Protein glycosylation by NleB and the secretion of therapeutic nanobody fusions

by

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Abstract

Escherichia coli and *Salmonella enterica* are Gram-negative bacteria that are among the leading causes of gastrointestinal tract infections. These bacteria interact with mammalian hosts by using a type III secretion system (T3SS) to secrete effectors into host cells. NleB and SseK are two *E. coli* and *Salmonella* effector orthologs that are unusual glycosyltransferases. They glycosylate host protein substrates on arginine residues with N-acetyl glucosamine (GlcNAc) to inhibit the function of host proteins involved in the innate immune response. Originally, it was thought that the effectors are inactive within the bacterium and fold into their active conformations only after being secreted. However, mass spectrometry experiments to identify glycosylation substrates of NleB orthologs challenged this dogma, providing the premise for the first part of this thesis. Mass spectrometry suggested that the septum site-determining protein used in cell division, MinC, and the peptidyl-prolyl cis-trans isomerase protein that promotes bacterial stress tolerance and virulence, FklB, were glycosylated by NleB on arginine residues R107 and R129, respectively. Data from *in vitro* assays suggested that NleB glycosylated the arginine residues of both bacterial substrates, but when tested further, MinC and FklB were not confirmed to be glycosylated in *Citrobacter rodentium*, *S. enterica*, enteropathogenic *E. coli* (EPEC), or enterohemorrhagic *E. coli* (EHEC). This could be due to the MinC and FklB bacterial proteins being false positives when the mass spectrometry experiments were completed. Despite this negative data, intra-bacterial glycosylation of the mammalian substrates, the fas-associated protein with death domain (FADD), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and tumor necrosis factor receptor type 1-associated death domain protein (TRADD), were then studied. Results detected that all three mammalian substrates were glycosylated by NleB on arginine residues R177, R197/R200, and R235, respectively, and that FADD was a stronger

glycosylation target of NleB than GAPDH and TRADD. The second part of this thesis also explores secretion through the T3SS, but instead of secreting a whole effector, camelid nanobodies were fused with signal sequences 20 amino acids in length from effectors that are naturally secreted and examined. An attenuated bacterium with an engineered T3SS was created to secrete the nanobody fusions, and *in vivo* analyses before and after precipitation were completed using *C. rodentium* and *E. coli*. Nanobodies fused with effector signal sequences upstream from the camelid signal sequences were expressed, but not secreted through the T3SS. This concludes that a 20 amino acid signal sequence does not promote the secretion of the nanobodies through a T3SS.

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Dedication

This study is dedicated to my loving family, fiancé, and fiancé's parents, who have been there for me through all the struggles, has shown me what comes from working hard, and who has given me strength when I thought of giving up; who has also provided continuous emotional support and who gave endless advice and encouragement to finish the study.

This dedication also goes to the almighty God for His guidance, strength, and skills that have been given to me throughout my time as a Masters student and throughout the study.

Chapter 1 - Introduction

1.1 Motivation

Medicine and technology are continuously advancing every day, yet microbial contamination and the complications that come with it remain a challenge around the world. Among some of the most significant microbes that cause public health problems are Gram-negative bacteria with the most prevalent group being the family Enterobacteriaceae (Oliveira and Reygaert, 2019). One reason for this is the high resistance to antibiotics. Treatment of serious nosocomial infections becomes more problematic as resistance arises and spreads, putting the population at risk to being refractory to treatment with many of the currently available antibiotics (Paterson, 2006). Another reason why the Enterobacteriaceae family is important is due to the high impact it has on public health, most notably seen through bacterial food-borne pathogens. Clinical microbiology, infectious diseases, and the community's well-being are all influenced greatly by some of the bacteria within this group (Baylis, Uyttendaele, Joosten, and Davies, 2011). These reasons alone would suggest that further research into the Enterobacteriaceae family would be merited.

Extensive research has focused on the Enterobacteriaceae family in the past, but recent advances in science have uncovered expansions in this area. This includes various mechanisms of the bacteria, emerging pathogenic strains, virulence characteristics, survival and persistence of the bacteria, and changes in the bacterial taxonomy (Baylis et al., 2011). One of the main focuses in science is how to prevent Enterobacteriaceae infection, and more specifically in this study, how to prevent *Escherichia coli* and *Salmonella* from infecting host cells and evading the host's immune response. To obtain the answer, different approaches on inhibiting the effectors of these microbes must be meticulously analyzed.

1.2 Background

The Host Immune Response. Knowledge of the immune system has been steadily increasing throughout recent years, yet its complexity and pervasiveness still leave room for new discoveries. The basis lies in the host's recognition of normal, structural components when abnormal cells are eliminated, and in the recognition of potential pathogens when protection against invading microbial infections is needed (Medzhitov, 2007). The immune system has three different stages that help with this basis of recognition and protection. The first mechanism of defense is physical barriers such as skin, mucus, and normal flora on and within the host.

The second defense is the innate immune response which responds immediately, reacts non-specifically, and does not improve with exposure or remember invading pathogens (Tizard, 2018). This is the natural immunity that a host would be born with. When a pathogen evades the primary physical barriers, innate immunity is triggered as an inflammatory reaction and takes only minutes to hours to be fully activated (Netea, Schlitzer, Placek, Joosten, and Schultze, 2019). In the early stages of infection, the innate immune response is critical in limiting microbial spread and replication. However, if the invading pathogen has a high amount of virulence, the initial removal of this pathogen can fail. The final defense, the adaptive immune response, would then be activated to eliminate the pathogen which takes about one to two weeks (Netea et al., 2019). The adaptive immune response reacts slower and with more specificity, enhances upon re-exposure, has immunological memory for the invading pathogens, and takes time to develop (Tizard, 2018). This is important for secondary infections so as to respond more effectively and efficiently to restimulation and includes specialized immune cells such as B cells

and T cells (Netea et al., 2019). All three of these stages of defense are required to create a balanced immune response that is critical for host health.

There are many aspects that play critical roles in the development of the innate immune response, including the nuclear factor-kappa B (NF-κB). NF-κB is a pathway of highly conserved transcription factors. This pathway regulates many important cellular behaviors, in particular, inflammatory responses, cellular growth, and apoptosis. Activation of various signaling pathways regulates the expression of both pro- and anti- inflammatory mediators to induce an inflammatory response (Lawrence, 2009). Receptors in these signaling pathways activates NF-κB when foreign microbial products enter the host cell. However, when proteins are specifically targeted by microbes within the host cell, inflammation is impaired by inhibiting NF-κB activation and expression of proinflammatory cytokines and chemokines (Lawrence, 2009). The NF-κB pathway is one of many mechanisms used in the innate immune response and serves as a general basis for the studies performed in this Master's Thesis.

Common Enteric Pathogens. Intestinal pathogens employ sophisticated strategies to colonize and infect many mammalian hosts worldwide. Most enteric infections are environmentally determined and typically enter the body through the oral route, which can occur through ingestion of contaminated food and water, by contact with animals and/or their environments, or by contact with an infected individual's feces (Petri, Miller, Binder, Levine, Dillingham, and Guerrant, 2008). Many children are affected due to the constant desire to insert things into their mouths, and at this young age, absorption of key nutrients is critical. This leads to substantial effects on intestinal absorption, childhood development, nutrition, and global morbidity and mortality (Petri et al., 2008). Many of these effects are due to the pathogen's

ability to cause persistent diarrhea and chronic, recurring enteric infections. Therefore, the study of the epidemiology, etiology, and pathophysiology of enteric infections is crucial.

Among the leading causes of gastrointestinal tract infections are Gram-negative bacteria such as *Escherichia coli* and *Salmonella*. *E. coli* O157:H7 is the most common type of Shiga Toxin-producing *E. coli* (STEC). Although most strains are harmless and live in the intestines of healthy cattle, this strain is a major foodborne pathogen that causes severe disease around the world in humans (Lim, Yoon, and Hovde, 2010). Common sources of disease have been associated with eating undercooked, contaminated ground beef, ingesting produce items that have been contaminated through contact with cattle feces, or through drinking raw milk or water contaminated by fecal material of cattle. There are five categories of *E. coli* strains based on pathogenic mechanisms that cause different diseases due to acquiring different virulence genes (Donnenberg and Kaper, 1992). These categories are: enteroinvasive (EIEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), and enteropathogenic (EPEC). EPEC is one of the more common types out of these categories and is important to this study along with EHEC.

Salmonella spp. are the second most common cause of diarrhea in the United States. The bacteria are commonly transferred through contaminated food or water either through animals or through a person, and occasionally through contact with animal or human feces (Aarestrup, Wiuff, Molbak, and Threlfall, 2003). Two types of *Salmonella* exist: 1) nontyphoidal, which is prevalent in the gastrointestinal tracts of many animals, and 2) typhoidal, where humans are the only known hosts, and it can cause enteric fever. Salmonella requires a certain set of genes encoded with Salmonella pathogenicity island 2 (SPI-2) to survive and replicate within host macrophages (Arpaia, Godec, Lau, Sivick, Mclaughlin, Jones et al. 2011). SPI-2 is involved in

intracellular bacterial replication and in initiation of systemic infections. SPI-2 also encodes a Type III Secretion System (T3SS) that aids in the translocation of SPI-2 effectors into the host cell and supports bacterial replication (Arpaia et al., 2011). This mechanism of infection by *Salmonella* forms one of the bases of this study.

Types of Secretion Systems. Many mechanisms are utilized by bacterial pathogens when invading mammalian hosts and inhibiting the immune response. The secretion of bacterial proteins across the phospholipid membrane can promote bacterial virulence and is an essential component of these mechanisms (Green and Mecsas, 2016). The proteins that are secreted are termed "bacterial effectors" and are commonly transported into the cells of their hosts through a Type III Secretion System (T3SS), a Type IV Secretion System (T4SS), or a Type VI Secretion System (T6SS). The T3SS, T4SS, and T6SS are only a few of the bacterial protein secretion apparatuses, which are divided into various classes based on their specificity, arrangement, and function (Green and Mecsas, 2016). These protein secretion systems are vital to bacterial effector transport and bacterial growth inside the host cell. The secretion apparatus of focus for the concurrent experiments is the T3SS.

T3SSs are found in a broad number of Gram-negative bacterial pathogens and symbionts. They act as injectosomes and "needle and syringe"-like apparatuses, due to their structure, to secrete effector proteins into the mammalian host cell (Green and Mecsas, 2016; Fig. 1). Protein substrates, such as NleB (*E. coli* and *Citrobacter rodentium*, a mucosal pathogen of mice that share several pathogenic mechanisms with *E. coli*) and SseK (*Salmonella*), are then secreted across both the inner and outer membranes of the bacterium. Anywhere from a few to several dozen of these effector proteins can be secreted depending on the pathogen that is involved

(Green and Mecsas, 2016). For a protein substrate to be recognized and secreted by the T3SS, a secretion signal is needed. These secretion signals are not cleaved during the process, are embedded within the N-termini of the substrate, and act as a "tag" that the T3SS recognizes and allows through for successful secretion (Green and Mecsas, 2016).

The effector protein first goes through the base complex of the T3SS, then through the needle component, and lastly the translocon. The needle has a tip complex on the outer portion that is important in the regulation of effector secretion and sensing contact with host cells (Green and Mecsas, 2016). The tip also inserts the translocon into the cell membrane where it is assembled upon contact with the host cells. A pore is then formed that aids in effector delivery through host cell membranes; however, this pore is not important for the secretion of effectors outside of the bacterial pathogen (Green and Mecsas, 2016). Once inside the host cell, the functions of the effectors can vary among different pathogens. Yet, many effectors can establish an infectious niche either at mammalian tissue sites or within the cell by remodeling normal cellular functions (Green and Mecsas, 2016).

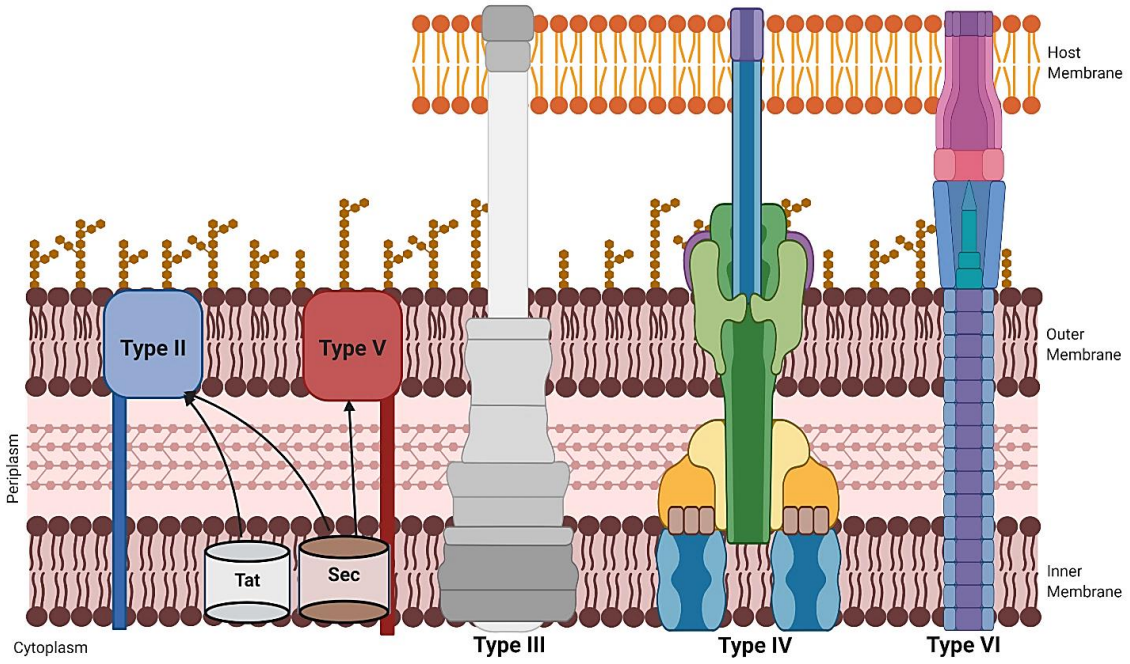

Figure 1 Gram-negative bacteria secretion systems.

This figure was created using BioRender and followed Green and Mecsas (2016) depiction of the secretion systems. Gram-negative bacteria have the ability to transport proteins across phospholipid membranes using various protein secretion systems. Depending on the secretion system, proteins can secrete across one or multiple membranes. Some proteins utilize the T2SSs and T5SSs and are secreted in a two-step Sec- or Tat-dependent mechanism. This means that Sec or Tat secretion pathways can aid in the secretion of proteins across the inner membrane and from there, the proteins are transported across the outer membrane employing a second secretion system. Other proteins utilize the T3SSs, T4SSs, and T6SSs, where a one-step Sec- or Tat-independent process is used to transport their proteins across both bacterial membranes and an additional host cell membrane. All of the pathways are able to secrete proteins from the cytoplasm outside the cell. However, the secretion through the additional host cell membrane is specific to only T3SSs, T4SSs, and T6SSs, and delivers secreted proteins directly into the cytosol to inhibit certain host immune responses in the cell.

Bacterial Effectors and the Host Cell. Pathogenic bacteria are dependent on virulence proteins, or effectors, with enzyme activity to efficiently colonize and propagate within their hosts and counteract host innate immunity. NleB and SseK are two of the many bacterial effectors that are highly conserved in a Type III Secretion System (T3SS) and are the focus of the resulting studies (Gao, Wang, Pham, Feuerbacher, Lubos, Huang, et al., 2013). NleB is encoded by attaching and effacing pathogens (*E. coli* and *Citrobacter rodentium*), whereas SseK is encoded by *Salmonella*. Effectors reach the cytosol through either translocation across endosomal membranes or they are expressed and secreted directly into the host cell through bacterial secretory apparatuses (Boyer, Paquette, Silverman, and Stuart, 2011). Once in the cell, effectors modify host defense mechanisms and manipulate a variety of cellular processes, thereby inhibiting the innate immune system.

Modulation of the innate immune system can be accomplished through *E. coli* T3SS effectors and can impact transcription factor NF-κB, which regulates various host cell responses (Gao et al., 2013). NleB is one of many effectors that plays an important role in the virulence of *E. coli* by suppressing NF-κB activation. NleB modifies mammalian glycolysis enzymes such as the FAS-Associated Death Domain protein (FADD), the Tumor Necrosis Factor (TNF) Receptor Type 1 (R1)-Associated Death Domain protein (TRADD), and the glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH) by functioning as a translocated glycosyltransferase (Gao et al., 2013). When NleB is secreted across the T3SS into the host cell, the effector uses N-acetyl-D-glucosamine (GlcNAc) to glycosylate target proteins on arginine residues. This then disrupts tumor necrosis factor receptor (TNFR)-associated factor (TRAF) signaling and effects downstream NF-κB signaling (Qaidi, Scott, Hays, Geisbrecht, Watkins, and Hardwidge, 2020). The pro-inflammatory NF-κB pathway is then inhibited which then blocks inflammation of the

innate immune system and catalyzes the addition of unusual post-translational modifications inside the host cell.

This background information along with previous experimental results is what provoked further investigation in this research study. Two different approaches were created based on the prior knowledge of bacterial pathogens infecting the host cell through the use of bacterial effectors and the T3SS. These approaches included the 1) possible inhibition of NleB glycosylation or 2) inhibiting NleB itself, which would ultimately allow the host cell to continue with its immune response and eventual elimination of the pathogen.

Chapter 2 - NleB Glycosylation of Bacterial and Host Proteins Inside the Bacterium

2.1 Rationale for the study

The focus of this study was on effectors NleB (*E. coli* and *C. rodentium*) and SseK (*S. enterica*). NleB was discovered to modify host protein substrates with N-acetyl glucosamine (GlcNAc) on arginine amino acid residues. This mechanism makes NleB an unusual glycosyltransferase since the guanidinium groups of arginine residues are poor nucleophiles (Gao et al., 2013; Qaidi et al., 2020). The normal functions of host target proteins that aid in the immune response are disrupted due to this post-translational modification.

The activity of chaperones keeps effectors in a structural state that allows for successful secretion, whereas T3SS effectors are inactive within the bacterium until they are injected and fold into their active conformations (Gao et al., 2013; Qaidi et al., 2020). With previous findings of NleB glycosyltransferase activity targeting GAPDH function to inhibit the NF-κB pathway (Gao et al., 2013), mass spectrometry was performed to identify various glycosylation substrates of NleB orthologs inside the bacterium. Qaidi et al. (2020) showed that glutathione synthetase (GshB) was glycosylated by NleB inside the bacterium on arginine residue R256. This served as the premise for the concurrent experiments with other glycosylation substrates MinC and FklB and with their arginine residues R107 and R129, respectively (Fig. 2). Another concurrent study focused on FADD, GAPDH, and TRADD and their arginine residues R117, R197/R200, and R235, respectively, for confirmation that human substrates can be glycosylated inside the bacterium. The purpose of this study was to see if effectors are active within the bacterium, which would go against previous studies that show effectors stay folded in their inactive conformations until secreted into the host cell, where they then fold into their active

conformations. If it is found that effectors are active inside the bacterium, then this would change previous thoughts and new areas of investigation on the extent to which other effectors with enzymatic activities that could be active within the bacterium may emerge.

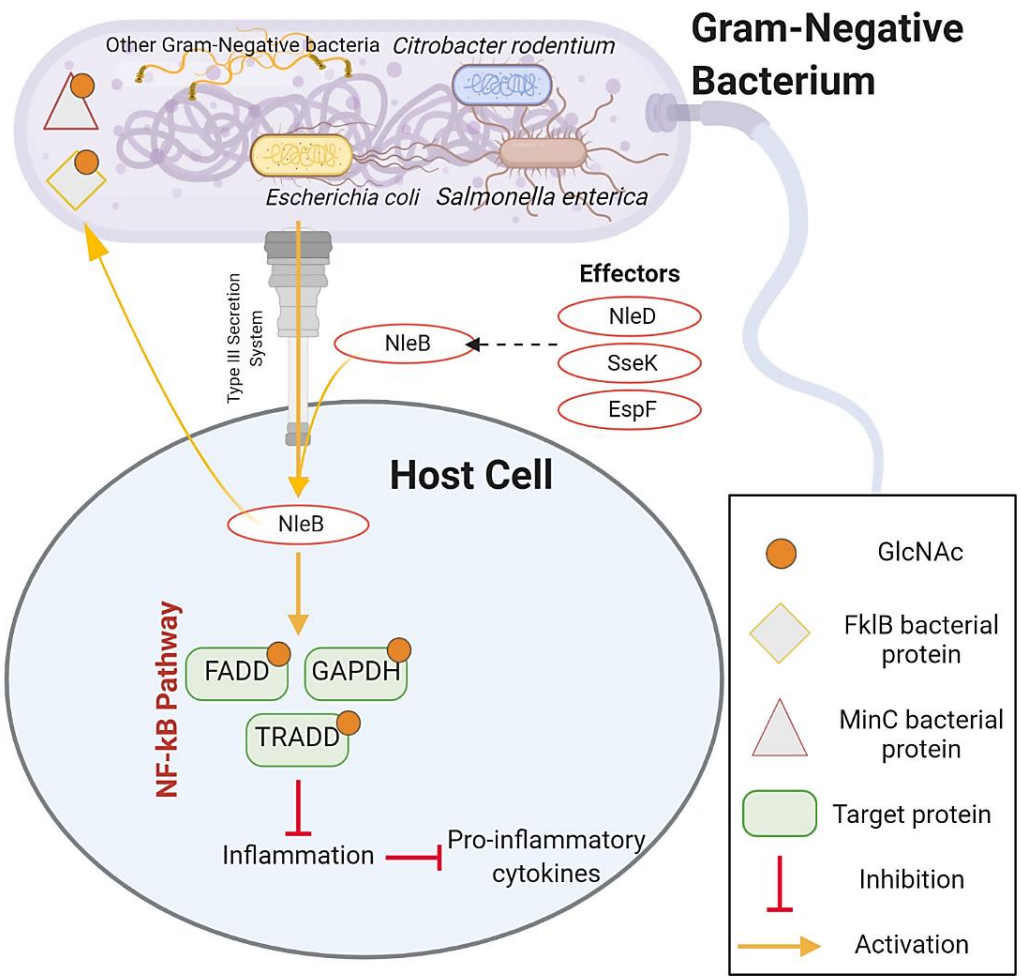

Figure 2 Schematics of NleB Glycosylation of Bacterial and Host Substrates.

This figure was created using BioRender. The *E. coli* effector, NleB, is secreted through the T3SS from the bacterium into the host cell to inhibit functions of host proteins. NleB acts as an unusual glycosyltransferase by using N-acetyl glucosamine (GlcNAc) to glycosylate the arginine residues of host substrates FADD on R117, GAPDH on R197/R200, and TRADD on R235. By doing this, NleB blocks the NF-κB pathway and overall inhibits inflammation and the innate immune response. In previous studies, it was found that NleB may be able to act within the bacterium on bacterial proteins. These bacterial proteins were discovered by completing mass spectrometry experiments. NleB was found to glycosylate MinC on R107 and FklB on R129 within the bacterium, and our studies are aimed at proving this concept.

2.2 Materials and Methods

Strains and Molecular Cloning. The plasmids and strains used in this study are listed in Table 1 (Appendix A). WT *nleB* (*Citrobacter rodentium*) and its derivative $DAD^{221-223}$/AAA were cloned into pET-42a. WT *minC* and its derivative *minC* R107A, and WT *fklB* and its derivative *fklB* R129A were cloned in pET-28a and pFLAG-CTC using restriction enzyme-based cloning. WT FADD, GAPDH, and TRADD and their derivatives FADD R117A, GAPDH R197A R200A, and TRADD R235A were cloned in pFLAG-CTC using ABC cloning (Qaidi and Hardwidge, 2019).

Construction of Mutant Strains. All mutant strains of WT *minC*, *fklB*, FADD, GAPDH, and TRADD were made by completing a PCR with the WT plasmids and specific primers to switch the arginine amino acid residue to an alanine amino acid residue. The PCR plasmid was then purified, and restriction enzyme-based cloning was completed, where restriction sites are determined based on the plasmid, cloning PCR is completed, the insert and vector plasmid is digested, then ligated together and transformed in DH5-α (NEB-α) *E. coli*.

Protein Expression and Purification. Proteins were expressed from *E. coli* BL21 (DE3) and induced with 0.5 mM IPTG when cultures reached an OD_{600} of 0.5. Cells were grown for an additional 4 h at 37 ºC and harvested using centrifugation. Cell pellets were resuspended in a 1/40 culture volume of 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8.0 supplemented with 0.5 mg/mL lysozyme and protease inhibitor mixed solutions (Thermo Scientific). Bacterial suspensions were incubated on ice for 30 min with occasional shaking. An equal volume of 50 mM Tris-HCl pH 8.0, 2 M NaCl, 8 mM imidazole, 20 % glycerol, and 1 %

Triton X-100 was added to the mix and an additional incubation on ice for 30 min with occasional shaking. Cell lysates were sonicated on ice and then centrifuged. Two mL Ni-NTA slurry (Qiagen) was added to the supernatant of the sonicated lysate and was gently rotated for 1 h at 4 ºC. The mixture was loaded on a Poly-Prep Chromatography Column (BioRad) and washed in 50 mM Tris-HCl pH 8.0, 600 mM NaCl, 60 mM imidazole, and 10 % glycerol. Proteins were eluted in 50 mM Tris-HCl pH 8.0, 600 mM NaCl, 250 mM imidazole, and 10 % glycerol.

Bacterial Strains and Growth Conditions. MinC and FklB, and MinC(R107A) and FklB(R129A), respectively, were electroporated with WT and mutant *C. rodentium*, *S. enterica*, enteropathogenic *E. coli* (EPEC), or enterohemorrhagic *E. coli* (EHEC). FADD, GAPDH, and TRADD, and FADD(R117A), GAPDH(R197A/R200A), and TRADD(R235A), respectively, were electroporated with WT and mutant *C. rodentium*. Two colonies from each strain were grown in LB medium with either carbenicillin (Cb 100; 100 μg/mL) (MinC, FklB, and FADD) or kanamycin (Kan 50; 50 μg/mL) (GAPDH and TRADD) and 0.5 mM IPTG to induce growth. Cultures were incubated overnight at 37 ºC while shaking.

Glycosylation Assays. *In vitro* glycosylation assays were performed as described previously (El Qaidi et al, 2017) using 200 nM of NleB1 or its orthologs with 1μM of either WT MinC or MinC R107A, or WT FklB or FklB R129A in 50 mM Tris-HCl pH 7.4, 1 mM UDP-GlcNAc, 10 mM $MnCl_2$, and 1 mM DTT. After 1.5 h incubation at room temperature, samples were subjected to western blotting using either an anti-HisTag monoclonal antibody (Abcam, Cambridge, UK) or anti-FlagTag monoclonal antibody (Abcam, Cambridge, UK) to detect

protein HisTag or FlagTag expression and an anti-R-GlcNAc monoclonal antibody (Abcam) to detect NleB glycosylation of proteins. *In vivo* glycosylation assays for bacterial proteins were performed using *C. rodentium* and *S. enterica* strains electroporated with plasmids expressing FLAG-His-MinC or FLAG-His-MinC(R107A), or FLAG-His-FklB or FLAG-His-FklB(R129A). *In vivo* glycosylation assays for host proteins were performed using the strain *C. rodentium* electroporated with plasmids expressing FLAG-His-FADD or FLAG-His-FADD(R117A), FLAG-His-GAPDH or FLAG-His-GAPDH(R197A R200A), or FLAG-His-TRADD or FLAG-His-TRADD(R235A). Transformed bacteria were grown overnight in the presence of 0.5 mM IPTG and harvested using centrifugation. His-tagged proteins were purified as described above and then subjected to western blotting using an anti-HisTag monoclonal antibody (Abcam, Cambridge, UK) and an anti-R-GlcNAc monoclonal antibody (Abcam, Cambridge, UK).

SDS-PAGE and Western Blot Analysis. WT MinC and MinC R107A, WT FklB and FklB R129A, WT FADD and FADD R117A, WT GAPDH and GAPDH R197A R200A, and WT TRADD and TRADD R235A cells for western blot analysis were harvested by centrifugation and suspended in 1 x sample SDS-loading dye (60 mM Tris-HCl pH 6.8, 1 % SDS, 1 % β-mercaptoethanol, 20 % glycerol, 0.01 % bromophenol blue). Cell extracts were then brought to a boil by sonication and heated at 98 ºC for 5 min, then centrifuged briefly. Cell samples were loaded onto a 15 % SDS-PAGE polyacrylamide gel and were ran using a mini-protean tetra system (Bio-Rad, Hercules, CA) at 25 mAmp per gel for 1.5 h. For SDS-PAGE assays, gels were then stained for 30 min while shaking with Coomassie brilliant blue and then distained as necessary. For western blot analysis, polyacrylamide gels were ran then transferred to a nitrocellulose filter membrane by following the semi-dry transfer unit (Hoefer Semi Phor)

and running at 100 V for 1 h using a mini-protean tetra system (Bio-Rad, Hercules, CA). After running the transfer, the first monoclonal antibody (anti-HisTag Mouse or anti-FlagTag mouse and anti-R-GlcNAc Rabbit) was added with a 1/5,000 dilution and either incubated overnight at 4 ºC shaking or incubated for 1 h at room temperature. The membrane was washed 3 x 10 minutes with Phosphate-Buffered Saline/Tween (PBST) and after, the second monoclonal antibody was added (goat anti-mouse and goat anti-rabbit) with a 1/5,000 dilution and incubated shaking at room temperature for 1 h. The membrane was then washed 3 x 10 minutes with PBST and detected using an Odyssey FC Imager (LI-COR, Lincoln, NE).

2.3 Results

MinC and FklB are glycosylated *in vitro* but are not glycosylated at endogenous levels of NleB. Mass spectrometry experiments were performed and used to identify new glycosylation substrates of NleB orthologs from EHEC strains. This was done by using EHEC strains that expressed NleB1 to infect HEK293T cells (Qaidi et al., 2020). To validate the mass spectrometry data, we conducted *in vitro* glycosylation experiments. MinC, the septum site-determining *E. coli* protein used in cell division, and FklB, the peptidyl-prolyl cis-trans isomerase *E. coli* protein that promotes bacterial stress tolerance and virulence, were first cloned in pET-28a for *in vitro* studies and pFLAG-CTC for further *in vivo* studies. The arginine to alanine mutants were also cloned in these plasmids to observe variation between the parent and mutant strains. Protein expression of MinC and FklB in pET-28a was detected using SDS-PAGE analysis (Fig. 3A).

C. rodentium NleB was used based off of the findings in Qaidi et al. (2020) with either MinC or FklB to investigate glycosylation within these proteins. *In vitro* glycosylation assays following protocols from El Qaidi et al. (2017) were conducted using the anti-HisTag and anti-R-GlcNAc monoclonal antibodies and by expressing recombinant forms of wild-type (WT) MinC and FklB or MinC(R107A) and FklB(R129A), respectively. NleB glycosylated the WT MinC and WT FklB, which was consistent with the mass spectrometry assays. However, the MinC(R107A) and FklB(R129A) mutants were also glycosylated by NleB (Fig. 3B). Within these assays, FADD was used as a positive control as a known NleB substrate (Qaidi et al. 2020). *C. rodentium* NleB was used to confirm that NleB was active in the bacterium. The *C. rodentium*

NleB $DAD^{221-223}$/AAA mutant was used as a negative control due to its lack of glycosylation activity.

We then generated a *minC* and *fklB* deletion in *C. rodentium* and complemented these strains with the cloned FLAG-tagged versions of either WT MinC or MinC(R107A), or WT FklB or FklB(R129A) for *in vivo* analysis. MinC, FklB and the arginine to alanine mutants were all expressed as inferred from SDS-PAGE and western blotting using an anti-FlagTag monoclonal antibody (Fig. 3C). However, using the anti-R-GlcNAc monoclonal antibody for detection established that these proteins were not glycosylated by NleB. We conducted similar analyses with *S. enterica*, EHEC, and EPEC, and similarly MinC or FklB were not glycosylated (Fig. 3D). For these assays, a *gshB* deletion in *C. rodentium* with a FLAG-tagged version of WT GshB was utilized as a positive control, while the *gshB* deletion in *C. rodentium* with FLAG-tagged GshB(R256A) was used as the negative control, both as confirmed NleB substrates in Qaidi et al. (2020).

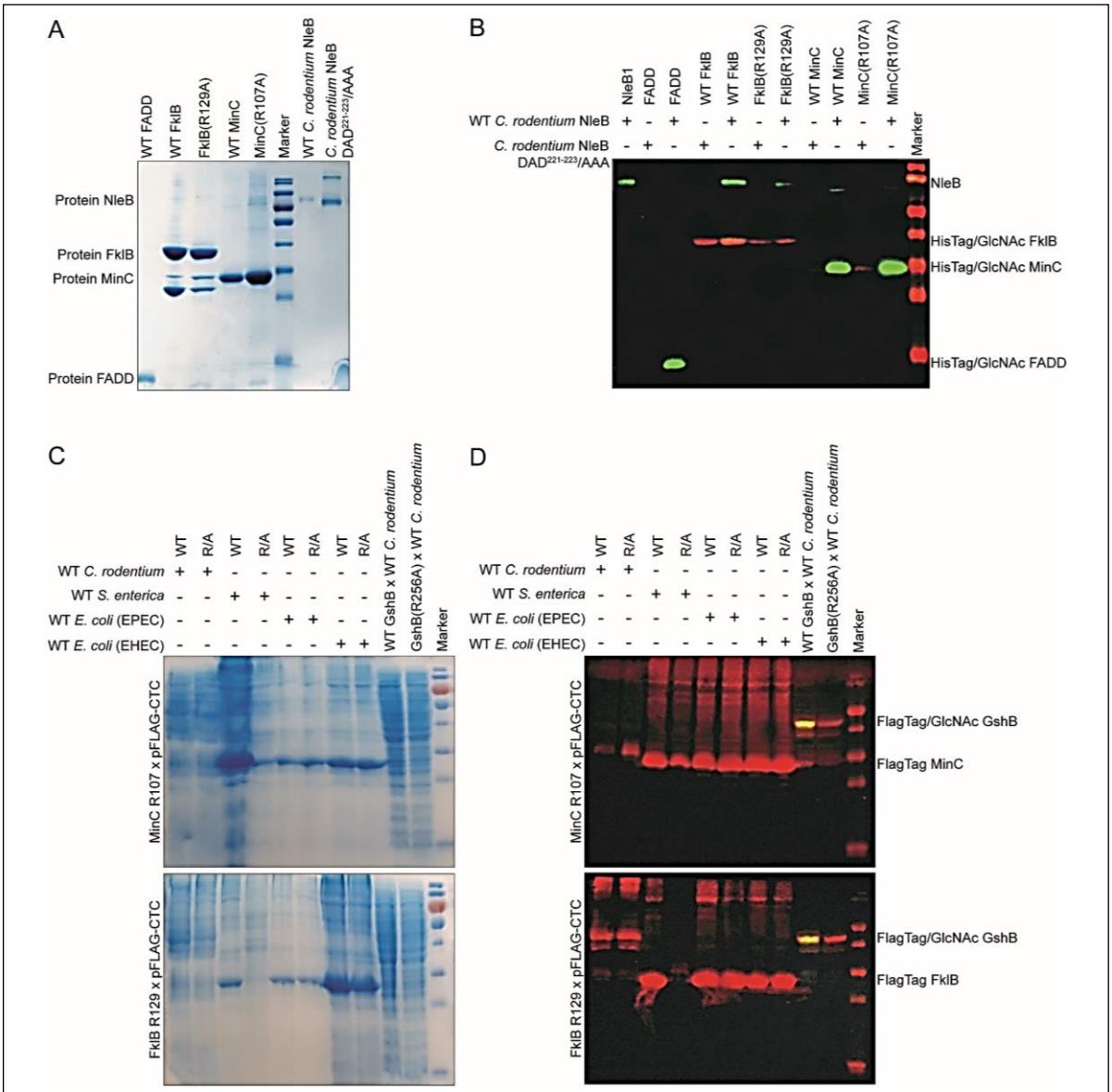

Figure 3 NleB glycosylates MinC R107 and FklB R129 *in vitro*, but not at endogenous levels of NleB.

(A) Coomassie blue staining and protein purification to detect expression of WT and point mutations MinC and FklB cloned in pET-28a. (B) MinC and FklB are glycosylated by NleB on their arginine residues *in vitro*. Point mutations from arginine to alanine was completed on arginine residues R107 and R129 and were also glycosylated by NleB *in vitro*. Anti-HisTag and anti-R-GlcNAc monoclonal antibodies were used for western blotting. (C) Coomassie blue staining after electroporation in *C. rodentium*, *S. enterica*, EPEC, and EHEC with WT and point mutations MinC and FklB in pFLAG-CTC protein expression was detected. (D) WT and point mutants MinC and FklB show FlagTag expression, but are not glycosylated by NleB within *C. rodentium*, *S. enterica*, EPEC, or EHEC. Anti-FlagTag and anti-R-GlcNAc monoclonal antibodies were used for western blotting. WT GshB in *C. rodentium* is the positive control (yellow).

NleB glycosylation verification through host proteins. Previous work has shown that NleB glycosylates mammalian substrates FADD, GAPDH, and TRADD inside the host cell (Park, Kim, Yoo et al et al., 2018). From this information, we attempted to determine whether these proteins could be glycosylated within the bacterium. We expressed FLAG-tagged versions of either WT FADD or FADD(R117A), WT GAPDH or GAPDH(R197A/R200A), or WT TRADD or TRADD(R235A) in WT *C. rodentium* and mutant *C. rodentium* NleB DAD$^{221-223}$/AAA and then confirmed protein expression through SDS-PAGE analysis (Fig. 4A). Protein expression was also confirmed through western blotting using an anti-HisTag monoclonal antibody. WT FADD, WT GAPDH, and WT TRADD were glycosylated by endogenous levels of NleB, detected using an anti-R-GlcNAc monoclonal antibody (Fig. 4B). Furthermore, mutating the relevant substrate arginine was found to be sufficient to eliminate *in vivo* glycosylation (Fig. 4C). For this set of assays, the *gshB* deletion in *C. rodentium* with a FLAG tagged version of WT GshB was utilized again as the positive control while the *gshB* deletion in *C. rodentium* with FLAG tagged GshB(R256A) served as the negative control. Both were confirmed NleB substrates in Qaidi et al. (2020). Thus, mammalian substrates of NleB are glycosylated within *C. rodentium*, reinforcing the notion that NleB is active within the bacterium.

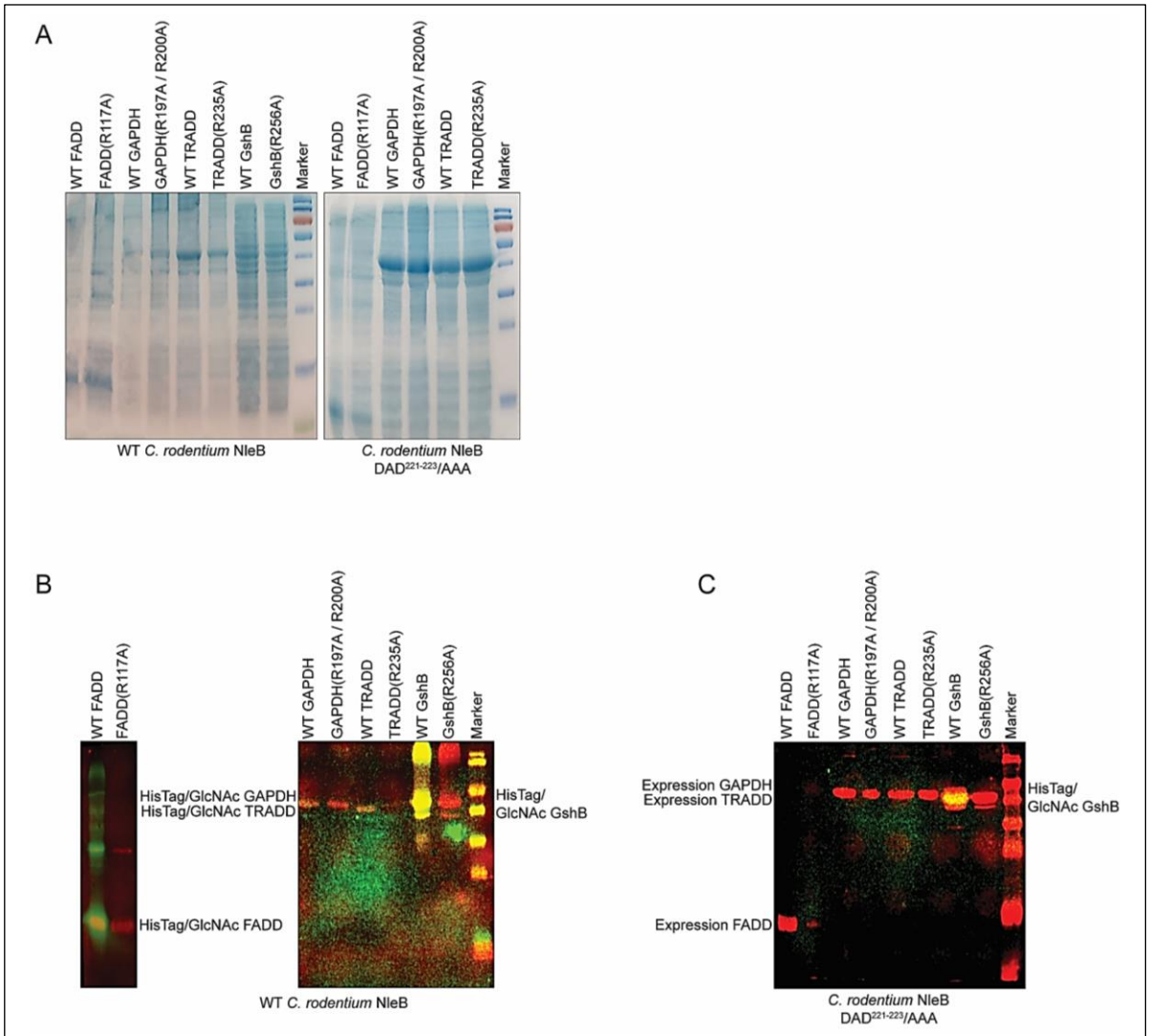

Figure 4 Mammalian substrates of NleB are glycosylated within *C. rodentium*.

(A) Coomassie blue staining after electroporation in WT *C. rodentium* and mutant, *C. rodentium* NleB DAD$^{221-223}$/AAA with host proteins WT FADD, GAPDH, and TRADD and their point mutants FADD(R117A), GAPDH(R197A/R200A), and TRADD(R235A), respectively, are expressed. (B) WT FADD, GAPDH, and TRADD are all glycosylated at endogenous levels of NleB within *C. rodentium*, and HisTag expression is detected for all proteins. Mutating the relevant substrate arginine is sufficient to eliminate *in vivo* glycosylation. Anti-HisTag and anti-R-GlcNAc monoclonal antibodies were used for western blotting. WT GshB within *C. rodentium* is glycosylated by NleB and is shown as the positive control (yellow) while GshB(R256A) is shown as a negative control (red). (C) Host proteins FADD, GAPDH, and TRADD are not glycosylated by NleB in mutant *C. rodentium* NleB DAD$^{221-223}$/AAA, as hypothesized. Again, WT GshB is shown as the positive control (yellow) and GshB(R256A) is shown as the negative control (red).

2.4 Discussion

NleB inhibits host pro-inflammatory immune responses mediated by NF-κB by glycosylating host regulatory proteins on arginine residues (Park et al., 2018). In recent studies completed by Qaidi et al. (2020), the bacterial protein GshB was also found to be glycosylated by NleB inside the bacterium on arginine residue R256. From this information, we decided to analyze two other bacterial proteins also found through mass spectrometry that were glycosylated by NleB on their arginine residues. In our assays, we found that the WT MinC and WT FklB were glycosylated by NleB, suggesting that they may be glycosylated within the bacterium and that *in vivo* assays were needed. However, when point mutations were used to change the relevant arginine residues to alanines, we still saw that glycosylation by NleB was possible (Fig. 3B). This was not expected as there was only one glycosylated arginine residue found in the mass spectrometry experiments, and so, this suggests that there may have been another arginine residue in the amino acid sequence that was being targeted by NleB. This can be confirmed by either creating a mutant library and mutating all the arginine residues to an alanine within the signal sequence or by performing another mass spectrometry experiment on MinC and FklB to see if there are other arginine hits that are identified during the experiment.

We then conducted *in vivo* studies on MinC and FklB, and their arginine to alanine point mutations, MinC(R107A) and FklB(R129A), respectively. The bacterial proteins were introduced into WT *C. rodentium* and western blot analysis was completed. Protein HisTag expression was detected for all proteins, WT MinC, WT FklB, MinC(R107A), and FklB(R129A) but NleB glycosylation was not seen for any of the proteins. We hypothesized that there may be a different bacteria that glycosylation would be more successful in. We introduced the same proteins into WT *S. enterica*, WT EPEC, and WT EHEC and western blotting was performed.

Protein HisTag expression was again detected. However, NleB glycosylation was not detected for any of the proteins (Fig. 4B). This strongly suggests that MinC and FklB are not glycosylated at the endogenous level of NleB and that these bacterial proteins may not be actual targets of NleB.

NleB/SseK activities in the host cell modifies host proteins involved in the innate immune response, such as FADD, GAPDH, and TRADD. We wanted to confirm whether these proteins could be glycosylated inside the bacterium. The host proteins FADD, GAPDH, and TRADD, and the mutated forms of the same proteins [FADD(R117A), GAPDH(R197A/R200A), and TRADD(R235A), respectively], were introduced into WT *C. rodentium* and western blotting was completed. Protein HisTag expression was detected for all host proteins. NleB glycosylation was observed for WT FADD, WT GAPDH, and WT TRADD, but not for their point mutated forms as hypothesized. We also observed that WT FADD had a much stronger glycosylation signal than WT GAPDH or WT TRADD, suggesting that FADD is a better NleB glycosylation substrate compared to GAPDH or TRADD. We confirmed that the results that we received were not experimental error through the use of our positive control, WT GshB in WT *C. rodentium*, and our negative control, GshB(R256A) in WT *C. rodentium*. No other experiments besides the previous GshB study by Qaidi et al. (2020) have seen these results, which makes this and the previous study the first evidence of activity within the bacterium. In summary, the bacterial proteins MinC and FklB are not glycosylated to endogenous levels of NleB, and this was confirmed through the *in vivo* NleB glycosylation of host proteins FADD, GAPDH, and TRADD inside the bacterium.

Chapter 3 - Secretion of Effector 1-20 Amino Acid Nanobody Fusions

3.1 Rationale for the Study

Collaboration with Dr. Luis Angel Fernandez on the utilization of nanobodies, and an earlier study by Charpentier et al. (2004) provoked the second study completed in this thesis. Nanobodies are derived from camelid single chain antibodies and are small recombinant binders that can bind to antigens similarly to antibodies (Brunner and Schenck, 2020). They have simple structures which make them fairly easy to work with in the laboratory setting and enables recombinant expression and simplistic cloning. They also act as blockers or modifiers of protein activity, hence their importance in the following experiments. In a previous article, a 1-20 amino acid (AA) signal sequence from an effector protein that would normally be secreted through a Type III Secretion System (T3SS) was fused upstream from TEM-1 β-lactamase, a protein that would not normally be secreted (Charpentier and Oswald, 2004). The effector 20 AA signal sequences that were used in this instance were derived from the Cif, Tir, Map, and EspF effectors. Successful secretion and translocation of the TEM-1 β-lactamase fused with the effector 20 AA signal sequence in a type III-dependent but chaperone-independent manner was observed (Charpentier and Oswald, 2004). Once secreted, the protein fused with the 20 AA signal sequence was able to block the G_2/M phase transition.

The study for this thesis followed similar protocols to identify if a camelid nanobody that would not normally be secreted through a functional T3SS could be secreted if fused to a T3SS secretion signal. Certain effectors could be modular proteins composed of an exchangeable N-terminal secretion and translocation signal (STS) and can be linked to a C-terminal effector domain. In this case, if a 20 AA signal sequence from an effector that is secreted is fused to the

nanobody and an attenuated bacterium with an engineered T3SS is created, then successful secretion may be seen. The attenuated bacterium is needed because it would be free of any toxins a normal bacterium may have, making it safe, and the T3SS would function as a normal T3SS. The 20 AA signal sequence would act as a tag specific to T3SSs so that the nanobody fusion could be injected through the T3SS into the host cell. The 20 AA signal sequence nanobody fusion would then be able to block NleB from targeting host proteins and stop NleB from inhibiting the host immune response. This served as the premise for the concurrent experiments with the therapeutic nanobody fusions and is further explained in Fig. 5.

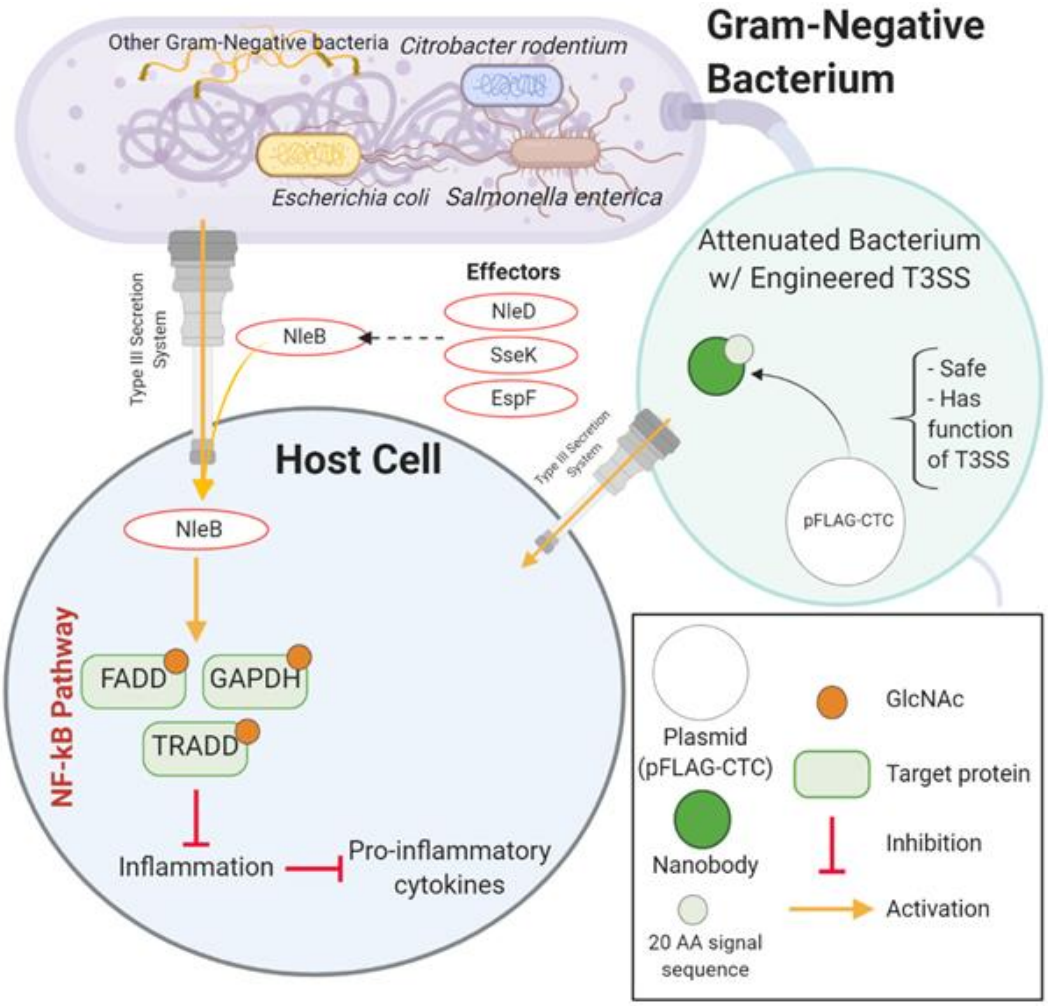

Figure 5 Schematics of the Secretion of 20 AA Signal Sequence Nanobody Fusions.

This figure was created using BioRender. The *E. coli* effector, NleB, is secreted through the T3SS from the bacterium into the host cell to inhibit functions of host proteins. NleB acts as an unusual glycosyltransferase by using N-acetyl glucosamine (GlcNAc) to glycosylate the arginine residues of host substrates by blocking the NF-κB pathway and overall inhibiting inflammation and the innate immune response. From a previous study, the idea was brought about that fusing a signal sequence from effectors that would normally be secreted through T3SSs to a substrate not normally secreted, then the signal sequence would promote secretion through a T3SS. For this study, an attenuated bacterium with an engineered T3SS that functions as a normal T3SS would be needed. A 20 AA signal sequence from certain effectors that were found to be secreted through T3SSs would then be fused to the nanobodies. Once secreted, the nanobody fusion could then act as a therapeutic to inhibit NleB from glycosylating target proteins and allow the host immune system to respond to the invading pathogen.

3.2 Materials and Methods

Construction of the 20 Amino Acid Signal Sequence Nanobody Fusion. Specific primers were constructed to create a plasmid that contained the camelid nanobody and the 20 AA signal sequence from a variety of effectors. The 20 AA signal sequence was added upstream towards the N-terminus of the nanobody sequence. T3SS signal sequences were derived from effectors EspF, Tir, NleB2, NleC, NleD, NleF, and NleH1. PCR was completed with nanobodies produced by Dr. Ramon Hurtado-Guerrero (Ruano-Gallego D, Álvarez B, and Fernández LÁ (2015) and specific primers to add on the 20 AA signal sequence from the effectors. Expression plasmids were created using ABC cloning (Qaidi et al, 2019) and are listed in Table 2 (Appendix B).

Bacterial Strains and Growth Conditions. Nanobodies fused with effector 1-20 AA signal sequence plasmids were electroporated with either the strain sent from Dr. Ramon Hurtado-Guerrero Synthetic Injector *E. coli* (SIEC) and its mutant SIEC P1 for one experiment, or WT and mutant *C. rodentium* for the other experiment. Two colonies from each strain were grown in either DMEM or LB broth with carbenicillin (Cb 100; 100 μg/mL) and 0.5 mM IPTG to induce growth. Cultures were incubated overnight at 37 ºC while shaking. Experiments were done with both DMEM and LB broth to help visualize if there was a significant difference in growth between the two.

Acetone Precipitation. Overnight cultures made from the 1-20 AA signal sequence nanobody fusions and electroporated with either SIEC and SIEC P1 or WT and Mutant *C. rodentium* were centrifuged at 4 ºC for 10 min. The culture supernatant (15 mL) was obtained

and filtered with a 25 mL luer-slip plastic syringe through a 0.20 μm sterile syringe filter (Corning, Inc., Corning, NY) into a 50 mL tube and the pellet was stored for later use at -20 ºC. Trichloroacetic acid (TCA) (5 %) was added to the filtered supernatant, mixed, and either precipitated on ice for 1 h or precipitated overnight at 4 ºC. The mix was then centrifuged for 30 min at 4 ºC and the maximum amount of supernatant was discarded by slowly inverting the tube. The pellet was washed 2 x in 5 mL of ice-cold acetone while scraping the sides of the tubes. After the last wash, the pellet was resuspended in 0.5 to 1 mL of ice-cold acetone and centrifuged at 4 ºC for 10 min. The maximum amount of supernatant was discarded by slowly inverting the tube and the pellet was resuspended in 20 μL of phosphate buffered saline (PBS) and 20 μL of 5 x sample SDS-loading dye (60 mM Tris-HCl pH 6.8, 1 % SDS, 1 % β-mercaptoethanol, 20 % glycerol, 0.01 % bromophenol blue).

Analysis of Expression and Secretion of Nanobody Fusion Proteins. A portion of the pellet was retrieved for sampling to visualize before and after the acetone precipitation, and 50 μL of 1 x SDS-loading dye was added. The pellet and precipitated supernatant cell extracts were brought to a boil through sonication and heated at 98 ºC for 5 min, then centrifuged briefly. Cell samples were electrophoresed on a 15 % SDS-PAGE polyacrylamide gel and were ran using a mini-protean tetra system (Bio-Rad, Hercules, CA) at 25 mAmp per gel for 1.5 h. For SDS-PAGE assays, gels were then stained for 30 min while shaking with Coomassie brilliant and then distained for 30 minutes while shaking. For western blot analysis, polyacrylamide gels were ran then transferred to a nitrocellulose filter membrane by following the semi-dry transfer unit (Hoefer Semi Phor) and running at 100 V for 1 h using a mini-protean tetra system (Bio-Rad, Hercules, CA). After running the transfer, anti-HisTag mouse monoclonal antibody was added

with a 1/5,000 dilution and either incubated overnight at 4 ºC shaking or incubated for 1 h at room temperature. The membrane was washed 3 x 10 minutes with Phosphate-Buffered Saline/Tween (PBST) and after, the second monoclonal antibody was added (goat anti-mouse) with a 1/5,000 dilution and incubated shaking at room temperature for 1 h. The membrane was then washed 3 x 10 minutes with PBST and detected using an Odyssey FC Imager (LI-COR, Lincoln, NE).

3.3 Results

Nanobodies with effector signal sequences are possible mediators of translocation. The nanobody is a single-domain antibody found in camelids and sharks that has the ability to bind to antigens just as an antibody would do, making them small, easy to obtain, and ideal for research. We used five nanobodies in the study that were produced and sent from Dr. Ramon Hurtado-Guerrero for this reason. Each nanobody started with "CAG" at the beginning of each sequence; "DYGS" is a recognition sequence for detection with an antibody that was also added into the nanobody sequences. To test the nanobodies as reporters for type III translocation, we fused each of the five nanobodies to the C-terminus of $EspF_{1\text{-}20}$ AA signal sequence, since EspF possesses an N-terminal STS, using PCR and specific primers. A 20 AA signal sequence was utilized because it was found in the studies of Charpentier and Oswald (2004) that a 20 AA sequence was optimal for successful secretion and translocation. After we completed the fusion, the $EspF_{1\text{-}20}$Nbs were cloned into pFLAG-CTC. Nanobody expression plasmids were electroporated into WT *C. rodentium* NleB, grown overnight, and then bacterial supernatants were acetone precipitated to concentrate the low concentration of the protein into a new pellet, and then the abundance of proteins in the supernatant was analyzed. We then completed SDS-PAGE analysis and western blotting. We detected the expression of all protein in the pellet, but the proteins in the supernatant were either very low in abundance or not detected at all (Figs. 6A-B).

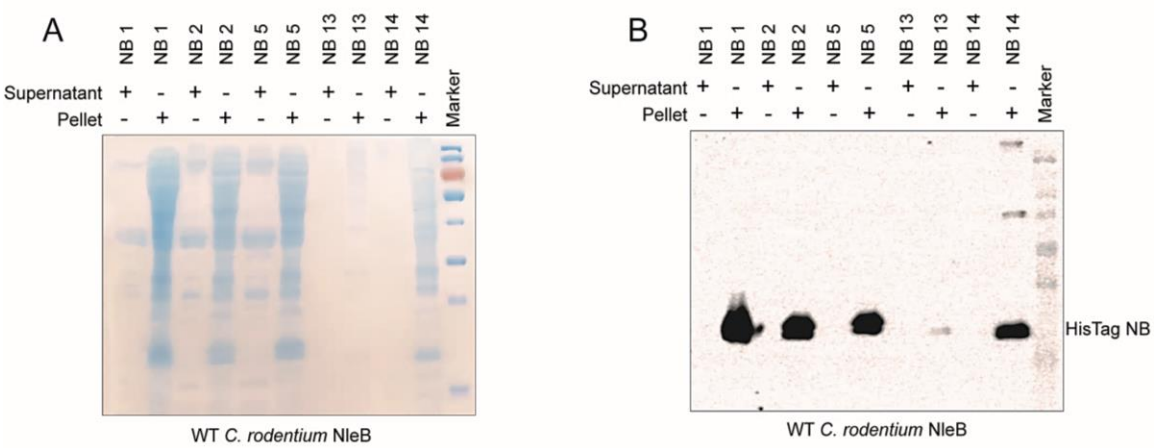

Figure 6 Expression of nanobody fusions is only detected within *C. rodentium* before precipitation.

(A) The effector EspF 20 AA signal sequence was fused to five nanobodies and were chosen to complete this study. Coomassie blue staining was completed and all $EspF_{1-20}$NB fusions were electroporated in WT *C. rodentium* and acetone precipitated to concentrate the low concentration of the protein into a new pellet. Then the abundance of proteins in the supernatant was analyzed. Expression was seen before precipitation for NBs 1, 2, 5, and 14, but low expression concentrations were seen after precipitation. NB 13 was not expressed. (B) $EspF_{1-20}$NB fusions were detected before precipitation, but not after precipitation. Nanobody fusions were not secreted through the T3SS within *C. rodentium*. An anti-HisTag monoclonal antibody was utilized for western blotting.

The first 20 codons of effectors do not mediate secretion of nanobodies. Once expression of the $EspF_{1\text{-}20}Nb$ in combination with WT *C. rodentium* but no secretion was observed, we decided to take one nanobody from the samples and complete further testing with various effectors that would also possess an N-terminal STS. We identified other STS in the first 20 codons of EPEC and EHEC effectors, then constructed fusions of the nanobody to the first 20 AA residues. The effectors identified and utilized were Tir (previously shown to have successful secretion in Charpentier and Oswald (2004)), NleB2, NleC, NleD, NleF, and NleH1. We also used the effector EspF again for verification and reproducibility. We then cloned them in pFLAG-CTC for *in vivo* preparation as in the first part of this study with EspF. Wanting to use a different Gram-negative bacteria to see if other bacteria may promote secretion more effectively, all nanobodies were electroporated into WT Synthetic Injector *E. coli* (SIEC) and its mutant, SIEC P1, strains. We grew the bacteria overnight and bacterial supernatants were acetone precipitated to concentrate the low concentration of the protein into a new pellet, and then the abundance of proteins in the supernatant was analyzed. Nanobody fusions were expressed in the WT SIEC and mutant SIEC P1 strains, but they were not secreted, as inferred from SDS-PAGE (Fig. 7A) and western blotting (Fig. 7B).

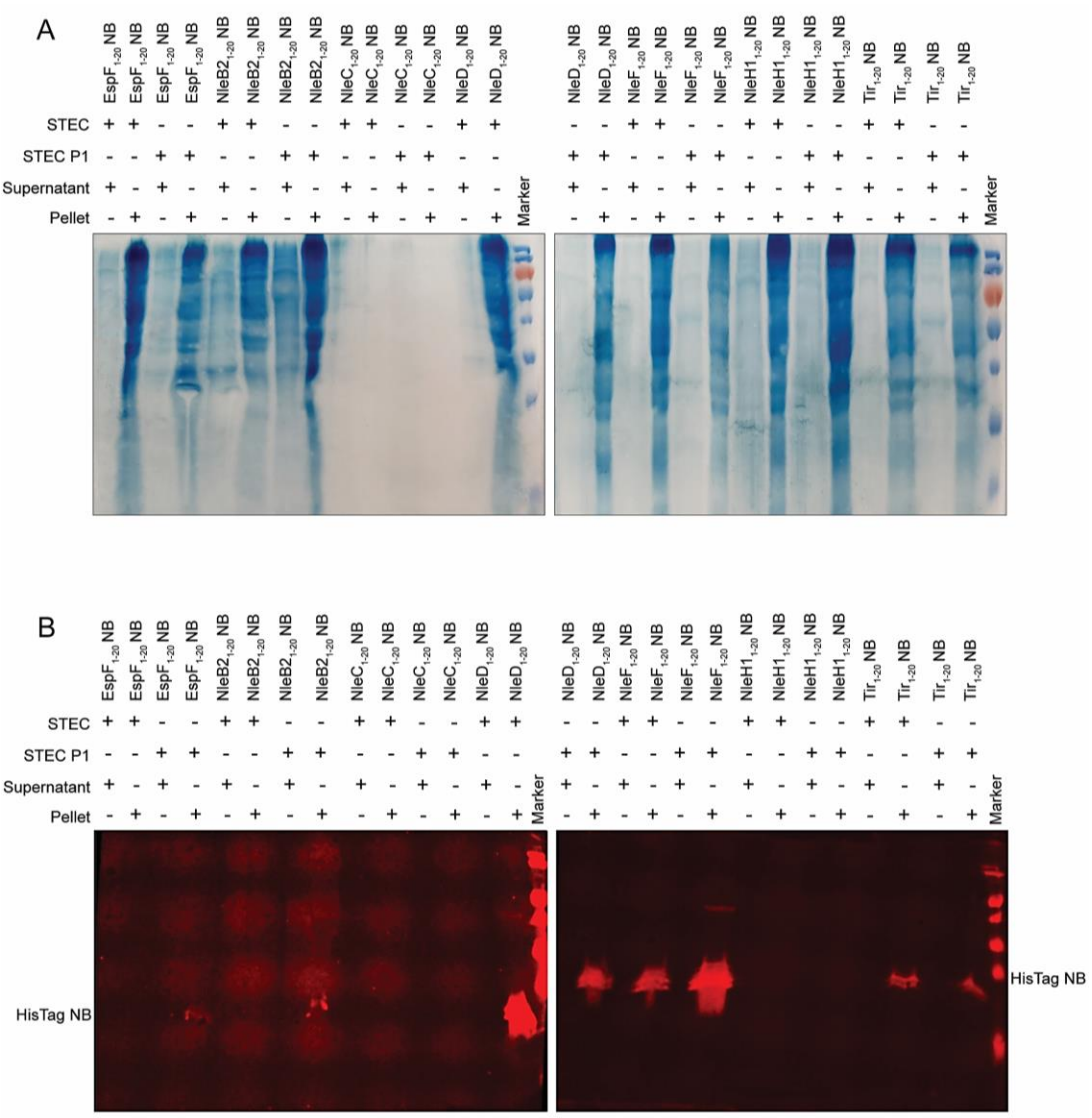

Figure 7 Some effector signal sequences work more efficiently than others, and nanobody fusions are expressed within *E. coli* but are not secreted.

(A) Nanobody 2 was used for the rest of the experiments since expression of the protein was found to be more abundant than the other nanobodies. Nanobody 2 was fused to signal sequences from effectors EspF, NleB2, NleC, NleD, NleF, NleH1, and Tir. Nanobody fusions were electroporated in WT SIEC and mutant SIEC P1 and acetone precipitated to concentrate the low concentration of the protein into a new pellet. Coomassie blue staining was completed and expression was seen before precipitation for all proteins except $NleC_{1\text{-}20}NB$, but low concentrations of expression was detected after precipitation (B) $EspF_{1\text{-}20}NB$, $NleD_{1\text{-}20}NB$, $NleF_{1\text{-}20}NB$, and $Tir_{1\text{-}20}NB$ fusion expressions were detected before precipitation, but no protein concentration was detected after precipitation. Anti-HisTag monoclonal antibody was utilized for western blotting. Nanobody fusions were not secreted through the T3SS within SIEC or SIEC P1.

We then tested WT *C. rodentium* and mutant *C. rodentium* NleB DAD$^{221\text{-}223}$/AAA with three of the nanobody fusions. The nanobody fusions Esp$F_{1\text{-}20}$Nb, Nle$F_{1\text{-}20}$Nb, and Tir$_{1\text{-}20}$Nb were chosen because they were expressed in greater abundance than the other fusions. The same protocol that was completed for SIEC and SIEC P1 was followed, and similar findings from the SIEC and SIEC P1 tests were found. Expression of all proteins in the pellet fraction were detected, but the proteins in the supernatant fraction after acetone precipitation were either very low in abundance or not detected at all. This suggests that there is no nanobody secretion through the T3SS (Figs. 8A-C).

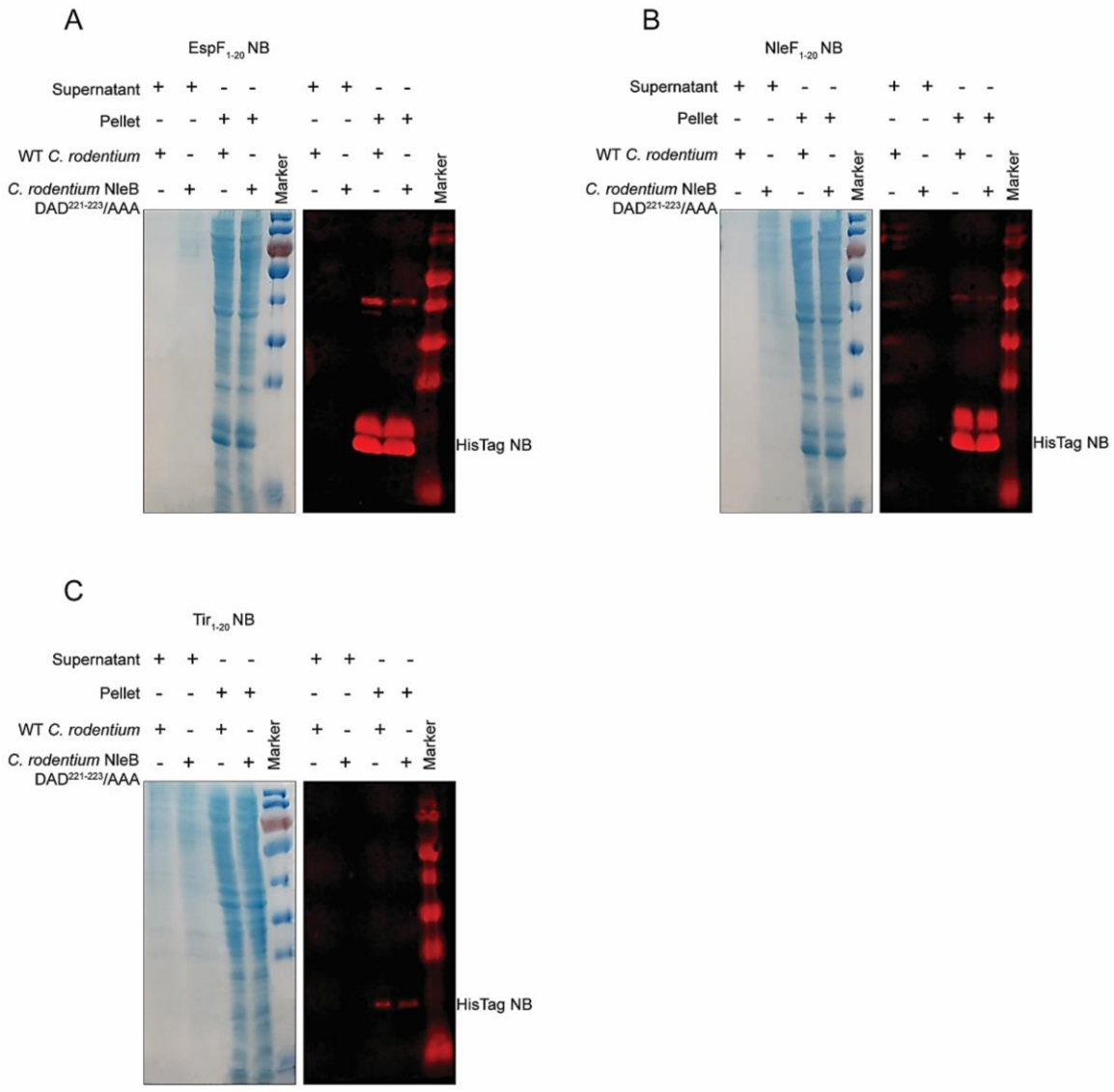

Figure 8 A 20 AA signal sequence does not promote secretion, and nanobody fusions are not secreted through the T3SS.

Coomassie blue staining, western blotting, and electroporation with WT *C. rodentium* and mutant *C. rodentium* NleB $DAD^{221-223}$/AAA was completed on the three nanobody fusions that had a higher abundance of protein throughout the study, (A) $EspF_{1-20}$NB, (B) $NleF_{1-20}$NB, (C) Tir_{1-20}NB. Nanobody fusions were acetone precipitated to concentrate the low concentration of protein. Expression before precipitation was detected through SDS-PAGE analysis and western blotting, but low to no concentration of protein was detected after precipitation. An anti-HisTag monoclonal antibody was utilized for detection in western blotting. Nanobody fusions were not secreted through the T3SS within WT or mutant *C. rodentium*, and the 20 AA signal sequence does not promote secretion of nanobody fusions through the T3SS.

3.4 Discussion

In a past experiment by Charpentier and Oswald (2004), the LEE-encoded effectors Tir and EspF had been studied to determine the domains utilized for translocation across a T3SS, and the signal sequences of both were confirmed to promote translocation and secretion of TEM-1 β-lactamase through a T3SS. EspF and Tir were also confirmed to contain an STS in their N termini, suggesting that LEE-encoded and non-LEE-encoded effectors could use the same molecular mechanisms to move from a bacterial cell to a host cell through a T3SS. In our study, we took a 20 AA signal sequence from EspF, Tir, NleB2, NleC, NleD, NleF, and NleH1 to create recombinant fusions of these T3SS signals to nanobodies. This would then determine if T3SS-dependent nanobody secretion would be promoted. Our decision to take the 20 AA signal sequence came from the Charpentier and Oswald (2004) study that revealed the first 16 N-terminal amino acid residues of an effector were sufficient for the secretion and delivery of protein into the host cell by WT EPEC strains. The first 20 N-terminal amino acid residues of each effector that marked proteins for secretion through their signals were fused to the nanobodies to examine if secretion of the nanobody through a T3SS was promoted. We hypothesized that, once inside the host cell, the nanobody fusions had the possibility to be utilized as a therapeutic method to inhibit NleB inside the cell so that an immune response against the pathogen would be encouraged.

Our first tests were completed with $EspF_{1\text{-}20}$Nb fusion in WT *C. rodentium* and the mutant *C. rodentium* strains. When the $EspF_{1\text{-}20}$ signal sequence was first observed in combination with the five different nanobodies, secretion through the T3SS was not seen, and suggested that a different effector signal sequence may be needed for delivery of the nanobody through the T3SS and that a different strain of *E. coli* may work better than *C. rodentium*. We

tested $Tir_{1\text{-}20}Nb$, $NleB2_{1\text{-}20}Nb$, $NleC_{1\text{-}20}Nb$, $NleD_{1\text{-}20}Nb$, $NleF_{1\text{-}20}Nb$, and $NleH1_{1\text{-}20}Nb$ fusions in WT Synthetic Injector *E. coli* (SIEC) and its mutant, SIEC P1. No secretion through the T3SS of the nanobody fusions was observed. Lastly, to confirm that secretion of the nanobody fusions was not possible, $EspF_{1\text{-}20}Nb$, $NleF_{1\text{-}20}Nb$, and $Tir_{1\text{-}20}Nb$ in WT *C. rodentium* and mutant *C. rodentium* NleB $DAD^{221\text{-}223}$/AAA was tested. Nanobody secretion was not observed, suggesting that a different method may be more apt for successful secretion into the host cell. It was also suggested to use an NleB deletion mutant instead of the inactive *C. rodentium*, which should be performed in future studies. Since we did not observe secretion of the nanobody fusions at any point, we did not examine translocation of the nanobodies. This suggestion is made since several other methods have been reported before to monitor the secretion and translocation through the T3SS of other proteins. It can also be proposed that somewhere in the acetone precipitation assay, part or all of the protein is not being fully precipitated, and more of the precipitated sample needs to be examined through western blot and SDS-PAGE analysis, since only 13 μL of each nanobody fusion was examined out of a total of 40 μL.

The studies of Charpentier and Oswald (2004) found that the full-length effector-protein fusion secreted and translocated through the T3SS more efficiently than the effector 16 AA signal sequence fused to the protein. These studies show that the N-terminal signal mediates secretion and translocation with a lower efficiency than the full length effectors, and suggests that in the case of our studies, using the full length of the effector signal sequence fused to the nanobodies would have been worth examining and should be examined in the future. Furthermore, the question remains if nanobodies are in fact efficient to be delivered through a T3SS or if another protein should be chosen to fuse the effector signal sequences to. Our positive controls were supposed to be the EspF and Tir 20 AA signal sequences since they had promoted

successful secretion in the Charpentier and Oswald (2004) study. However, the effectors fused to the nanobodies did not promote secretion for the nanobodies. In the future, it would be essential to include the EspF and Tir AA signal sequences fused to TEM-1 β-lactamase, as proven to be secreted in the previous study, as a positive control for future studies and should have been included in this thesis' studies.

Chapter 4 - Conclusion and Future Studies

Escherichia coli and *Salmonella enterica* are still among the leading causes of gastrointestinal tract infections. Over 200 million cases of gastroenteritis per year are reported in the United States alone, with children, the immunocompromised, and the elderly most affected. *E. coli* and *S. enterica* interact with their mammalian hosts by secreting effectors, such as NleB and SseK, through a type III secretion apparatus into the host cells. There, they target host proteins to inhibit host protein function that is involved in the innate immune response, which in turn impairs inflammation through blocking NF-κB activation.

The first part of these studies focused on NleB and SseK acting as unusual glycosyltransferases that glycosylate host protein substrates on arginine residues with N-acetyl glucosamine (GlcNAc) to block the function of host proteins. These effectors were initially thought to be inactive within the bacterium, however, were later found through mass spectrometry experiments to glycosylate substrates inside the bacterium. One of these identified substrates, GshB, was confirmed to be glycosylated on arginine residue R256 by Qaidi et al. (2020). Another two bacterial proteins, MinC and FklB glycosylated on arginine residues R107 and R129, respectively, were also postulated to be glycosylated by NleB. Two main conclusions were drawn from these experiments. One was that MinC and FklB were glycosylated *in vitro* on their arginine residues but were also found to be glycosylated on their arginine to alanine point mutants, which was not hypothesized as there was only one glycosylated arginine residue originally reported. The second was that even though *in vitro* analyses presented glycosylation of MinC and FklB on their arginine residues, these bacterial substrates were not found to be glycosylated at endogenous levels of NleB. This study was then verified through successful glycosylation of host proteins FADD, GAPDH, and TRADD on arginine residues R117,

R197/R200, and R235, respectively, by endogenous levels of NleB. As hypothesized, mutating the relevant substrate arginine was sufficient to eliminate *in vivo* glycosylation. These results concluded that mammalian substrates of NleB are glycosylated within *C. rodentium*, which reinforced the idea that NleB is active within the bacterium.

The second part of these studies focused on the secretion of a nanobody fused to a 20 AA signal sequence from effectors that would normally be secreted through a T3SS. Once secreted, the nanobody fusion could then act as a therapeutic to inhibit NleB from glycosylating target proteins and allow the host immune system to respond to the invading pathogen. In our first experiment, we tested the protein concentrations of $EspF_{1\text{-}20}Nb$ fusions in WT *C. rodentium* NleB before and after acetone precipitation. Our results revealed that there was no successful secretion through the T3SS. The next experiment was based on trying to find an effector that may aid in nanobody secretion more effectively and using a different Gram-negative bacterium to see if secretion was possible. $Tir_{1\text{-}20}Nb$, $NleB2_{1\text{-}20}Nb$, $NleC_{1\text{-}20}Nb$, $NleD_{1\text{-}20}Nb$, $NleF_{1\text{-}20}Nb$, and $NleH1_{1\text{-}20}Nb$ fusions were tested in WT Synthetic Injector *E. coli* (SIEC) and its mutant, SIEC P1, and protein concentrations in the pellet and after precipitation was measured. Yet again, no successful secretion through the T3SS was seen. Our last experiment was completed to confirm that the nanobody fusions were not secreted by Gram-negative bacteria. Three nanobody fusions were chosen as a result of their expression being in greater abundance than the other fusions; $EspF_{1\text{-}20}Nb$, $Tir_{1\text{-}20}Nb$, and $NleF_{1\text{-}20}Nb$ nanobody fusions were examined in WT *C. rodentium* NleB and its mutant, *C. rodentium* NleB $DAD^{221\text{-}223}$/AAA, before and after precipitation. No secretion of the nanobody fusions was observed when examining their protein abundance after precipitation. This concluded that the 20 AA signal sequence from effectors EspF, Tir, NleB2, NleC, NleD, NleF, and NleH1 fused upstream at the N-terminus of the

nanobody does not promote secretion through the bacterial T3SS. Since the nanobodies fused to the 20 AA signal sequence of effectors were not secreted, translocation was not examined.

Further studies are needed to fully understand the roles of both glycosylation of bacterial substrates inside the bacterium by NleB (Chapter 2) and of the secretion of effector AA signal sequence fusion to nanobodies, or another protein that may work better than the nanobody, through the T3SS (Chapter 3). For the first part of the set of studies, more bacterial substrates can be tested from the mass spectrometry list of arginine amino acid residue hits. The various changes on the functions that NleB glycosylation causes inside the bacterium would also be worthwhile to explore in future experiments, as our experiments did not get this far due to the *in vivo* results. For the second part of the set of studies, it is presumed that the number of proteins translocated by the T3SS is underestimated, and more studies of other possible secretion substrates would have to be conducted as there may be a better protein for fusion and secretion through the T3SS. It should also be noted that in our experiments, only a 20 AA signal sequence from the effectors was utilized. It would be beneficial to take the same effectors with the Gram-negative bacteria tested and fuse the whole signal sequence of the effector to the nanobody to see if translocation through the T3SS is possible. This idea was brought forth in the studies of Charpentier and Oswald (2004) when it was stated that the whole effector sequence fused to the protein seemed to secrete the fusion more efficiently than with the 20 AA signal sequence fusion. Further studies should be completed with the EspF and Tir 20 AA signal sequences fused to TEM-1 β-lactamase as a positive control since it has been proven to be secreted through a T3SS and it was not added in our experiments. Lastly, more experiments should also be performed to identify other T3SS effector substrates, as this would introduce new areas of investigation and aid in our understanding of EHEC and EPEC-mediated diseases.

Overall, the work completed in this thesis indicates that, with more research and positive results, these ideas have the potential to reduce the prevalence of *E. coli* and *S. enterica* in our society. By using the strategies listed in this thesis, the possibility of either targeting and inhibiting glycosylation through NleB of proteins inside the bacterium or targeting NleB directly inside the host cell through inhibitor protein fusions remains intact. If the studies with these two ideas were successful, they would show the second evidence of protein glycosylation inside the bacterium and the first evidence of nanobody fusions being secreted and translocated to inhibit NleB. However, since neither of these ideas were successful and with the confirmation that glycosylation within the bacterium is possible, future studies are needed. Ultimately, these studies bring us one step closer toward designing effective treatment and prevention strategies against food-borne pathogens.

Chapter 5 - References

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Appendix A - NleB Glycosylation of Proteins Plasmids and Strains

Table 1 Plasmids and strains used in Chapter 2.

Plasmids	Relevant Features	Source/ Reference
FLAG-NleB (*C. rodentium*)	WT *C. rodentium* used for electroporation	Gao et al. (2013)
FLAG-NleB (*C. rodentium*) ($DAD^{221-223}$/AAA)	Mutant *C. rodentium* used for electroporation	Gao et al. (2013)
pFlag-CTC	Cloning plasmid for *in vivo* analysis	This study
pET-28a	Cloning plasmid for *in vitro* analysis	This study
His-FADD	HisTagged FADD, positive control for *in vitro* assays	Qaidi et al. (2020)
FLAG-FADD (*H. sapiens*)	WT FADD x pFLAG-CTC	This study
FLAG-FADD(R117A) (*H. sapiens*)	FADD(R117A) x pFLAG-CTC	This study
FLAG-GAPDH (*H. sapiens*)	WT GAPDH x pFLAG-CTC	This study
FLAG-GAPDH(R197A/R200A) (*H. sapiens*)	GAPDH(R197A/R200A) x pFLAG-CTC	This study
FLAG-TRADD (*H. sapiens*)	WT TRADD x pFLAG-CTC	This study
FLAG-TRADD(R235A) (*H. sapiens*)	TRADD(R235A) x pFLAG-CTC	This study
His-MinC	WT MinC HisTagged x pET-28a	This study
His-MinC(R107A)	MinC(R107A) HisTagged x pET-28a	This study
FLAG-MinC (*C. rodentium*)	WT MinC x pFLAG-CTC	This study
FLAG-MinC(R107A) (*C. rodentium*)	MinC(R107A) x pFLAG-CTC	This study
His-FklB	WT FklB HisTagged x pET-28a	This study
His-FklB(R129A)	FklB(R129A) HisTagged x pET-28a	This study
FLAG-FklB (*C. rodentium*)	WT FklB x pFLAG-CTC	This study

FLAG-FklB(R129A) (*C. rodentium*)	FklB(R129A) x pFLAG-CTC	This study
Strains		
C. rodentium DBS100	Electroporation strain for *in vivo* assays	Qaidi et al. (2020)
C. rodentium DBS100 D*nleB*	Electroporation strain for *in vivo* assays	Gao et al. (2013)
C. rodentium DBS100 D*nleB*/pFLAG-CTC-*nleB*	Electroporation strain for *in vivo* assays	Gao et al. (2013)
C. rodentium Δ*gshB*/pFLAG-CTC *gshB*	Positive control for *in vivo* assays	Gao et al. (2013)
C. rodentium Δ*gshB*/pFLAG-CTC *gshB*(R256A)	Negative control for *in vivo* assays	Qaidi et al. (2020)
C. rodentium/FLAG-FADD	WT FADD x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-FADD(R117A)	FADD(R117A) x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-GAPDH	WT GAPDH x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-GAPDH(R197A/R200A)	GAPDH(R197A/R200A) x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-TRADD	WT TRADD x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-TRADD(R235A)	TRADD(R235A) x pFLAG-CTC/*C. rodentium*	This study
C. rodentium Δ*minC*/FLAG-*minC*	WT MinC x pFLAG-CTC/*C. rodentium*	This study
C. rodentium Δ*minC*/FLAG-*minC*(R107A)	MinC(R107A) x pFLAG-CTC/*C. rodentium*	This study
C. rodentium Δ*fklB*/FLAG-*fklB*	WT FklB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium Δ*fklB*/FLAG-*fklB*(R129A)	FklB(R129A) x pFLAG-CTC/*C. rodentium*	This study
S. enterica	Electroporation strain for *in vivo* assays	Qaidi et al. (2020)

E. coli BL21(DE3) x pET-28a-*minC*	MinC strain used for protein purification and *in vitro* analysis	This study
E. coli BL21(DE3) x pET-28a-*minC*(R107A)	MinC(R107A) strain used for protein purification and *in vitro* analysis	This study
E. coli BL21(DE3) x pET-28a-*fklB*	FklB strain used for protein purification and *in vitro* analysis	This study
E. coli BL21(DE3) x pET-28a-*fklB*(R129A)	FklB(R129A) strain used for protein purification and *in vitro* analysis	This study
E. coli BL21(DE3) x pET-42a-*nleB*	Active NleB for *in vitro* assays	Qaidi et al. (2020)
E. coli BL21(DE3) x pET-42a-*nleB* $DAD^{221-223}$/AAA	Inactive NleB for *in vitro* assays	Qaidi et al. (2020)
E. coli BL21(DE3) x pET42a-*nleB1* (EHEC)	Electroporation for *in vivo* assays	El Qaidi et al. (2017)
E. coli BL21(DE3) x pET42a-*nleB1* (EPEC)	Electroporation for *in vivo* assays	El Qaidi et al. (2017)

Appendix B - Nanobody Fusion Secretion Plasmids and Strains

Table 2 Plasmids and strains used in Chapter 3.

Plasmids	Relevant Features/ Genotype	Source/ Reference
FLAG-NleB (*C. rodentium*)	WT *C. rodentium* used for electroporation	Gao et al. (2013)
Residues 1-20 of EspF fused to Nanobody 1	$EspF_{1-20}$-NB x pFLAG-CTC	Charpentier et al. (2004), This study
Residues 1-20 of EspF fused to Nanobody 2	$EspF_{1-20}$-NB x pFLAG-CTC	Charpentier et al. (2004), This study
Residues 1-20 of EspF fused to Nanobody 5	$EspF_{1-20}$-NB x pFLAG-CTC	Charpentier et al. (2004), This study
Residues 1-20 of EspF fused to Nanobody 13	$EspF_{1-20}$-NB x pFLAG-CTC	Charpentier et al. (2004), This study
Residues 1-20 of EspF fused to Nanobody 14	$EspF_{1-20}$-NB x pFLAG-CTC	Charpentier et al. (2004), This study
Residues 1-20 of Tir fused to Nanobody 2	Tir_{1-20}-NB x pFLAG-CTC	Charpentier et al. (2004), This study
Residues 1-20 of NleB2 fused to Nanobody 2	$NleB2_{1-20}$-NB x pFLAG-CTC	This study
Residues 1-20 of NleC fused to Nanobody 2	$NleC_{1-20}$-NB x pFLAG-CTC	This study
Residues 1-20 of NleD fused to Nanobody 2	$NleD_{1-20}$-NB x pFLAG-CTC	This study
Residues 1-20 of NleF fused to Nanobody 2	$NleF_{1-20}$-NB x pFLAG-CTC	This study
Residues 1-20 of NleH1 fused to Nanobody 2	$NleH1_{1-20}$-NB x pFLAG-CTC	This study
Strains		

C. rodentium DBS100	Electroporation strain for *in vivo* assays	Qaidi et al. (2020)
C. rodentium DBS100 D*nleB*	Electroporation strain for *in vivo* assays	Gao et al. (2013)
WT Synthetic Injector *E. coli* (SIEC)	Electroporation strain for *in vivo* assays	Dr. Ramon (collaborator)
Mutant Synthetic Injector *E. coli* (SIEC P1)	Electroporation strain for *in vivo* assays	Dr. Ramon (collaborator)
C. rodentium/FLAG-EspF$_{1-20}$-NB Nanobody 2	EspF$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-Tir$_{1-20}$-NB Nanobody 2	Tir$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-NleB2$_{1-20}$-NB Nanobody 2	NleB2$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-NleC$_{1-20}$-NB Nanobody 2	NleC$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-NleD$_{1-20}$-NB Nanobody 2	NleD$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-NleF$_{1-20}$-NB Nanobody 2	NleF$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study
C. rodentium/FLAG-NleH1$_{1-20}$-NB Nanobody 2	NleH1$_{1-20}$-NB x pFLAG-CTC/*C. rodentium*	This study