure 3.10. Western blot for p62 and LC3 in NCTC10/84 WT and ∆cylE infected hBMEC. (A) Representative blot showing decrease in p62 levels in WT infected cells (B) Semi-quantitative analysis of p62 levels compared to GAPDH. **, p < 0.005; ****, p < 0.0005. (C) Analysis of LC3-II/LC3-I ratio.

Figure 3.11. Intracellular survival assay of WT GBS (COH1) or ∆cylE to ATG5−/− KO and ATG5+/+ WT MEFs. Percent survival of (A) WT GBS and (B) the ΔcylE mutant in ATG5 WT and KO MEFs. *, p < 0.05; ** p < 0.005; ***, p < 0.0005.

mutant would be able to survive better in autophagy-deficient MEFs. There is an increase in ∆cylE survival in ATG5−/− KO MEFs (Figure 3.11B), similar to the results found in WT GBS. However, the percent survival between WT GBS and ΔcylE are very similar as well. This indicated that β-h/c is not the only important bacteria factor. There is also the
possibility that GBS lacking the β-h/c activity may be eliminated through another pathway, like the endocytic pathway.
CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

It is already known that GBS can invade brain endothelial cells and transcytosis through to gain access to the brain [15]. However, its trafficking and mechanisms of intracellular survival have not been well studied. Other intracellular pathogens have been shown to hijack or evade host degradation pathways to promote its survival inside the host cell [16, 27, 32, 33]. In my research, I focused on whether GBS is capable of evading or utilizing these host defense mechanisms to allow it to persist within the brain endothelium. I also described GBS factors that are important for its trafficking through the endocytic pathway and for autophagy activation.

4.1 GBS TRAFFICKING THROUGH THE ENDOCYTIC/PHAGOLYSOSOMAL PATHWAY

Intracellular bacterial pathogens, such as GAS, have been shown to associate with Rab5 and Rab7 GTPases, markers of the endocytic pathway [17]. Through TEM, I have been able to visualize GBS within membrane bound vesicles, which indicated that intracellular GBS might also be shuttled through this pathway as well. Using immunofluorescence, intracellular GBS was shown to co-localize with Rab5 (early endosomes) and Rab7 (late endosomes). Also, a portion of intracellular GBS also associated with acidic compartments; however, it is still not clear whether GBS is in fact degraded by the lysosome. GBS encodes for multiple factors involve in acid tolerance that allow it to persist in phagocytic cells [5, 8, 9]. It is possible that GBS prefers to be shuttled to these acidic lysosomes and possibility it is this subset of intracellular GBS are the viable bacteria that is actually recovered. Therefore, it would be interesting to examine if viable GBS can be recovered from lysosomes. The Rab11-labeled recycling endosomes could also play a role in how GBS is trafficked through hBMEC. Recycling endosomes regulate vesicle trafficking in polarized cells [34, 35]. Recently, this pathway was characterized in the shuttling of Clostridium perfringens toxin to the plasma membrane [36].
The CiaR response regulator of GBS is an important factor allowing GBS to potentially aid in evading trafficking to the lysosome or in its ability to persist in acidic environments. The $\Delta$ciaR mutant was trafficked more readily to the lysosomes and possibly degraded. CiaR was also shown to not have an impact on trafficking to the early and late endosomes. The CiaR-regulated gene, SAN_2180, was shown to play a role in GBS persistence in hBMEC and has a 42% homology to a Lactococcus protein, which is involved in acid/multi-stress tolerance [5]. Therefore, this protein may have the same function in GBS. In order to further study this protein, a complete deletion of this gene needs to be made. It would also be interesting to determine if there is an increase in co-localization of this mutant with lysosomes and if they are being degraded more readily.

4.2 ROLE OF THE GBS HEMOLYSIN PROTEIN IN AUTOPHAGY INDUCTION

Autophagy has been described in targeting classically intracellular bacterial pathogens, such as GAS, Listeria, and Shigella, which can damage the membrane it resides in and become cytosolic, for lysosomal degradation [17, 37-39]. Autophagy has also been described as targeting Mycobacteria and Salmonella-containing vesicles [37, 38]. Bacterial trafficking to Rab7-positive endosomes has also been shown to be important for autophagy progression to occur [40, 41]. Some intracellular pathogens that either escape or damage the vesicle they reside in can be ubiquitinated and subsequently targeting them for degradation via autophagy through various adaptor proteins, like p62, nuclear dot protein 52 (NDP52), and optineurin, allowing for LC3-II recruitment [27, 42].

However, I have seen instances when the vesicular membrane is disrupted, but GBS is not observed in the cytosol. I have also shown that certain bacterial factors elicit a different autophagic response. The GBS factors that have been shown to be important in autophagy induction are the $\beta$-h/c and the invasion factors PilB and IagA, which helps anchor LTA to the cell surface. When either of these factors is absent, autophagy is not induced to the same level as seen with WT GBS. The adaptor protein p62 was also shown to be involved during GBS infection. It is possible that GBS can damage the vesicle it resides in and subsequently be ubiquitinated, then labeled by p62, which can bind to both ubiquitin and LC3-II. Additionally, utilizing the autophagy-deficient MEFs, I have observed that GBS
intracellular survival increased indicating that autophagy is a crucial host defense mechanism that limits GBS persistence in host cells.

4.3 Summary and Future Direction

In summary, my findings indicate that GBS can be trafficked through the endocytic/phagolysosomal pathway and elicits an autophagic response from the brain endothelium. However, it is still unclear if GBS can be trafficked through a pathway that does not lead to fusion with a lysosome or if it can damage vesicle membranes. I have also demonstrated that the bacterial factors CiaR and a CiaR regulated gene, and the β-h/c are important contributors to lysosomal trafficking and autophagy activation, respectively. Future studies will focus on the further elucidation of autophagy and the host factors involved in its initial activation, along with the interaction of certain bacterial factors.
REFERENCES


APPENDIX A

CONSTRUCTION OF GFP-EXPRESSING GBS
To better visualize intracellular GBS, I constructed Green-Fluorescent Protein (GFP)-expressing GBS strains. The GFP plasmid, pDESTerm GFP, was a generous contribution from Victor Nizet (UCSD) that is codon-optimized for Gram-positive bacteria. The pDESTerm backbone is similar to pDCerm and antibiotic selection for the plasmid is 5μg/ml Em. Table A.1 lists all the strains’ corresponding isogenic mutants that the GFP plasmid was transformed in to using the protocol describe earlier. GFP-expressing strains for COH1, NCTC 10/84 and A909 wild-type were also constructed.

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**Table A.1. GFP-GBS Strains and Mutants Created**

A.1 GROWTH STUDY

Growth studies were performed to determine if the presence of the plasmid affected bacterial growth. Growth curves were performed under a variety of conditions for each GFP-GBS strain made. GBS containing pDESTerm GFP was grown in either normal THB or THB containing 5μg/ml Em. As a control, GBS without the plasmid was grown at the same
time. At specified time points, bacterial growth was monitored by OD\textsubscript{600} measurements. Figure A.1 shows that the addition of the bacteria has not affected the normal sinusoidal growth curve. All strains tested had similar growth rates as the one depicted in Figure A.1.

![Graph showing bacterial growth](image)

**Figure A.1.** Representative growth curve of COH1 WT with pDESTerm GFP, which shows the plasmid does not dramatically alter bacterial growth. COH1+pDESTerm GFP showed no difference in growth when antibiotic selection was used. All other GFP-GBS strains also had this similar growth pattern.

### A.2 Fluorescence Study

To assess GFP fluorescence, a small aliquot of the GBS culture was removed and placed on a slide for viewing under the Zeiss fluorescent microscope. The GBS aliquots were taken from the cultures that were measured for the growth study. The GFP expression in both growth conditions was strong (Figure A.2). Visually, there did not seem to be a noticeable difference when the antibiotic selection for the plasmid was used. Therefore, GBS does not need to be under antibiotic selection to retain the GFP plasmid.

However, for a more quantitative analysis of GFP fluorescence, FACS was utilized. GFP-GBS grown with or without 5μg/ml Em plasmid selection was examined. GBS not containing the plasmid was included as a control. After 30 minutes of growth, there was no difference in the population of GFP-positive GBS (Figure A.3). However, after 3 hours of
Figure A.2. Representative fluorescent images of COH1 WT with pDESTerm GFP. GFP-GBS was grown in media with (A) or without (B) Em. After specified time points, a wet mount of the culture was made and examined under Alexa488 filtered light. Images were taken under 630X magnification.

growth, there is a distinct shift in GFP-positive GBS population between the two growth conditions. When Em is included in the growth media, there is a higher population of GBS expressing GFP when compared to those grown in THB without Em. This information indicates that GFP-GBS does need to be grown up in antibiotic selection to prevent the plasmid from being lost. Based on this data, for all experiments using GFP-GBS, overnight cultures were grown in THB with 5μg/ml Em. Also, to ensure that we start with a high population of GFP-GBS, all subcultures used for immunofluorescence were grown to mid-log phase in THB with 5μg/ml Em.
Figure A.3. Representative FACS analysis of COH1 WT with pDESTerm GFP. (A) After 30 minutes of growth, the population of GFP-positive GBS is the same for both growth conditions. (B) After 3 hours of growth, there are less GFP-positive GBS when it is not grown in Em selection.
APPENDIX B

TEM ANALYSIS OF INTRACELLULAR GBS
Figure B.1 is additional TEM images taken from the time course experiment of GBS-infected hBMEC prepared by a previous lab member. Figures B.2 and B.3 are additional images of hBMEC infected with various GBS mutants and pre-treated with bafilomycin.

Figure B.1. Additional TEM images of time-course experiment. (A) 6 hours post-infection. (B) 24 hours post-infection. Scale bar = 500nm.
Figure B.2. Additional TEM images of WT GBS infected hBMEC. (A) hBMEC infected with COH1 WT and (B) hBMEC pre-treated with bafilomycin and infected with COH1 WT. (C) Uninfected hBMEC pre-treated with rapamycin and bafilomycin depicting double or multiple-membrane vesicles. Scale bar = 500nm.
Figure B.3. TEM images of hBMEC infected with various GBS mutants. (A) hBMEC infected with COH1 ΔcylE. (B) hBMEC pre-treated with bafilomycin, then infected with COH1 ΔcylE. (C) hBMEC pre-treated with bafilomycin, then infected with HY106 (capsule-deficient mutant). (D) hBMEC pre-treated with bafilomycin, then infected with COH1 ΔciaR. Scale bar = 500nm.