Intrabacterial activity of type 3 secretion system effectors

by

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B.S., Mawlana Bhashani Science and Technology University, 2014
M.S., Kansas State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Abstract

Gram-negative enteric pathogens are a major source of infection and disease all over the world. Understanding the pathogenesis mechanisms of enteric pathogens is critical for developing strategies to reduce dissemination, infection, and mortality. The sophisticated secretion system known as the Type 3 Secretion System (T3SS) is one of the key virulence strategies shared by major gram-negative enteric pathogens. T3SS is used by pathogens to deliver special effector proteins to the host cell. These effector proteins, as the term implies, target many eukaryotic cellular pathway components to modulate host response during infection, resulting in increased pathogen survivability.

T3SS effectors are presumed to be inactive until they are injected into host cells and fold into active conformations. We previously discovered that the T3SS effectors NleB and SseK1 glycosylate *Citrobacter rodentium* and *Salmonella enterica* proteins, respectively, increasing resistance to environmental stress. A new class of T3SS effectors was investigated for potential intrabacterial activity in Chapter 2. The purpose of this study was to determine whether the T3SS effector proteases NleC, NleD, and EspL are active in *C. rodentium*. To accomplish this, we expressed in *C. rodentium* the best-characterized mammalian substrate of NleC, the NF-B p65 subunit; the substrate of NleD, JNK; and the substrates of EspL, RIPK1 and RIPK3 and monitored their proteolytic cleavage as a function of effector activity. NleC cleaved p65 intra-bacterially. As a result, we conclude that NleC, in addition to NleB, is enzymatically active within *C. rodentium*. Furthermore, T3SS effector EspL cleaved recombinant RIPK1, whereas NleD failed to cleave recombinant JNK protein, indicating that EspL is enzymatically active inside the pathogen, but NleD is not. The presence or absence of N-terminal Signal Peptides in the tested effectors was identified as a potential indicator of intrabacterial activity. To identify the potential target of NleC,

we used a recombinant target protein cleavage assay, RNA sequencing, and a unique proteomics-based technique.

Our group discovered intrabacterial activity and several bacterial targets of the T3SS effector NleB and its ortholog SseK1. The previously known intrabacterial activity of the T3SS effector glycosyltransferase was expanded in Chapter 3 to identify a novel target - the two-component response regulator OmpR. We show that the *Salmonella* T3SS effector glycosyltransferase SseK1 glycosylates the bacterial two-component response regulator OmpR on R15 and R122 arginine residues. OmpR Arg-glycosylation reduces the expression of *ompF*, a major outer membrane porin gene. Glycosylated OmpR has a lower affinity for the *ompF* promoter region than unglycosylated OmpR. Furthermore, the *Salmonella ΔsseK1* mutant strain had higher bile salt resistance and biofilm formation capacity than WT *Salmonella*, linking OmpR glycosylation to several important aspects of bacterial physiology.

Our healthcare system faces a significant challenge from enteric gram-negative pathogens. Given the recent emergence of several multidrug-resistant enteric gram-negative strains, as well as other unique disease conditions where antibiotic administration is not feasible, novel alternatives to traditional antimicrobials are desirable. T3SS effector intrabacterial activity provides us with a unique opportunity to develop antivirulence compounds that can target T3SS effectors and effectively reduce their capacity to sustain infection. We previously performed high throughput screening assays to identify novel small molecule inhibitors of the T3SS effector NleB and its orthologs. Even though many of the effectors demonstrated promising in vitro results, they had limitations that prevented them from being considered viable therapeutics. Several of the identified inhibitors, for example, were poorly soluble, expensive to bulk synthesize, or lacked any prior safety and efficacy data. In Chapter 4, we discovered a potential activity for avasimibe, a

previously identified acyl-coenzyme A:cholesterol acyltransferase inhibitor, in inhibiting the NleB and SseK arginine glycosyltransferases from *Escherichia coli* and *Salmonella enterica*, respectively, using high-throughput screening assays. Avasimibe inhibited the activity of the *Citrobacter rodentium* NleB, *E. coli* NleB1, and *Salmonella enterica* SseK1 enzymes without affecting the activity of the human serine/threonine N-acetylglucosamine (O-GlcNAc) transferase. At reasonably high dose, avasimibe was neither toxic to mammalian cells nor was it bactericidal. A dose of 10 μM avasimibe was sufficient to reduce *S. enterica* abundance in RAW264.7 macrophage-like cells. We conducted in vivo efficacy experiments using a mouse model in this study. Avasimibe intraperitoneal injection significantly reduced *C. rodentium* survival in mice, whether administered pre- or post-infection. We propose that avasimibe or related synthetic derivates may be useful in preventing or treating bacterial infections by inhibiting T3SS effector arginine glycosyltransferases, which are essential for virulence.

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Approved by:

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Acknowledgements

My gratitude and appreciation for my advisor, Prof. Dr. Philip R. Hardwidge, run deep and I cannot express them enough. Throughout my Ph.D. study, his unwavering guidance, patience, and encouragement have been a beacon of hope that brought me back from the depths of despair. When I was lost and couldn't find my way, he was there to light my path.

To my other committee members, Dr. T. G. Nagaraja, Dr. Brian Geisbrecht, and Dr. Kathryn Reif, I owe a debt of gratitude that can never be fully repaid. Their assistance and advice on my research and thesis were invaluable. Their belief in me and my work gave me the strength to persevere when the going got tough.

And to my lab members, Dr. Samir El Qaidi and Mike Hays, I extend my heartfelt thanks for their valuable suggestions and selfless support. Dr. Samir El Qaidi, in particular, has been an inspiration to me. His rich knowledge and extensive experience have guided me into the field of microbiology.

Finally, I want to express my deepest appreciation to all my friends and my parents for their unwavering support during my PhD journey. Their love and encouragement sustained me through the long, arduous days and nights of research. Without them, I could not have made it this far.

Chapter 1 - Introduction

Gram-negative enteric pathogens represent a significant public health concern, with a global impact on morbidity and mortality. In addition to the United States, numerous other countries struggle with the prevalence of these pathogens, which are estimated to be responsible for up to 1.5 million deaths annually [1]. The economic burden of these infections is also substantial, with estimates indicating that costs in the United States exceed $10 billion [2]. *Campylobacter jejuni, Escherichia coli, Salmonella, Shigella*, and *Vibrio cholerae* are among the most common gram-negative enteric pathogens in the United States, with *E. coli* O157, *Salmonella*, and *Shigella* posing urgent threats to the healthcare system, according to the Centers for Disease Control and Prevention (CDC) [3]. These pathogens cause a range of illnesses, including diarrhea, foodborne illnesses, and systemic infections.

Moreover, in addition to posing a threat to human health, enteric pathogens also have a significant impact on animal health. For instance, *Salmonella* is a leading cause of foodborne illnesses in both humans and animals, with an estimated 1.2 million cases of human salmonellosis occurring annually in the United States [4]. The issue of *Salmonella* contamination is significant in the food production industry, particularly in the poultry and meat industries, where it can result in severe economic losses [5]. Similarly, infections caused by Enterohemorrhagic *E. coli* (EHEC) and Enteropathogenic *E. coli* (EPEC) result in substantial losses in the pig production industry every year [6]. Understanding the complex relationship between gram-negative enteric pathogens and both human and animal health is essential for developing effective prevention and control strategies.

Pathogenic *E. coli* strains

Pathogenic *Escherichia coli* strains have serious public health implications and pose a global threat to food safety [9]. These bacteria are a diverse group that can cause a wide range of illnesses, from mild gastroenteritis to severe, life-threatening infections like hemolytic uremic syndrome [8]. Pathogenic *E. coli* strains are classified based on their virulence factors and clinical manifestations [9]. The most well-known pathogenic *E. coli* strain is *E. coli* O157:H7, which has been linked to foodborne illness outbreaks and has resulted in several large-scale food recalls [7,11]. Other pathogenic *E. coli* strains include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC). These strains are spread through contaminated food or water, person-to-person contact, or contact with animals or the environment [9].

Enterohemorrhagic *E. coli* (EHEC) is a pathogenic strain of *E. coli* that can cause severe gastrointestinal illness in humans [10]. The virulence of EHEC is due to the production of a potent Shiga toxin [10]. Shiga-like toxin (SLT), also termed verotoxin and encoded by *stx* genes, is the main virulence factor in EHEC [10]. The toxin is synthesized as a single polypeptide chain that is then cleaved into an A subunit and five B subunits [10]. The A subunit enters the cell after the B subunits bind to the receptor on the surface of the cell. When the A subunit enters the cell, it inhibits protein synthesis, causing cell death [10].

EHEC also produces several other virulence factors that help it colonize and infect the host [10]. Fimbriae are hair-like structures on the bacteria's surface that allow them to attach to intestinal cells, and a Type 3 Secretion System allows the bacteria to inject proteins directly into host cells, modulating the host immune response and facilitating colonization [10]. EHEC infection is typically diagnosed through stool culture, in which the bacteria can be isolated and

identified [10,15,16,17]. Molecular techniques such as polymerase chain reaction (PCR) can also be used to detect the presence of the Shiga toxin gene or other virulence factors in clinical samples [10,13,14]. EHEC infection treatment is primarily supportive, with hydration and electrolyte replacement being critical components of management. Antibiotics are generally not advised because they raise the risk of kidney failure [12,18-21].

Enteropathogenic *Escherichia coli* (EPEC) is a bacterium that is typically spread through contaminated food or water. Infection can cause severe diarrhea and dehydration, especially in vulnerable populations such as infants, the elderly, and those with compromised immune systems [25]. The bacterium attaches to the intestinal epithelium, causing an attaching and effacing lesion (A/E lesion), which results in the destruction of microvilli and the formation of actin-rich pedestals that promote bacterial adherence and colonization [22].

The presence of the locus of enterocyte effacement (LEE) pathogenicity island, which encodes a type III secretion system (T3SS) and a suite of effector proteins that manipulate host cell functions, mediates EPEC virulence [23]. The T3SS is responsible for injecting effector proteins into host cells, which causes changes in the actin cytoskeleton, cell signaling pathways, and immune responses. Intimin, which mediates bacterial adhesion to host cells, and Tir, which is translocated into host cells and acts as an intimin receptor, are two major effector proteins. Other effector proteins regulate apoptosis, cytokine secretion, and tight junction formation in host cells [26].

EPEC infection causes a strong innate immune response in the host, including the release of proinflammatory cytokines like interleukin-8 (IL-8) and the recruitment of neutrophils to the infection site. EPEC, on the other hand, is capable of subverting host defenses via the action of its effector proteins, which disrupt host cell signaling and inflammatory pathways. The effector

protein NleH1 can inhibit the activation of the nuclear factor kappa B (NF-kB) transcription factor, which is required for the expression of many proinflammatory cytokines and immune response genes [24].

ETEC is a leading cause of traveler's diarrhea in developing countries with poor sanitation and hygiene. The bacteria spread through the fecal-oral route, which is frequently contaminated food or water. ETEC is also a major cause of diarrhea in children under the age of five in these areas, where it is a leading cause of death. ETEC infections are less common in developed countries, but they can occur in travelers returning from endemic areas [47].

ETEC causes disease by releasing two types of enterotoxins: heat-labile toxin (LT) and heat-stable toxin (HST) (ST). LT is an AB5 toxin that binds to the GM1 ganglioside receptor on the host cell surface and is structurally similar to cholera toxin. Once inside the host cell, the toxin's A subunit activates adenylate cyclase, causing an increase in intracellular cyclic AMP (cAMP). Increased cAMP levels then activate protein kinase A, causing chloride channels in the host cell membrane to open and chloride ions to be secreted into the intestinal lumen. The resulting osmotic gradient causes water and electrolytes to be secreted into the lumen, resulting in diarrhea [29].

In contrast, ST is a smaller peptide that binds to a different host cell surface receptor, guanylate cyclase C. (GC-C). ST activates GC-C, increasing intracellular cyclic GMP (cGMP) levels. Increased cGMP levels then activate protein kinase G, causing chloride channels to open and water and electrolytes to be secreted into the intestinal lumen, resulting in diarrhea [27].

Salmonella enterica

Salmonella enterica belongs to the Enterobacteriaceae family, which also includes pathogenic bacteria such as *E. coli* and *Shigella* spp. It is a species of the genus *Salmonella*, which

contains over 2,500 serotypes. Based on biochemical and genetic characteristics, *Salmonella enterica* is further subdivided into six subspecies. *S. enterica* subsp. enterica, *S. enterica* subsp. salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. houtenae, and *S. enterica* subsp. indica are among the subspecies [30]. *Salmonella* causes a variety of diseases in humans and animals, such as gastroenteritis, typhoid fever, and bacteremia [31]. This bacterium has a complicated pathogenesis that includes colonization, invasion, intracellular survival, and dissemination. *Salmonella enterica* first colonizes its host's intestinal tract. Adhesins such as FimH, PefA, and SiiE help the bacterium attach to the mucosal surface of the small intestine [32]. *Salmonella* can establish a foothold in the host's gut by recognizing and binding to specific receptors on the surface of intestinal epithelial cells [33]. Once attached, the bacterium begins to proliferate and form microcolonies on the intestinal mucosa. Invasion of the intestinal epithelium is the second stage of *Salmonella* pathogenesis. T3SS-1, or "triggering of host cell entry," is a process by which the bacterium induces its own uptake into epithelial cells. This system introduces bacterial effector proteins into the host cell, where they manipulate the cytoskeleton to induce bacterial endocytosis. SopE and SopE2 T3SS-1 effectors activate host GTPases, causing actin cytoskeleton rearrangements that promote bacterial uptake [34]. The bacterium lives inside the host cell in a membrane-bound vacuole known as the *Salmonella*-containing vacuole (SCV) [35]. *Salmonella* enterica has developed the ability to survive and replicate within host cells such as epithelial cells, macrophages, and dendritic cells. Once inside the SCV, *Salmonella* manipulates the host cell to create a niche that favors bacterial survival and replication. Through its T3SS-2, the bacterium injects a suite of effector proteins that modulate the host cell machinery, including the formation of a lipid-rich vacuolar environment, acidification resistance, and autophagy evasion. The effector proteins SseJ and SifA are crucial for the maturation of the SCV, the

formation of *Salmonella*-induced filaments (SIFs), and the inhibition of phagolysosomal fusion [36]. The final stage of *Salmonella* pathogenesis is dissemination from the intestine to other organs. The bacterium can enter the bloodstream through damaged intestinal mucosa after crossing the intestinal epithelium, or it can be taken up by dendritic cells and transported to regional lymph nodes. *Salmonella* can then spread to other organs, such as the liver and spleen, causing systemic disease. The bacterium moves through the host tissue and invades new host cells with its flagella, which contributes to the formation of new bacterial foci [37].

Citrobacter rodentium

Citrobacter rodentium is a Gram-negative bacterium in the Enterobacteriaceae family. It's a rod-shaped bacterium that is about 1-3 micrometers long and 0.5-1 micrometers wide. *C. rodentium* is an important model organism for studying enteric infections, and it has been extensively used in research to better understand the pathogenesis of infections caused by enteropathogenic and enterohemorrhagic *E. coli. Citrobacter rodentium* is most commonly found in rodents, specifically mice, rats, and other rodents. Human infection is uncommon, but it has been reported in people who have encountered infected rodents or who have consumed contaminated food or water. The bacterium can be spread through direct contact with infected animals or by consuming contaminated food or water.

The bacterium colonizes the colonic mucosa, where it forms attaching and effacing lesions (A/E lesions) on epithelial cells. This is accomplished through the secretion of bacterial virulence factors such as EspA, EspB, and EspD into the host cell via a type III secretion system (T3SS) [38]. Once inside the host cell, the virulence factors promote the formation of actin-rich pedestals, allowing the bacteria to adhere to the epithelial cell surface in proximity.

C. rodentium infection also causes a strong innate and adaptive immune response in the host, which is critical for infection control. Toll-like receptors (TLRs) are activated during the innate immune response to recognize pathogen-associated molecular patterns (PAMPs) on the bacterial surface [40]. This results in the production of proinflammatory cytokines such as interleukin-1 (IL-1) and IL-6, as well as chemokines such as CXCL1 and CXCL2. The cytokines and chemokines attract neutrophils and other immune cells to the infection site, where they aid in the removal of bacteria.

During *C. rodentium* infection, the adaptive immune response is also activated, resulting in the production of specific antibodies against the bacterium. These antibodies, particularly immunoglobulin A (IgA), play an important role in preventing bacterial colonization and promoting bacterial clearance [39]. Colonic hyperplasia caused by *C. rodentium* infection is also characterized by the proliferation of colonic crypts and an increase in the number of goblet cells. This is thought to be a host defense mechanism aimed at increasing mucin production, which aids in the trapping and removal of bacteria from the gut [41].

Pathogenesis of gram-negative enteric pathogens

Given the importance of gram-negative enteric pathogens in Human health and agriculture industry and food safety, understanding the fundamental mechanisms of pathogenesis is of great significance. The pathogenesis mechanism of Gram-negative pathogens is quite diverse.

Pilus and non-pilus adhesins:

Adhesins are a class of proteins that are produced by many bacterial species and enable the bacterium to adhere to host cells or other surfaces. Pili and non-pilus adhesins are important virulence factors that allow bacteria to adhere to host cells or other surfaces, allowing bacteria to

colonize host tissues and evade host immune responses. The structure and function of these two types of adhesins are what distinguishes them. Pili are proteins that are made up of repeating protein subunits and can be classified into different types based on their structure and function, whereas non-pilus adhesins are a diverse group of proteins that are not made up of repeating protein subunits and can be classified into several categories based on their structure and function.

Pili:

Pili are hair-like appendages found on the surfaces of many bacteria, including *E. coli*, *N. gonorrhoeae*, and *Pseudomonas aeruginosa*. Pili is made up of a repeating protein subunit called pilin and are classified according to their structure and function [43].

One type of pilus, known as type 1 pilus, is involved in host cell adherence and biofilm formation. Type 1 pili are made up of a single pilin subunit and are anchored to the bacterial outer membrane by the chaperone-usher pathway. A receptor-binding domain at the tip of the type 1 pilus interacts with host cell receptors, allowing the bacterium to adhere to the host cell surface [46]. Type 4 pilus is another type of pilus that is involved in a variety of functions such as adherence, twitching motility, and DNA uptake. Type 4 pili are composed of multiple pilin subunits and are anchored to the bacterial outer membrane by a protein complex called the type 4 pilus assembly machinery. A specialized adhesin at the tip of the type 4 pilus allows the bacterium to bind to specific host cell receptors or extracellular matrix components [42].

Pilus adhesins have been studied extensively in Gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa*. The type 1 pilus adhesin in *E. coli* oversees attachment to host cells and extracellular matrix components. Mannose residues on host cells and extracellular matrix components are recognized and bound by type 1 pilus adhesins [44]. PilY1 is the pilus adhesin in

P. aeruginosa that is involved in attachment to host cells and extracellular matrix components. PilY1 binds to sialic acid residues on host cells as well as extracellular matrix components [43].

Additionally, there are other types of pili found in different bacteria, such as type 2 pili and conjugative pili, that have distinct structures and functions [45].

Non-pilus Adhesins:

Non-pilus adhesins are surface proteins that mediate bacterial attachment to host cells or extracellular matrix components without the use of pili. Non-pilus adhesins are found in both Gram-negative and Gram-positive bacteria and play several roles, including adhesion, invasion, and colonization [48].

The fibronectin-binding protein (FnBP) of *Staphylococcus aureus* is one of the most well-studied non-pilus adhesins. FnBP is a surface protein that allows *S. aureus* to attach to host cells and extracellular matrix components. FnBP binds to fibronectin, a glycoprotein found in many tissues that plays a role in cell adhesion, migration, and wound healing. FnBP is a key virulence factor of *S. aureus* that plays a role in the pathogenesis of several infections, including endocarditis, osteomyelitis, and sepsis [50].

Streptococcus pyogenes M protein is another well-studied non-pilus adhesin. The M protein is a surface protein that allows *S. pyogenes* to attach to host cells and extracellular matrix components. The M protein interacts with several host proteins, including fibrinogen, fibronectin, and immunoglobulin G. The M protein is a key virulence factor of *S. pyogenes* that plays a role in the pathogenesis of several infections, including pharyngitis, impetigo, and necrotizing fasciitis [49].

Secretion systems:

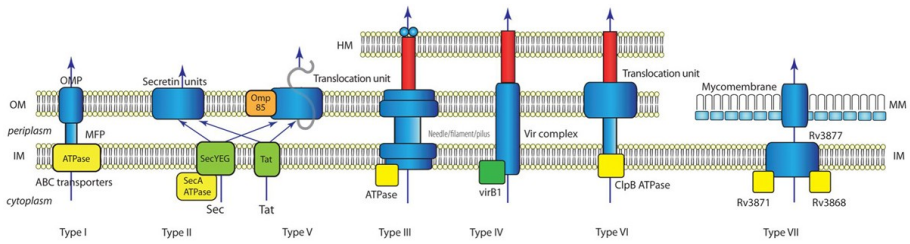

Figure 1 A schematics of different secretion systems.
A simplified schematics of each secretion system are sketched here. HM: Host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases and chaperones are shown in yellow.
Note. Reprinted from "Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology", by Tseng, TT, 2009 *BMC Microbiol* **9** (Suppl 1). [99]

One of the key aspects of gram-negative enteric pathogens pathogenesis is the use of unique secretion systems to deliver a variety of cargo to different targets. These secretion systems, along with their cargo, show great diversity. For example, so far, 8 unique secretion systems have been identified for gram-negative enteric pathogens. These secretion systems can deliver proteins, DNA, and various small molecules to either the host cell or the pathogen's outside environment. Gram-negative bacteria use a variety of secretion systems, including the type I, II, III, IV, V, and VI secretion systems. A schematics of different secretion system is shown in Figure 1.

The type I secretion system is the most basic of the secretion systems and is found in a wide range of Gram-negative bacteria, including *E. coli* and *P. aeruginosa*. This system is made up of a single protein complex that spans the bacterium's inner and outer membranes. An ABC transporter recognizes the protein to be secreted, which is then pumped through the complex and released into the extracellular environment. [53]

Gram-negative bacteria with the type II secretion system include *Vibrio cholerae* and *Legionella pneumophila*. This system is made up of complex machinery that spans the bacterium's inner and outer membranes. The secreted protein is first transported across the inner membrane via the Sec or Tat pathway, and then translocated across the outer membrane via the type II secretion machinery. A wide variety of proteins, including enzymes, toxins, and virulence factors, are secreted by the type II secretion system. [52]

Among the known secretion systems, the T3SS is the most relevant, and arguably the most well-studied one. The type III secretion system is a specialized secretion system used by a variety of Gram-negative pathogens, including *Salmonella enterica* and *Yersinia pestis*. This system is made up of complex machinery that spans the bacterium's inner and outer membranes. The type III secretion system is used to deliver bacterial effector proteins directly into the cytosol of the host cell. These effector proteins manipulate host cell signaling pathways, allowing bacteria to invade and survive [51, 54].

Gram-negative bacteria also use type IV secretion systems, which are ancestrally related to conjugation machines, for intercellular transfer of macromolecules [52]. Additionally, gram-negative bacteria use type V and VI secretion systems, which are involved in the secretion of autotransporters and bacteriocins, respectively [53]. Various studies have been conducted to understand the structure and mechanism of these secretion systems [55,56].

The type IV secretion system is a complex machinery found in a variety of Gram-negative bacteria, including *Helicobacter pylori* and *Agrobacterium tumefaciens*. This system is responsible for the secretion of numerous molecules, including proteins, DNA, and lipids. Like the type III secretion system, the type IV secretion system is used to inject bacterial effector proteins into host

cells. The type IV secretion system, on the other hand, is more versatile and can be used for a variety of functions such as bacterial conjugation, DNA transfer, and pathogenesis [57,58,59,60].

Gram-negative bacteria with the type V secretion system include *Bordetella pertussis* and *Klebsiella pneumoniae*. This system is made up of a protein complex that spans the bacterium's inner membrane and is used to transport proteins across the outer membrane. A wide variety of proteins, including enzymes, toxins, and virulence factors, are secreted by the type V secretion system [61].

The type VI secretion system is a complex machinery found in a variety of Gram-negative bacteria, including *V. cholerae* and *P. aeruginosa*. Toxic effector proteins are delivered into target bacterial or eukaryotic cells using this system. The type VI secretion system plays several roles, including bacterial competition, biofilm formation, and virulence [62,63,64,65,66].

T3SS effector proteases:

The main purpose of T3SS secretion apparatus is to deliver its cargo proteins known as effectors to host cell. T3SS Effectors are the “worker” molecules of the whole T3SS system. It is known that the *Salmonella* genome encodes for a diverse array of effectors, with some strains containing more effectors than others. For example, the *Salmonella enterica* serovar Typhimurium strain SL1344 has been reported to encode for 39 effectors. Effectors are generally enzymes that targets host proteins to alter their function. The result of these alterations typically results in increased survivability of the pathogen on or in the host cell, which generally are achieved by the reduction of immune response or modification host cellular microenvironment [67,68,69,70].

The Type III Secretion System (T3SS) proteases effectors are crucial for the virulence of Gram-negative bacterial pathogens such as *Pseudomonas aeruginosa, Salmonella enterica*, and *Yersinia pestis*, as they can cleave specific host proteins [71]. Zinc metalloprotease T3SS effectors

can cleave host proteins involved in immune signaling and cytoskeletal rearrangement, among other functions [72]. The well-studied *P. aeruginosa* effector ExoS is an example of a bifunctional effector with ADP-ribosyltransferase and protease activity, which can cleave host proteins involved in cytoskeletal regulation and immune signaling to evade the host immune response [73]. The zinc metalloprotease activity of T3SS effectors relies on a conserved zinc-binding motif, HxxEH, which is found in the active site of the protease domain [74]. Six zinc metalloprotease T3SS effectors have been identified, including GogA, GtgA, and PipA from *Salmonella*, NleC and NleD from *Citrobacter rodentium*, and RipAX2 from *Ralstonia solanacearum* [75]

The cleavage of various subunits of the nuclear factor kappa B (NF-κB) transcription factor complex has been established with GogA, GtgA, PipA, and NleC [76]. Although these proteins have low sequence identity, they all cleave the N-terminal domain (NTD) of p65. NleC mainly cleaves p65 between residues Cys-38 and Glu-39, whereas GtgA, GogA, and PipA cleave between residues Gly-40 and Arg-41. Furthermore, GtgA, GogA, and PipA cleave RelB but not p105/p50 or p100/p52, whereas NleC cleaves cRel, RelB, and p50 [77]. The crystal structure of NleC shows that its catalytic core retains the structural topology of other members of the Zincin superfamily [78] However, the mechanism by which GtgA, GogA, PipA, and NleC recognize and cleave the NTD of NF-κB subunits is not fully understood.

NleD is another zinc-dependent protease that rapidly inactivates JNK and p38 by cleaving them in their TXY motif between the X and Y residues [79]. Unlike JNK and p38, ERK remains unaffected by NleD. Thus, by employing NleD, bacteria can inactivate p38 and JNK, which are required for the host immune response, without affecting the ERK-dependent host cell viability [80]. In EPEC infection, NleD cleavage and inactivation of the MAP kinases c-Jun amino-terminal

kinase (JNK) and p38 contribute modestly to the inhibition of IL-8 production and UV-induced apoptosis [81.

During EPEC infection, the type III secretion system (T3SS) effector EspL breaks down RHIM-containing proteins, including RIPK1, RIPK3, TRIF, and ZBP1/DAI [83]. A tripartite cysteine protease motif in EspL consisting of Cys47, His131, and Asp153 is required for this process, which cleaves within the RHIM of these proteins [84]. EspL expression leads to the rapid inactivation of RIPK1, RIPK3, TRIF, and ZBP1/DAI, inhibiting necroptosis and inflammatory signaling induced by TNF, LPS, or poly(I:C) [83].

Moreover, EPEC infection also inhibits the phosphorylation and plasma membrane localization of MLKL, induced by TNF. The cysteine protease activity of EspL contributes to persistent colonization of mice by the EPEC-like mouse pathogen *Citrobacter rodentium* in vivo. In Chapter 2 we investigated the intrabacterial activity of these protease effector and tried to understand the molecular basis of activity/inactivity of an effector inside the pathogen.

Intrabacterial activity of T3SS effectors

Although the role of T3SS effector has been recognized for some time, its function within the bacterium, other than during its infectious lifestyle, has remained unknown. T3SS effectors are thought to be linked to their chaperones until secreted [85]. The chaperone attachment allows the effector to maintain an unfolded configuration, which improves its adaptability for secretion via the T3SS apparatus. As a result, the chaperone bound T3SS effectors are rendered inactive within the pathogen. Our research group was the first to show that T3SS effectors are active within the pathogen and play a role in the pathogen's various stress resistance and survival strategies.

For example, the effectors NleB from *E. coli* and *C. rodentium*, as well as SseK from *S. enterica*, are glycosyltransferases that modify host protein substrates by attaching N-acetyl

glucosamine (GlcNAc) to arginine residues [86]. This glycosylation event disrupts the normal function of host immune response proteins, allowing the bacterium to survive within the host. During our mass spectrometry experiments aimed at identifying glycosylation substrates of NleB orthologs, we serendipitously discovered that the bacterial glutathione synthetase (GshB) is glycosylated by NleB on arginine residue R256 [87]. This discovery is particularly intriguing because GshB is essential for maintaining redox homeostasis in bacterial cells, particularly under oxidative stress conditions. GshB activity increased because of NleB-mediated glycosylation, resulting in increased production of glutathione, a critical antioxidant molecule that neutralizes reactive oxygen species. Furthermore, glycosylation of GshB increased *C. rodentium* survival under oxidative stress, emphasizing the importance of this modification in bacterial pathogenesis (Fig 2).

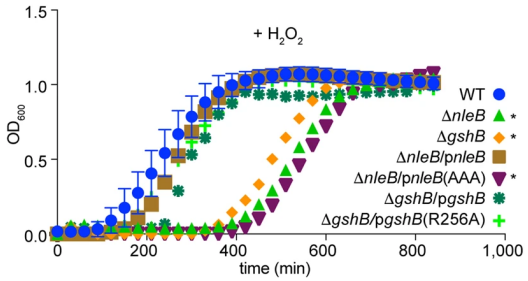

Figure 2 Intrabacterial activity of T3SS effector NleB.
C. rodentium growth assays. Quantification of *C. rodentium* growth (OD_{600}) as a function of time (min) in the presence of 2.4 mM H_2O_2.
Note. Reprinted from "An intra-bacterial activity for a T3SS effector", by El Qaidi, S., 2020 *Sci Rep* ***10****, 1073*. [87]

A second example of intrabacterial activity comes from our discovery of the potential activity of NleB orthologs within *Salmonella enterica*. Our group discovered that SseK1 and SseK3 can confer methylglyoxal resistance via intrabacterial activities. SseK1 can modify four proteins involved in methylglyoxal detoxification by glycosylating specific arginine residues:

GloA (R9), GloB (R190), GloC (R160), and YajL. (R149) [88]. SseK1 has been shown to increase the catalytic activity of these four proteins by Arg-glycosylation, providing a significant example of intra-bacterial activity of type three secretion system (T3SS) effector proteins. Our study revealed that SseK1 glycosylates specific arginine residues in two regulatory proteins, NagC and GlmR [89]. NagC glycosylation increases its DNA-binding affinity, which increases the expression of NagC-regulated genes *glmU* and *glmS*. Furthermore, glycosylation of GlmR by SseK1 improves its ability to enhance GlmS activity. We also found that NagC directly stimulates *glmR* expression. *Salmonella* lacking SseK1 produced significantly less UDP-GlcNAc than SseK1-expressing *Salmonella*. Our findings suggest that SseK1 regulates UDP-GlcNAc synthesis by increasing GlmS activity via GlmR glycosylation and augmenting NagC's DNA-binding activity. These regulatory activities could have evolved to ensure adequate UDP-GlcNAc levels for bacterial cell wall synthesis while SseK1 modifies bacterial and host targets, especially during infection and environmental changes.

In another recent study, conducted by Xue *et al.* found several bacterial proteins being glycosylated during infection. The two-component response regulator PhoP was found to be glycosylated at multiple arginine residues and resulted in significant reduction of the ability of PhoP to bind to DNA [90]. Arginine glycosylation of PhoP decreased the activity of PhoP and reduced the expression of its downstream genes. In Chapter 3 we identified yet another intrabacterial activity of the *Salmonella* T3SS effector SseK1.

T3SS effectors as target for antivirulence compound development

Antimicrobial Resistance (AMR) is a growing concern around the world and is regarded as one of the most pressing healthcare issues of our time. It is estimated that AMR infections cause millions of deaths each year (91). Gram-negative enteric pathogens are a major contributor to this

problem, with several common antibiotics losing efficacy against these pathogens due to the development of significant antimicrobial resistance (92). Enterobacteriaceae bacteria such as *E. coli, Salmonella, Shigella, Enterobacter,* and *Klebsiella* are particularly problematic, as they share many different classes of AMR elements (93).

Carbapenem-resistant Enterobacteriaceae (CRE) and ESBL-producing Enterobacteriaceae (ESBL) are particularly concerning, with the former classified as an "urgent threat" and the latter as a "serious threat" by the CDC (94). Carbapenems and colistin are considered last-resort antibiotics, but resistance to these antibiotics has also emerged in the last decade. β-lactamases and carbapenemases are becoming more common in *E. coli* isolates, and *mcr*, an emerging Antibiotic Resistance Gene (ARG) against colistin in Enterobacteriaceae, has been identified (95).

Antimicrobial resistance in enteric gram-negative pathogens is thought to be primarily due to horizontal gene transfer (HGT) (96). HGT can occur between a wide variety of bacteria, including those from different species and phylogenies, and is most accomplished through conjugative elements. With as many as 28 different plasmid types circulating within this bacterial group, the Enterobacteriaceae are especially vulnerable to resistance development via HGT. Antimicrobial resistance strategies developed for one species could potentially be applied to many other enteric gram-negative AMR pathogens.

However, the development of new antibiotics has been stagnated for a long time, owing primarily to the pharmaceutical industry's lack of profitability because bacterial infections are usually not chronic. Furthermore, the development of antimicrobials with mode of action like current ones- killing microbes indistinguishably, would almost certainly result in resistance development. As a result, developing antivirulence molecules that specifically target pathogen virulence factors has emerged as a promising strategy. This method lowers the risk of developing

other opportunistic infections, such as *C. difficile* infections, caused by the initial antibiotic disruption of commensal bacteria. Furthermore, there are several other situations in which traditional antibiotics may not be desirable, such as in the case of HUS (Hemolytic Uremic Syndromes), cross-reactivity with other antibiotics, and so on.

Our discovery of T3SS effector intrabacterial activity provided us with a unique opportunity to develop alternatives to traditional antimicrobials. We hypothesized that they are excellent targets for the development of novel antimicrobials because we now know that these effectors are active in pathogens and are required not only for their virulence within the host but also before they even contact the host cells. A high-throughput screening method was used to identify small molecules that inhibit NleB/SseK activity [97]. Following the screening, two compounds, 100066N and 102644N, were identified as effectively inhibiting the activities of NleB1, SseK1, and SseK2. When these compounds were added to mammalian cell cultures, they inhibited NleB1 mediated glycosylation of tumor necrosis factor receptor type 1-associated DEATH domain protein. Furthermore, these compounds inhibited *S. enterica* strain ATCC 14028 replications in mouse macrophage-like cells. Notably, the inhibitors were non-toxic to mammalian cells and showed no significant in vitro cross-reactivity with mammalian O-linked N-acetylglucosaminyltransferase.

In a related investigation, Zhu *et al.* undertook a study featuring the inhibitor YM155, revealing that sepantronium bromide effectively impedes the activity of *E. coli* NleB1, *Citrobacter rodentium* NleB, and both *Salmonella enterica* SseK1 and SseK2 [98]. Moreover, YM155 was demonstrated to be non-toxic to mammalian cells, and did not exhibit any cross-reactivity with the mammalian O-linked N-acetylglucosaminyltransferase (OGT). The compound demonstrated efficacy in reducing *Salmonella* survival in mouse macrophage-like cells, without concomitant

impact on bacterial growth rates. These findings suggest YM155 may represent a promising anti-virulence inhibitor. Adding to these findings, In Chapter 4 we investigated the potential repurposing of an FDA approved ACAT inhibitor -Avasimibe, to be used as an antivirulence compound.

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Chapter 2 - Intrabacterial Activity of T3SS Effector Proteases of *C. rodentium* .

I contributed to the figures 3-11 and tables. Figure 12,13,14,15 is contributed by NE Scott, U. Melbourne in Chapter 2. A modified version of this chapter including figures 3,4,5, and 6 is published in Pathogens (Hasan MK, El Qaidi S, & Hardwidge PR. (2021).

Hasan MK, El Qaidi S, Hardwidge PR. The T3SS Effector Protease NleC Is Active within *Citrobacter rodentium*. Pathogens. 2021 May 12;10(5):589. doi: 10.3390/pathogens10050589. PMID: 34065796; PMCID: PMC8151275.

Introduction:

Gram-negative bacteria interact with host cells and inject virulence factors (effectors) through type III secretion system (T3SS) machineries [1]. Many *Escherichia coli* and *Citrobacter rodentium* T3SS effectors inhibit pro-inflammatory pathways, including NleB [2], NleH [3], EspL [4], NleD [5], and NleE [6].

NleC is a zinc metalloprotease T3SS effector that cleaves the NF-κB p65 subunit and thus inhibits p65 nuclear translocation. This dampens the host innate immune response, particularly the production of inflammatory cytokines [7–11]. NleC proteolytic activity is lost upon mutagenesis of the consensus zinc metalloprotease motif $_{183}$HEIIH$_{187}$ [11]. Although NleC is secreted through the T3SS machinery, it also has the capacity to behave as a short-trip toxin and be internalized independently of the T3SS [12]. Other substrates of NleC other than p65 have been described and include the Rel family member p50 [8] and the acetyltransferase p300 [13]. It is thought that NleC specifically targets the NF-κB pathway instead of the MAPK pathway since it shows no activity against STAT or ERK [7,9].

Although two distinct p65 recognition and cleavage sites were initially described [7,11,14], it is now established that the cleavage site is C38/E39 [15,16]. NleC recognition of its substrates is also influenced by residues that are distant from the cleavage site [15,17].

The traditional view of T3SS effector secretion is that the effectors are inactive until injected into host cells, where they then fold into their active conformations and interact with host targets [18]. This is understood to be true since effectors are bound by chaperones before they are secreted [23-29]. However, our recent work has challenged this dogma. We discovered that the T3SS effector NleB is active not only within the host, but also within *C. rodentium*. NleB activity with *C. rodentium* results in glycosylation of the glutathione synthetase (GshB) to enhance GshB activity and cause increased production of glutathione, leading to protection from oxidative stress [19]. We also observed that the NleB ortholog SseK1 is active within *Salmonella enterica*, where it enhances methylglyoxal detoxification [20]. As these data suggested that T3SS effectors may be active within the bacterium, here we desired to investigate the potential intra-bacterial effector of effectors with a different enzymatic activity. *C. rodentium* T3SS effector proteases NleC, NleD, and EspL plays a significant role in during host pathogen interaction and are fairly similar in regard to their availability, host targets, and significance in pathogenesis in several gram-negative enteric pathogens. We choose these T3SS effector proteases as our next candidate effectors to investigate intrabacterial activity.

Materials and Method:

Strains and Molecular Cloning

Plasmids were constructed in pET15 and pET28 backgrounds by using ABC cloning [31]. p65 and NleC point mutants were created by using site-directed mutagenesis. *C. rodentium* Δ*nleC* and Δ*nleD* strains were constructed by using lambda red recombination [32] with pKD3 and pKD119

plasmids. A recombination cassette harboring a selectable marker flanked by ~50 bp of complementary sequences from the 5′ and 3′ ends of the gene to be deleted was created by using PCR with pKD3 as a template. The *nleC* recombination cassette was amplified by using forward (5′-GCTCCCATTGCTCCTAATCGTGCTGAAAATGCCTATGCGGATGAAATCTAACAATGCGCT) and reverse (5′-GCCAATCCAGGAAAATCATGTTGCTGGATAAATCCGTATCGGTCGAGGTGGCCCGGCTCC) primers. The *nleD* recombination cassette was amplified by using forward (5′-GCTCAATGTCAGATACAGATATCGAGTCTCTTGTAAAAGCAAGCCACTGGAGCACCTCAA) and reverse (5′-CGGGTAGGAGGTTCTACGGGGCATCCCAATCTCTTCGTGGACGGGGAGAGCCTGAGCAAA) primers. Plasmids were introduced into *C. rodentium* and *S. enterica* strains by using electroporation.

Western Blotting

Strains were grown in LB media with appropriate antibiotics (kanamycin and/or carbenicillin) for 4 h at 37 °C and then induced with 0.5 mM IPTG with growth for an additional 4 h at 30 °C. Cell lysates were derived from 1 mL of bacterial cultures via centrifugation and lysed in 100 µL SDS loading buffer by boiling for 5 min. Samples (10 µL) were used in Western blot assays with anti-His (1:1000 dilution; H1029, Sigma-Aldrich, St. Louis, SL, USA) and anti-FLAG (1:5000 dilution; F7425, Millipore, Burlington, USA) primary antibodies. IRDye 800CW goat-anti-mouse IgG (926-32210, LI-COR) and IRDye 680RD goat-anti-rabbit IgG (926-68071, LI-COR) secondary antibodies were used at 1:10,000 dilutions.

Edman Degradation

The C-terminal p65 cleavage product generated from WT *C. rodentium* was purified using standard His-tagged protein purification methods and processed according to the sample preparation guidelines for Edman degradation by the Protein Facility of the Iowa State University Office of Biotechnology.

RNA sequencing

RNA sequencing was done in K-State College of Veterinary Medicine RNA sequencing facility. Samples were grown in DMEM at 30ºC. Samples are prepared from 1.5 ml cultures grown anaerobically for 8 h and OD600 of 0.8. Estimated number of organisms ~ 10^9; with 100 µg total RNA; and an expected 30% yield amounting to 30 µg RNA. Samples were saved in 500 µl RNA*later* (Invitrogen) and supplied to the sequencing facility. 4 replicates of each sample were used to generate the RNA sequencing data. Galaxy Server (https://usegalaxy.org.au/root) was used to perform data analysis on the read counts. Lima-voom package in the Galaxy Servers was used to identify differentially expressed genes. Genes with higher than logFC of 2.0 and adjusted p-value< 0.05 was considered to be significantly differentially expressed genes.

HUNTER proteomics analysis sample preparation

Samples were resuspended in 200µl of 4% SDS, 50mM HEPES pH 8.0 by boiling for 10 minutes at 95ºC. Samples were then quantified by BCA and 40µl (equaling 200µg of samples) reduced/alkylated with DTT 10mM and IAA 50mM. Reduced/alkylated samples were then clean up using 2mg of SP3 beads washed with H20 three times. Samples were precipitated onto beads with 100% ethanol, final concentration 80%, by shaking for 25 minutes. Samples were then washed three times with 80% ethanol. Beads were resuspended in 60µl of 2M gdm, 100mM HEPES pH 8.0 and 10mM TCEP. Samples were then dimethylated with 30mM formaldehyde and

15mM sodium cyanoborohydride twice for one hour with additional formaldehyde and sodium cyanoborohydride added. Active NleC samples were labelled with light formaldehyde (+28Da) while inactive NleC *C. rodentium* samples were labelled with CD2O (+32Da). After 2 hours of labelling was quenched with Tris (final 600mM) overnight. Samples were combined and cleaned up again using SP3 with the addition of a further 1mg of beads (5mg beads final per replicate) by precipating on bead for 20 minutes with ethanol (80%final) and washing three times with 80% ethanol. Samples were then resuspend in 120µl of 200mM HEPES pH 8.0 and digested with 2.5µg of solu-trypsin (1:80 trypsin:protein) overnight. After overnight digestion 12µl (1/10 of the sample ~40µg) was taken and used for C18 clean up as input controls. 60µl (~200µg) of the remaining sample used for HUNTER N-terminal enrichment. 60µl of labelled sample was mixed with 50µl of 100% ethanol, 10µl of undecanol and 5µl of 1M sodium cyanoborohydride. pH was checked to be 7.0 and labelling allow to proceed for 3 hours. After 3hr samples were acidified with 400µl of 50% ethanol and 0.5% TFA (final concentration 50% ethanol, 0.5% TFA). Sample were pass through a conditioned tC18 SEPPAK and the flow through collected, then dried down. After drying the samples were then cleaned up using SDB-RPS columns.

LC-MS

Proteomic analysis of the pre-hunter and hunter enriched samples were undertaken on a Orbitrap 480. Pre-hunter samples were separated using a 95-minute analytical runs. 120k resolution MS1 events were followed by up to 3 seconds of MS2 HCD (30K, at least a AGC of 450% and a maximum fill time of 80ms). Data files were searched using Maxqunt (v2.0.2.0) using the *Escherichia coli* O157:H7 proteome (Uniprot: UP000002519, 5270 Proteins) allowing for demethylation of the N-termini and lysines as well as oxidation on Methionine, pyro-Q and pyro-E as variable modification. The resulting data files were then processed using Perseus to undertake

statical analysis and Pearson correlation analysis. The resulting glycopeptide data was plotted using ggplot in R.

Results:

NleC is active inside *Citrobacter rodentium*:

To investigate NleC activity inside *C. rodentium*, we cloned its known eukaryotic substrate p65 into a prokaryotic expression vector. Since the NleC cleavage site of p65 is near the N-terminus, we added a glutathione S-transferase (GST) tag to the N-terminus of p65 to increase the size of cleavage products. We also added a FLAG tag to the N-terminus to further aid detection of the cleavage product, as well as a C-terminal His-tag to aid with purification. Thus, the final recombinant p65 construct has a His-tag at the C-terminus and GST- and FLAG tags at the N-terminus. If cleavage by NleC occurs, the recombinant p65 protein is expected to be cleaved into two fragments: a 30 kDa N-terminal FLAG-tagged fragment and 26 kDa C-terminal His-tagged fragment (Figure 3A).

To investigate the NleC mediated cleavage of p65, p65 expression plasmids introduced into wild-type (WT) *C. rodentium*, or into deletion strains lacking NleC or NleD (Δ*nleC*), and (Δ*nleD*) strains. We observed that p65 was cleaved into the two expected fragments of appropriate molecular weights in the WT strain, but no such cleavage products were seen in the Δ*nleC* strain. Additionally, the same cleavage products were also seen in the Δ*nleD* strain, a strain lacking the T3SS effector NleD, a zinc metalloprotease that cleaves JNK [5] (Figure 3B). Thus, p65 cleavage within *C. rodentium* appears to be dependent upon NleC.

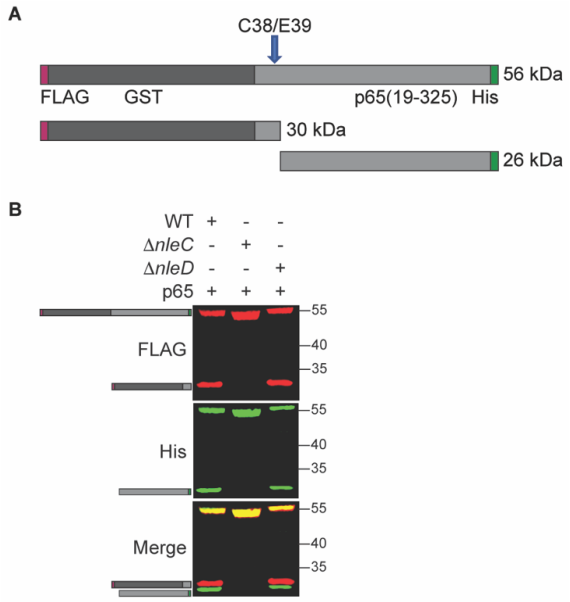

Figure 3 NleC cleaves p65 in *C. rodentium*.

(A). Schematic. p65 (residues 19-325) was cloned and expressed as a recombinant fusion to N-terminal FLAG (red)- and GST (grey)-tags and a C-terminal His-tag (green). The cleavage site is indicated with an arrow; the N-terminal and C-terminal cleavage products with epitope tags and their expected sizes are also shown. (B). p65 cleavage is dependent upon NleC. p65 was expressed in WT, Δ*nleC*, or Δ*nleD C. rodentium*. Protein lysates were analyzed using Western blotting. FLAG, His, and merged blot images are shown. Cartoons indicate the expected full-length and cleavage products as depicted in panel A.

NleC enzymatic activity is significantly reduced in the host cell when the critical NleC residue E184 around the Zn^{2+} binding pocket is mutated to A184 [7]. To further evaluate p65 cleavage specificity and its dependence upon NleC activity, we co-transformed Δ*nleC C. rodentium* with a p65 expression plasmid and either WT NleC or NleC E184A complementation plasmids. The recombinant p65 was cleaved by WT NleC but not by NleC E184A (Figure 4).

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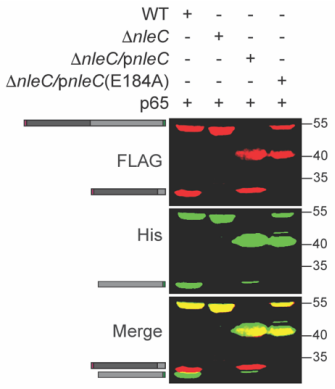


Figure 4 Inactive NleC does not cleave p65.

p65 was expressed in WT, *ΔnleC*, or *ΔnleC* complemented with either WT NleC or NleC E184A *C. rodentium*. Experiments were conducted as described in Figure 3 panel B. The 40 kDa protein band is the NleC protein produced via plasmid complementation.

p65 is cleaved by NleC between C38 and E39 [15]. To determine whether this cleavage specificity was retained within *C. rodentium*, we mutated the p65 expression plasmid to change C38/E39 to alanine residues. We expressed this plasmid in WT *C. rodentium* and observed that the p65 C38A, E39A mutant was no longer cleaved by NleC (Figure 5). In addition, we also purified the His-tagged (C-terminal) fragment of the cleavage product (Figure 5, lane 1) and performed Edman degradation analyses. The five N-terminal amino acids we identified corresponded to the expected sequence (EGRSA) of the C-terminal cleavage product (data not shown). Thus, we concluded that NleC is active within *C. rodentium*.

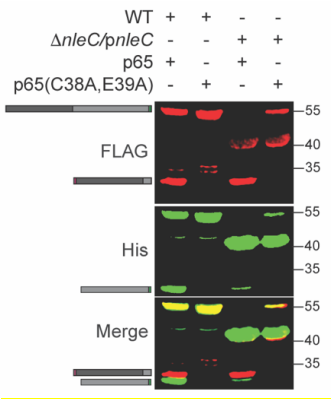


Figure 5 NleC does not cleave the p65 (C38A/E39A) mutant.
NleC does not cleave the p65 (C38A/E39A) mutant. WT and mutant p65 were expressed in WT *C. rodentium*. Experiments were conducted as described in Figure 3 panel B.

Salmonella enterica also induces p65 cleavage in host cells [21, 22]. We tested whether p65 is cleaved inside *S. enterica* in a similar fashion to *C. rodentium*. We expressed the recombinant WT and C38A/E39A p65 constructs in *S. enterica* and observed cleavage of only the WT p65 construct, similarly to what was observed for *C. rodentium* (Figure 6). *S. enterica* harbors at least three distinct T3SS effectors, GogA, PipA, and GtgA, that cleave p65 inside host cells [22]. At least one of these effectors may be active within *S. enterica*.

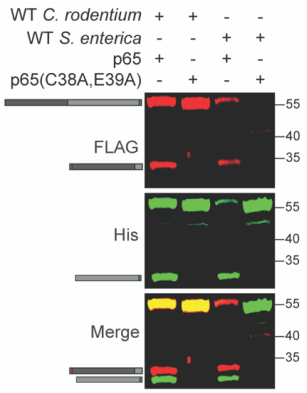


Figure 6 p65 cleavage occurs within *S. enterica*.
p65 cleavage occurs within *S. enterica*. WT and mutant p65 were expressed in WT *S. enterica*. Experiments were conducted as described in Figure 3 panel B.

NleD is not active inside *Citrobacter rodentium*:

After determining that NleC is active within the pathogen, we investigated whether NleD—another T3SS effector metalloprotease—is active or not. As previously described for the NleC intrabacterial activity test, we created a recombinant fusion protein of JNK (the eukaryotic target of NleD) and GST. Figure 7A depicts the construct. We used western blot technique to detect if JNK is cleaved inside *Citrobacter*. We observed no cleavage product of expected sizes (Fig 7B). Whereas p65 construct was cleaved and worked as a positive control for the experiment (Fig 7B lane 1).

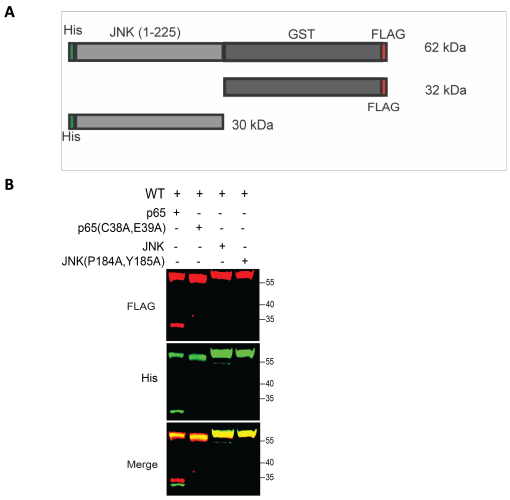


Figure 7 JNK is not cleaved inside *C. rodentium*.

(A). Schematic. JNK was cloned and expressed as a recombinant fusion to N-terminal FLAG (red)- and GST (grey)-tags and a C-terminal His-tag (green). The N-terminal and C-terminal cleavage products with epitope tags and their expected sizes are also shown. (B). JNK cleavage was not observed. p65 or p65 (C38A,E39A) and JNK or JNK (P184A, Y185A) was expressed in WT *C. rodentium*. Protein lysates were analyzed using Western blotting. FLAG, His, and merged blot images are shown.

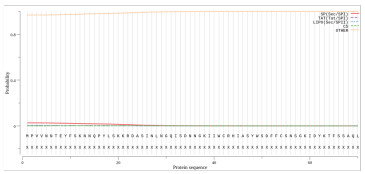
NleD Signal peptide prevents cleavage of its target JNK:

The traditional understanding of T3SS effector secretion is that the effectors are bound with chaperone after translation and thus remain inactive until translocated. To understand the possible mechanisms for the selective activity of different effectors, we hypothesized that the presence of a chaperone binding domain inside the bacteria is an indicator of effector activity or inactivity. To identify potential chaperone binding domains of the effectors, we used a computational analysis tool SignalP to analyze their amino acids sequences. Our findings show that only NleD has a predicted signal peptide sequence at the N-terminus of all three proteases (Figure 8). Interestingly,

one our best characterized T3SS effector with intrabacterial activity -NleB also had no predicted Signal peptide sequence at its N-terminus.

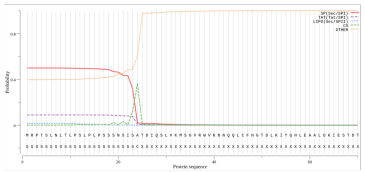
EspL

Protein type	Signal peptide (Sec/SPI)	TAT signal peptide (Tat/SPI)	Lipoprotein signal peptide (Sec/SPII)	Other
Likelihood	0.0263	0.0017	0.004	0.968



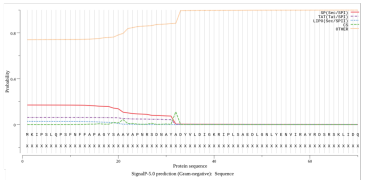
NleD

Protein type	Signal peptide (Sec/SPI)	TAT signal peptide (Tat/SPI)	Lipoprotein signal peptide (Sec/SPII)	Other
Likelihood	**0.4951**	0.0887	0.0145	0.4017



NleC

Protein type	Signal peptide (Sec/SPI)	TAT signal peptide (Tat/SPI)	Lipoprotein signal peptide (Sec/SPII)	Other
Likelihood	0.1691	0.0609	0.0264	0.7436



NleB

Protein type	Signal peptide (Sec/SPI)	TAT signal peptide (Tat/SPI)	Lipoprotein signal peptide (Sec/SPII)	Other
Likelihood	0.0156	0.0046	0.003	0.9768

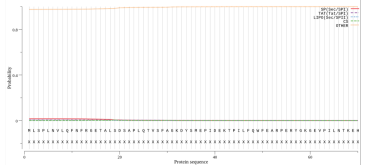


Figure 8 Signal peptides prediction of the T3SS effectors tested for intrabacterial activity.
SignalP 5.0 webtool was used to predict the presence of N-terminal signal peptides in the T3SS effectors under investigation. Among all the effectors analyzed, only NleD has a predicted Signal Peptide.

To test our hypothesis, we created a recombinant version of NleD without its N-terminal Signal Peptide and co-expressed it with the recombinant JNK construct to check for the presence of cleavage products. Our results show that NleD without the Signal Peptide does cleave the recombinant JNK. Though the efficiency of cleavage is lower than NleC mediated cleavage of its target recombinant P65 (Fig 9).

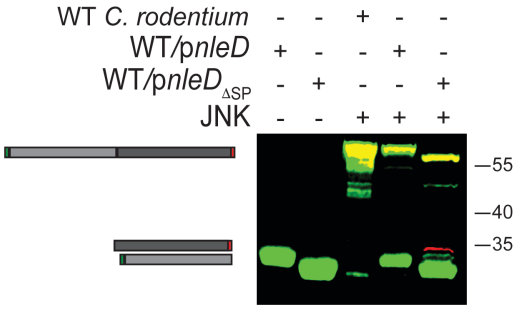


Figure 9 NleD without N terminal Signal peptide cleaves JNK.
JNK cleavage was observed with NleD without signal peptide. JNK was co-expressed with WT NleD or NleD w/o SP in WT *C. rodentium*. Protein lysates were analyzed using Western blotting. FLAG, His, and merged blot images are shown.

To further validate our results, we also created a recombinant version of NleC with the NleD signal peptide grafted at its N-terminal. We then co-expressed the recombinant Signal Peptide grafted NleC with the recombinant P65 into the *ΔnleC Citrobacter* and cheeked for the cleavage. Matching with our hypothesis, when NleD signal peptide was grafted in the N terminal of NleC, NleC failed to cleave P65.

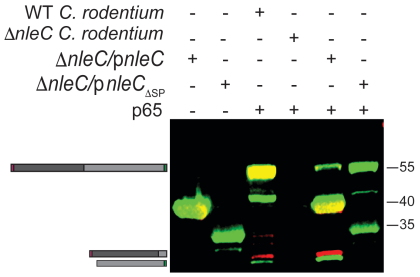


Figure 10 Substrate cleavage assay of recombinant NleC with NleD signal peptide.
p65 was expressed in WT, *ΔnleC C. rodentium* complemented with either WT *nleC* or *nleC* with Signal Peptide. Experiments were conducted as described in Figure 3 panel B.

EspL is active inside *Citrobacter rodentium*:

Because our data indicates that an effector lacking a signal peptide has the potential to be active within the pathogen, we investigated whether EspL, which lacked a predicted signal peptide, is active within the pathogen. We investigated EspL intrabacterial activity using a method similar to that described previously for testing NleC and NleD intrabacterial activity. EspL has two eukaryotic targets known as RIPK1 and RIPK3. We engineered recombinant RIPK1 and RIPK3 with His tags on both ends (Fig 11A and B). We decided against using a GST tag because the proteins are quite large. We then expressed the recombinant targets in WT *Citrobacter* for cleavage assay. Our findings indicate that RIPK1 is cleaved within *Citrobacter* (Fig 11C). We were unable to confirm whether RIPK3 is cleaved inside the pathogen because the size difference between cleaved and un-cleaved products was too small to detect in our assay. The presence of a putative signal peptide at the N terminal end of an effector may indicate its potential intrabacterial activity, according to these finding.

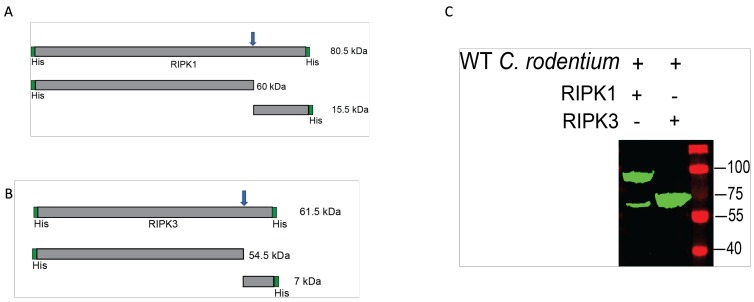


Figure 11 EspL cleaves its target RIPK1.
(A). Schematic. RIPK1 and (B) RIPK3 was cloned and expressed as a recombinant fusion to a N-terminal and a C-terminal His-tag (green). The cleavage site is indicated with an arrow; the N-terminal and C-terminal cleavage products with epitope tags and their expected sizes are also shown. (C). RIPK1 cleavage is observed in WT *C. rodentium*. Protein lysates were analyzed using Western blotting.

Identification of bacterial targets of NleC:

Because NleC is active within the pathogen, we wanted to identify any bacterial targets of NleC. We used a variety of methods to accomplish this. To begin, we used RNA sequencing to identify potential NleC targets. Because the best characterized target of NleC p65 is a transcription factor and recent studies also indicates NleC mimics the double strand of DNA to interact with its target, we hypothesize that the bacterial targets of NleC could also be transcription factors. We hypothesize that if NleC target is a transcription factor, we can identify it by comparing WT and *ΔnleC C. rodentium* transcripts using RNA sequencing experiments. According to the results of our RNA seq experiments, there are two transcripts that are upregulated in the *ΔnleC C. rodentium* and 9 that are downregulated.

GeneID	Gene name	Description	logFC	AveExpr	adj.P.Val
RS17380		DUF1778 domain-containing protein	-2.318773855	6.407647279	0.0005
RS16815	*Cts2U*	DotU component of T6SS	-2.310621098	3.762563376	0.0399
RS18065		phage tail protein R	2.090410533	2.552012306	0.0367
RS20445		hypothetical protein	2.162133988	3.091500921	0.0231
RS10180		Putative DNA binding protein	2.505401856	5.490252101	0.0036
RS02330	*ribH*	Riboflavin synthase Beta chain	2.535322403	4.013212298	0.0048
RS04625		phage tail assembly protein	2.56692898	3.122079376	0.0256
RS23275	*lys*	Phage lysozyme	2.607882754	3.114558025	0.0392
RS11205	*mdtQ*	multidrug resistance outer membrane protein	2.753979525	3.855342129	0.0161
RS08205	*nleC*	type III secretion system effector zinc metalloprotease NleC	3.67815324	7.864438193	1.37E-05

RS27450	*nleB* (pseudo)	non-LEE encoded effector protein NleB	4.083279338	5.105385898	0.0038

Table 1 Differentially expressed genes identified by RNA sequencing.

Among the detected downregulated genes with the lowest adjusted p-value was *nleC*. The *nleB* pseudogene had the greatest fold change. We ended up choosing genes with an adjusted p-value less than 0.05 and a LogFC greater than 2.0. Table 1 lists the genes that meet those criteria. The heatmap and MD plot of the top ten differentially expressed genes between the WT and *C. rodentium ΔnleC* are shown in Fig 12. *mdtQ* and *ribH* are two other genes that are downregulated in *ΔnleC Citrobacter*. MdtQ is a potential efflux pump for multidrug resistance. Furthermore, MdtQ amino acid sequence analysis indicates that it is a Rnd efflux pump involved in increased drug resistance to puromycin, acriflavine, and tetraphenylarsonium chloride [33]. The gene *ribH* encodes lumazine synthase, an enzyme in the riboflavin biosynthesis pathway. Riboflavin, also known as Vitamin B2, is a biochemical precursor for the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), both of which are involved in redox reactions in all organisms. In addition to these two downregulated genes, we discovered the prophage gene *lys* to be downregulated in *C. rodentium ΔnleC*. Lys is a prophage-encoded lysozyme that promotes viral release by lysis of host cells or facilitates infection. Not surprisingly, the majority of the highly differentially expressed genes were of prophage origin and T6SS components. Given that NleC is a prophage encoded effector protease, it is possible that NleC is involved in the regulation of genes of prophage origin.

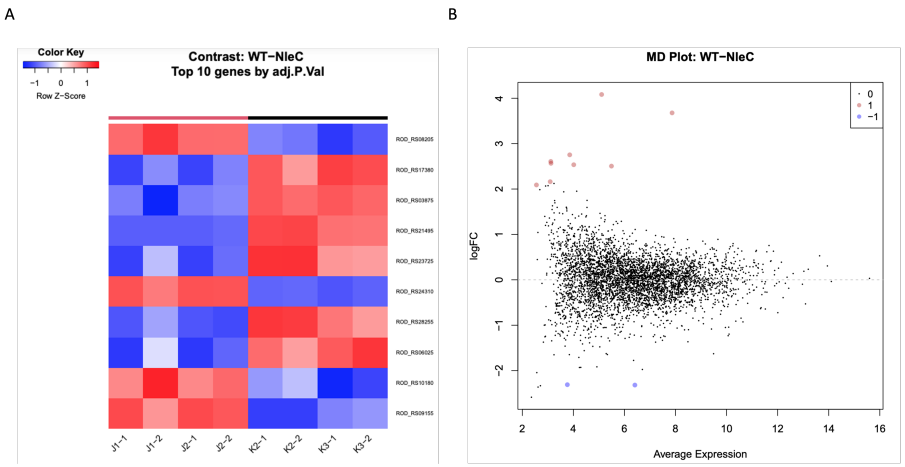


Figure 12 Heatmap and MD plot of RNA sequencing differentially expressed genes.
(A) Heatmap of normalized read counts of the top 10 differentially expressed genes detected by Limma-voom analysis ranked by their adjusted p value. Left 4 samples are WT replicates. Right 4 samples are replicates of *ΔnleC C. rodentium.* Red represents higher expression and blue represents lower expression. (B) MD-plot of significantly (Padj < 0.05 and fold change > 2) up-regulated (red) and downregulated (blue) genes in WT vs. Δ*nleC C. rodentium*. Red represents upregulated in WT and blue represents downregulated genes in WT. Black represents (NS) expressed genes.

Following that, we used a cutting-edge proteomics-based analysis to identify potential NleC cleavage products. We used a HUNTER (High-efficiency Undecanal-based N-Termini EnRichment) proteomic assay to detect cleavage products of NleC. HUNTER is a sensitive, reproducible, and automated method for identifying and quantifying N termini in microscale samples such as cells, tissues, and liquid biopsies using mass spectrometry. During HUNTER analysis, neo-formed N-termini are selectively enriched and identified as potential protease substrates by mass-spec analysis based on protease cleavage sites [30].

Prior to HUNTER analysis, a standard mass-spectrometry analysis of the samples was performed to validate sample quality. The samples are pre-HUNTER analyzed to establish a baseline proteomics against which the HUNTER analysis results can be compared. Furthermore, Pre-HUNTER analysis can detect differential levels of proteins in the sample if they exist. This

also allows for a baseline correction of the HUNTER analysis. *C. rodentium* Δ*nleC* expressing either active NleC or inactive NleC (E184A) grown in the same conditions where p65 cleavage was observed was used to collect sample after 8 Hours of growth. The samples were then lysed, and proteins of the cytoplasmic fractions were precipitated. Samples were sent to Dr. Nicholas Scott at Melbourne University, Australia for HUNTER analysis. After determining that the pre-HUNTER analysis of the sample proteomics was of acceptable quality, we proceeded with the HUNTER analysis of the samples. Pearson correlation provides a "bird's-eye view" of the datasets and how similar they are to one another. Figure 13 shows that the samples had good correlation.

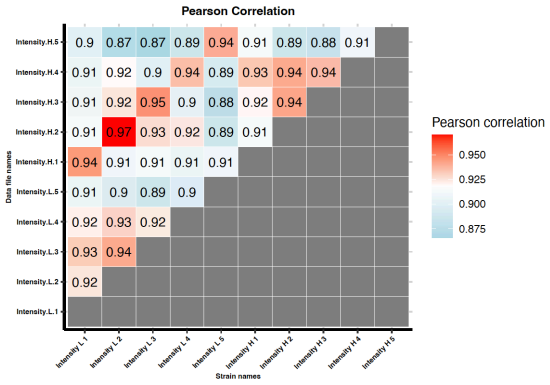


Figure 13 Pearson correlation of the samples used in the HUNTER assay.
Correlation is generally good across samples, no clear clustering of WT vs *ΔnleC C. rodentium*.

Our Pre-HUNTER analysis reveals a few peptides that are differentially enriched between the samples. For example, the N-termini peptide of the outer membrane porin protein OmpF is found to be highly enriched in samples expressing active NleC as opposed to inactive NleC (Fig 14).

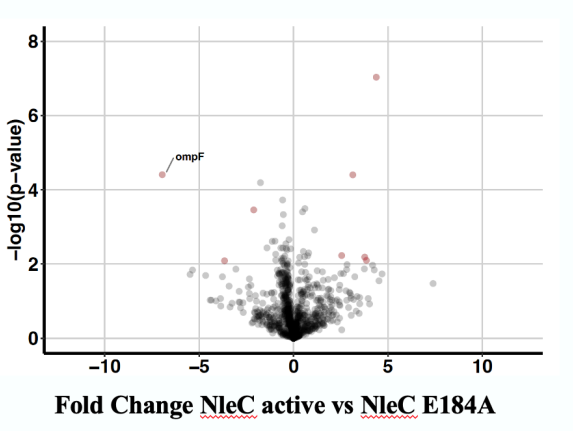


Figure 14 Pre-HUNTER proteomics analysis of the samples.
Pre-HUNTER analysis of the samples shows overall consistent level of identified proteins and a few proteins with differential level of availability. OmpF is highlighted.

The HUNTER analysis results show that the samples have differential level of N-termini peptides availability. Table 2 lists the N-termini that are differentially available between the samples. SecA, ClpB, and Tig (Trigger Factor) were among the most differentially enriched N-termini. SecA, ClpB, and Trigger Factors are chaperone proteins that are primarily responsible for high molecular weight protein folding and integration in membrane. These findings are significant because they suggest that NleC may have bacterial targets involved in cellular protein translation and translocation pathways.

N: Student's T-test Difference pNleC_vs_pNleC_E184A	N: Student's T-test Test statistic pNleC_vs_pNleC_E184A	T: Sequence	T: Gene Names	T: Protein Names
11.1022	8.3848	YAQKDPKQEYKR	*secA*	Protein translocase subunit SecA
10.9497	7.30119	YVGYEEGGYLTEAVR	*clpB*	Chaperone protein ClpB
7.81416	5.93926	FGEVDILVNNAGITR	*fabG*	

7.55409	5.91941	LSGVPAAGDEVTVVR	*infB*	Translation initiation factor IF-2
8.04391	5.75461	YMQKEIYEQPNAIKNTLTGR	*glmS*	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]
7.49199	5.66194	YNFEDSILVSER	*rpoB*	DNA-directed RNA polymerase subunit beta
8.81237	5.62113	YQPQDATTNPSLILNAAQIPEYR	*talB*	Transaldolase
8.58109	5.61761	FIDTPGAYPGVGAEER	*accA*	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha
7.73231	5.30992	YAEEGIGLAATQVDIHQR	*def*	Peptide deformylase
7.27556	5.29994	YSHGFNIVEVGEQIR	*ilvC*	Ketol-acid reductoisomerase
6.19979	5.28713	IVQLEDGVQISSGDTLAR	*rpoC*	DNA-directed RNA polymerase subunit beta'
8.02004	5.27837	IVYFDLDKYDIR	*pal*	
6.65077	5.27516	FLTDPDSVDANWR	*sucA*	
7.5673	5.24619	AYSSGKPAIGVGAGNTPVVIDETADIKR	*adhE*	Aldehyde-alcohol dehydrogenase
6.98314	4.94523	HAVTEASPMVKAKDER	*rpsF*	30S ribosomal protein S6
7.29185	4.87568	LGQFNLDGINPAPR	*dnaK*	Chaperone protein DnaK
7.54308	4.83007	YVVDTSDFGNSGNWR	*ydgH*	
7.69868	4.82123	VTDPASIESVLEKIR	*fabG*	
12.0502	4.80187	FTGSVDGEEFEGGKASDFVLAMGQGR	*tig*	Trigger factor
6.45483	4.78795	LTGLEGEQLGIVSLR	*infC*	Translation initiation factor IF-3
6.93462	4.76005	LNNAPADSWR	*yhgF*	
7.32726	4.7295	MDSSFTPIEQMLKFR	*mprA*	
6.32511	4.71618	AIVDTNSDPDGVDFVIPGNDDAIR	*rpsB*	30S ribosomal protein S2
7.79708	4.58159	WCDLLEENSVDAVKVR	*rpoC*	DNA-directed RNA polymerase subunit beta'

7.75021	4.54465	YSSGKPAIGVGAGNTPVVIDE TADIKR	*adhE*	Aldehyde-alcohol dehydrogenase

Table 2 List of differentially enriched peptides from HUNTER analysis.

The volcano plot image of the HUNTER analysis trends shows that expression of the active NleC significantly increases the amount of detected neo-N-termini indicating that several *C. rodentium* proteins are cleaved by NleC (Fig 15). Among the detected Neo-N terminals several of them begin at either N terminal Methionine or the 2nd amino acid from the N terminal. We decided that these could not be the potential targets of NleC since the cleavage site would be too close to N-terminal and removed from our Table of potential NleC targets (Table 2).

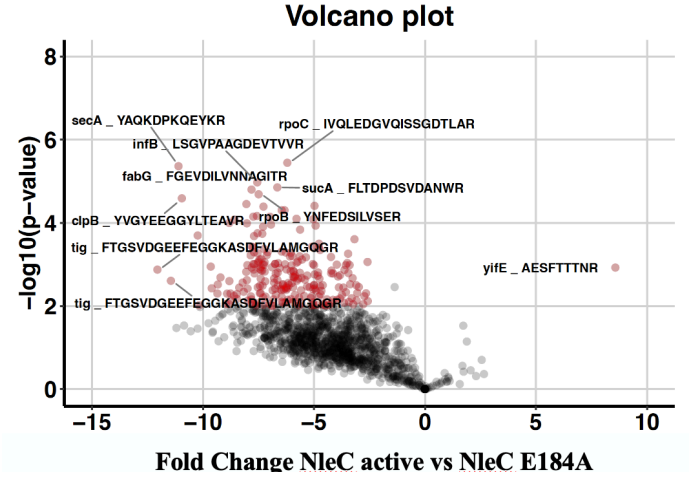


Figure 15 Volcano Plot of the HUNTER analysis results.

HUNTER analysis of the samples shows more abundant peptides detected in samples expressing active NleC. A few of the most abundant peptides are highlighted.

Discussion:

We have discovered the potential activity of several T3SS effectors. In this section of the study, we investigated the protease effectors of *C. rodentium* for their activity inside the pathogen. Additionally, we explored the potential role of NleC in *C. rodentium* by RNA-seq analysis. To evaluate the protease activity of NleC in *C. rodentium* we used HUNTER proteomics analysis to detect NleC mediated cleavage events in *C. rodentium*.

NleC is a zinc metalloprotease that cleaves the NF-kB p65 subunit and dampens the immune response of the host cell. Despite the traditional view of T3SS effectors being active only after their translocation into the host, here we demonstrated that the *C. rodentium* T3SS effector NleC and EspL is active inside the bacterium. The implication of this novel finding is far-reaching. As demonstrated by our previous finding of *C. rodentium* T3SS effector NleB glycosylating the bacterial substrate GshB and therefore contributing to the increased survival of *C. rodentium* under environmental stress [19] NleC and EspL may have their own endogenous bacterial targets. Compared with Locus of Enterocyte Effacement (LEE)-encoded effectors, non-LEE-encoded effectors are potentially more recent acquisitions to the pathogens and their roles as effectors are still evolving [21]. This indicates that non-LEE effectors potentially have a dual role in modulating both pathogen and host cellular pathways.

To our knowledge, no chaperone for NleC has been identified, indicating that NleC might not be chaperoned before its secretion, potentially explaining its activity inside the bacteria. Similarly, no chaperone has been identified for NleB1 [19]. The potential intra-bacterial targets of NleC are currently unknown, and this absence of knowledge potentially limits the biological impact of our findings.

The role of Signal peptide in NleD inactivity is intriguing. Just like NleC, no NleD chaperone has been identified either. However, the presence of Signal Peptide as a determinant of intrabacterial activity necessitates further investigation of the phenomenon. One possible explanation is that the identified signal peptide sequences have intrinsic properties that disrupt the 3D structure of the effectors, rendering them inactive. Indeed, several T3SS effectors has to be purified without their signal peptide so they could be folded properly before getting crystal structures.

The identification of bacterial targets of effector proteases is critical for understanding the exact role that they play in bacteria. Our efforts to determine the role of NleC in the pathogen have revealed a plethora of potential pathogen pathways that could be altered by NleC. NleC significantly upregulates/downregulates several genes in *C. rodentium*, according to our RNA-seq analysis. We discovered that a large number of differentially expressed genes are of prophage origin. Because they are related to bacterial virulence, these prophage-borne genes may play an important role in *C. rodentium* pathogenesis and virulence. Many pathogens' physiological fitness during environmental stress is also influenced by prophage genes. Thus, NleC's alternative expression of these genes may suggest a role for NleC in the regulation of *C. rodentium* virulence and physiological fitness.

The HUNTER proteomics assay was used to identify peptides that are more abundant in enzymatically active NleC expressing bacteria versus inactive NleC expressing bacteria. Several peptides are significantly more abundant in samples expressing active NleC, according to our findings. These proteins could be NleC's direct proteolytic targets, or they could be differentially cleaved by other protease/proteases that are regulated in some way by NleC. More research is needed to determine how NleC mediates the differential enrichment of certain groups of peptides.

Surprisingly, the most differentially enriched peptides in NleC-expressing samples are mostly from translation machines and chaperones. This suggests that NleC may be involved in fundamental cellular processes such as membrane protein translation and translocation. Given that NleC is a phage protein, it has a high potential to subvert its host cellular machineries. One intriguing finding from our HUNTER analysis is that we detected a significantly higher amount of OmpF protein in samples expressing active NleC in Pre-HUNTER analysis. This finding suggests that NleC may play a role in the expression or modulation of major outer membrane porins. This could be accomplished by directly targeting the OmpF transcriptional regulatory pathway, such as OmpR, Lrp, or CpxR. Another possibility is NleC expression causes an increase in OmpF accumulation in the cytosolic fraction of prepared sample. Indeed, a study to determine the role of chaperone proteins in cytosolic protein aggregation discovered a link between Trigger factor expression and OmpF accumulation in the cytosolic fraction of samples [34]. Tig is one of the highly enriched peptides detected in our HUNTER assay. This could imply that the observed increased expression of OmpF is caused by a difference in Trigger factor levels. Even though our preliminary HUNTER analysis suggests that NleC may be involved in cell translation and chaperone pathways, further validation of the findings is required by individually testing the identified proteins for cleavage by NleC.

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Chapter 3 - A Novel Intrabacterial Activity of T3SS Effector Glycosyltransferase in *Salmonella*

I contributed to all the tables and figures, except for Fig 17A, 17B which is contributed by NE Scott, U. Melbourne and Figure 20B which is contributed by SE Qaidi, KState in Chapter 3. A modified version of this chapter is submitted for publication in Scientific Reports.

Introduction:

Salmonella is responsible for around 1.35 million infections in the USA each year [1]. Identifying virulence factor mechanisms involved in pathogenesis and environmental persistence is essential to find better approaches to reduce *Salmonella* dissemination and infection. *Salmonella* uses a highly specialized secretion system named Type Three Secretion System (T3SS) to inject effector proteins inside the host cells [2,3,4]. These effector proteins, once inside the host cell, targets different cellular pathways to modify host immune response to better accommodate the pathogens infectious lifestyle. *Salmonella* T3S effector SseK and its ortholog NleB targets host cell immune response pathways to reduce host inflammatory response [5]. SseK and NleB are glycosyltransferase that target several host proteins and interfere with their physiological function [6,7,8]. For example, SseK1 modifies the death domain containing protein TRADD with N acetylglucosamine (GlcNAc) on specific arginine residues [9,10]. This modification in turn disrupts the death domain interactions leading to disruption of TNF alpha complex assembly and resulting in reduced TNF signaling. Besides death domain containing proteins, NelB1, an SseK1 ortholog, modifies arginine residues of GAPDH [11]. Arginine glycosylation of GAPDH prevents its interaction with TRAF2 and induction of NFkB signaling, leading to reduced proinflammatory response by host cell.

In addition to their known host targets, our group and others have recently demonstrated that NleB/SseK orthologs also target bacterial proteins. For example, the *Citrobacter rodentium* effector NleB Arg-glycosylates the glutathione synthase GshB, leading to enhanced glutathione synthase catalytic activity and consequently increased resistance to oxidative stress [12]. *Salmonella* T3S effector SseK1 also display an intrabacterial activity which plays a significant role in methylglyoxal detoxification by glycosylating GloA, GloB, GloC, and YajL proteins in this pathway [13]. Additionally, our latest study on SseK1 intrabacterial activity demonstrates that SseK1 upregulates UDP-GlcNAc synthesis by glycosylating NagC and GlmR [14].

Two-component response regulators are key cellular components used by bacteria to sense and respond accordingly to the surrounding environment [15,16,17]. Two-component systems are comprised of a membrane bound kinase and a corresponding response regulator that exerts the effect of the external stimuli typically by regulating transcription of target genes [18]. It was recently described that SseK3-mediated Arg-glycosylation also plays an important role in modulating the DNA-binding activity of *Salmonella* PhoP, a two-component response regulator. Another critical two component response regulator of *Salmonella* is the EnvZ-OmpR system. The EnvZ-OmpR system is historically known for its regulatory effects on the major outer membrane porins OmpF and OmpC in response to extracellular pH and osmolarity change [20,21,22]. With increasing osmolarity, EnvZ phosphorylates the response regulator protein OmpR. Once phosphorylated OmpR's binding affinity to target promoters increases to consequently upregulate target genes transcription [23,24]. Additionally, OmpR can also non-canonically regulate transcription of its target genes under its non-phosphorylated state [21]. Besides regulating several stress related genes [25-30], OmpR also regulates expression of effector genes within *Salmonella*

Pathogenicity Island 2 (SPI2) which is especially relevant to the intracellular lifestyle adaptation of *Salmonella* [24,31-35].

In our prior effort to identify novel host target of effector glycosyltransferase, we came across several putative SseK/NleB bacterial targets. Among those was the Two-component response regulator OmpR. Thus, we investigated whether OmpR is a target of *Salmonella* T3SS effector SseK paralogs. We found that OmpR is glycosylated by SseK1. Glycosylation of OmpR leads to decreased expression of its target gene *ompF*, presumably by reducing the binding affinity of OmpR to the *ompF* promoter region. We also found that whereas a *Salmonella ΔompR* mutant has a significant growth defect in bile salts and has a reduced capacity to form biofilms, a *Salmonella ΔsseK1* mutant has the opposite phenotype, indicating an overall repression of OmpR transcriptional activity through SseK1 mediated- glycosylation.

Materials and Methods:

Plasmids, strains, and cloning:

The plasmids and strains used in this study are listed in Tables 1 and 2, respectively. Wild type *sseK1* (*Salmonella enterica*) and its derivative H244A E255A N256A, were cloned into pET42a using ABC cloning [36]. *ompR* and *qseF* were cloned in pTac using ABC cloning [36]. *ompR* and *sseK1/ompR* deletions were constructed using lambda red recombination with the pKD3 and pKD119 plasmids [37]. Mutants were screened on LB medium supplemented with 10 µg/ml chloramphenicol and mutations were confirmed by PCR and DNA sequencing. Protein purification was performed as described previously [12]. For the purification of glycosylated OmpR, His-tagged OmpR was co-expressed (or not) with FLAG-tagged SseK1 and His -tagged proteins were purified as described previously [13].

Glycosyltransferase Assay:

In vitro glycosylation assays were carried out as previously described[7]. 200 nM SseK1 or SseK1 HEN were incubated in 50 mM Tris-HCl buffer pH 7.4, 1 mM UDP-GlcNAc, 10 mM MnCl2, and 1 mM DTT with 1 mM OmpR. After 2 hours of incubation at room temperature, samples were blotted with anti-R-GlcNAc and anti-His tag monoclonal antibodies (Abcam, Cambridge, MA, USA). Western blot images were captured in a LI-COR (LI-COR Biosciences, Lincoln, NY, USA) imager.

Digest of gel-separated proteins:

Affinity-purified proteins were separated by SDS-PAGE, fixed, and then visualized with Coomassie staining. Bands of interest were excised and Coomassie staining removed by destaining with 50 mM NH_4HCO_3, 50 % ethanol for 20 minutes at room temperature with shaking at 750

rpm. Destained samples then dehydrated with 100 % ethanol, before being reduced by being rehydrated with 10mM DTT in 50 mM NH_4HCO_3. Samples were reduced for 1 h at 56 °C with shaking and then washed twice in 100 % ethanol for 10 minutes to remove DTT. Reduced dehydrated gel bands were then rehydrated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 and allowed to alkylate in the dark for 45 minutes at room temperature. Alkylation buffer was removed, and the gel samples washed with 50 mM NH_4HCO_3, followed by two rounds of 100 % ethanol before being vacuum dried. Alkylated samples were then rehydrated with 20 ng/µl of trypsin (Promega) in 40 mM NH_4HCO_3 at 4 °C for 1 h. Excess protease was removed, gel pieces were covered in 40 mM NH_4HCO_3 and incubated overnight at 37 °C. Peptides were collected, desalted using homemade R3/C18 stage tips as previously described [40] before analysis by LC-MS.

Reverse phase LC-MS/MS:

Peptide samples were resuspended in Buffer A* (2 % MeCN, 0.1 % TFA) and separated using a two-column chromatography set on a a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific). Samples were first concentrated on a PepMap100 C18 20 mm x 75 µm trap at 5 µl/min for 5 minutes with Buffer A (0.1 % formic acid, 2 % DMSO) and then separated on a PepMap C18 500 mm x 75 µm analytical column (Thermo Fisher Scientific). Separated peptide were infused into a Orbitrap Eclipse Mass Spectrometer (Thermo Fisher Scientific) at 300 nl/minute for 65-minutes by altering the buffer composition from 2 % Buffer B (0.1 % formic acid, 77.9 % acetonitrile, 2 % DMSO) to 28 % B over 35 minutes, then from 28 % B to 4 % B over 10 minutes, then from 40 % B to 80 % B over 5 minutes. The composition was held at 100 % B for 5 min, and then dropped to 2 % B over 1 min before being held at 2 % B for another 9 min. The Eclipse Mass Spectrometer was operated in a data-dependent mode, acquiring one full precursor scan (resolution

120,000; 375-2000 m/z, AGC target of 1×106) followed by up to 3 seconds of data-dependent HCD MS-MS events (using three collision energies of 25, 30, and 35; resolution 15k AGC target of 250% with a maximum injection time of 22 ms).

Mass spectrometry data analysis:

Identification of Arg-glycosylation events was accomplished using MaxQuant (v1.6.17.0) [41]. The predicted amino acid sequences for OmpR were combined into a database with the *Escherichia coli* K12 proteome (Uniprot accession: UP000000625) the *Salmonella Typhimurium* SL1344 OmpR-his sequence and searched, allowing carbamidomethylation of cysteine set as a fixed modification and the variable modifications of oxidation of methionine and Arg-GlcNAcylation (H13C8NO5; 203.0793 Da to Arginine). Searches were performed with Trypsin cleavage specificity, allowing 2 mis cleavage events with a maximum false discovery rate (FDR) of 1.0 % set for protein and peptide identifications. The resulting modified peptide output was processed within the Perseus (v1.4.0.6) [42] analysis environment to remove reverse matches and common protein contaminants. To ensure high data quality assigned glycopeptides were manually assessed and the HCD spectra assigned to each unique glycopeptide annotated with the Interactive Peptide Spectral Annotator [43] (http://www.interactivepeptidespectralannotator.com/PeptideAnnotator.html).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [44] partner repository with the dataset identifier PXD039412.

mRFP assay:

A low-copy number plasmid (pHG165) carrying *ompF* promoter transcriptional fusions to mRFP (monomeric red fluorescent protein) was electroporated into *Salmonella enterica* serovar Typhimurium. Two hundred µl of LB media with Carbenicillin was used to grow the transformed bacteria in 96 well clear bottom black walled assay plates. mRFP expression levels were measured every 20 minutes of growth by a synergy H1 microplate reader at an excitation and emission wavelength of 584 nm and 607 nm, respectively. mRFP data were presented as an average of RFU (Relative Fluorescence Units)/OD_{600} ratio.

EMSAs:

A 5' Alexa-fluor labeled DNA corresponding to the *Salmonella ompF* promoter region was amplified by PCR from *Salmonella* gDNA using the oligonucleotides: 5' Alexa-fluor - tttttacgtcacactcaaggccagctatgctg-3' and 5'- ttattaccctcattggtttttttatatgac-3'as forward and reverse primers respectively. Two nanomoles of purified PCR product were incubated for 10 min at room temperature with either OmpR or OmpR-GlcNAc in 10uL of buffer containing 50 mM HEPES, 100 mM K glutamate (pH 8.0), and 0.5 mg/ml BSA. Samples (10 µl) were loaded on 0.5% agarose gels and subjected to electrophoresis in 0.5X TBE buffer. DNA–protein complexes were visualized by using a Li-COR Odyssey.

Bile salt resistance and Biofilm assays:

Overnight culture of *Salmonella typhimurium* strains was diluted 1:100 to start a growth assay in LB with 0.6% or 0.3% Sodium deoxycholate in a 96 well plates with OD_{600} values measured every 3 h. For biofilm assays, overnight culture of *Salmonella* strains was inoculated at

1:100 dilution into LB without sodium chloride into 96 well polystyrene plates. The plate was incubated at 30 ˚C without any agitation. After 36 h of growth, the planktonic cells were removed, and wells were washed 3 times with PBS. Biofilm was fixed by adding 200uL of methanol to the wells and incubating for 20 minutes at room temperature. 150uL of 1% (w/v) crystal violet solution was added to the wells and incubated for 15 min. Wells were rinsed with PBS and air-dried. 150uL of 30% (v/v) acetic acid was added to the wells and the plate was shaken gently to solubilize the crystal violet. The OD_{570} was measured to quantify biofilm.

Results:

SseK1 glycosylates OmpR:

Salmonella enterica serovar Typhimurium harbors three SseK glycosyltransferase paralogs named SseK1, SseK2, and SseK3. We expressed OmpR in wild type, single, double, and triple *sseK* mutants. To investigate which of the three SseK paralogs glycosylates OmpR, we used anti R- GlcNAc antibody to conduct western blot analysis of cell lysates expressing recombinant His-tagged OmpR. Western blot results shows that only SseK1 modifies OmpR (Fig16A). Additionally, another two-component response regulator QseF was not labelled by any of the SseK paralogs, serving as a negative control for the assay (Fig 16A). For further confirmation, our *in vitro* glycosylation assay using purified OmpR and SseK1 demonstrates that OmpR is labelled by wild type Ssek1 whereas a catalytically inactive version of SseK1 (Ssek1 HEN mutant) failed to label OmpR with GlcNAc (Fig 16C).

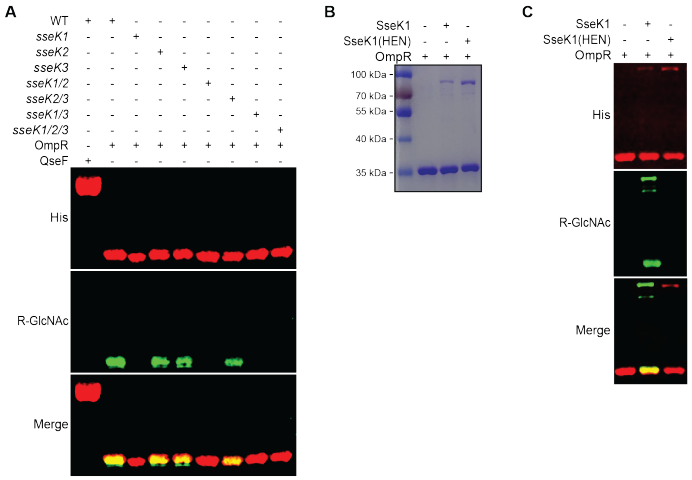

Figure 16 SseK1 Arg-glycosylates OmpR.

(a) Western blot analysis of intrabacterial glycosylation of OmpR and QseF in different *Salmonella* sseK backgrounds; (b) SDS PAGE image of the enzymes and substrate used in the in vitro glycosylation reaction; (c) Western blot analysis of in vitro glycosylation of OmpR in the presence of active or inactive (HEN) forms of SseK1.

After confirming that OmpR is a bacterial SseK1 target, we wanted to identify the OmpR arginine residues that are subject of SseK1-mediated glycosylation. Mass spectrometry sugar analysis detected two potential OmpR arginine residues, R15 and R122 (Figs. 17A and 17B). We validated the mass spec findings by creating recombinant OmpR proteins with R15A, R122A, and R15, R122A point mutations. We expressed the recombinant OmpR point mutants in WT *Salmonella*. Western blot analysis of cell lysates indicated that OmpR R12A is glycosylated, albeit at a reduced level whereas the R122A and R15, R122A point mutants were not glycosylated, indicating that R122 is the primary SseK1 target residue (Fig 17C).

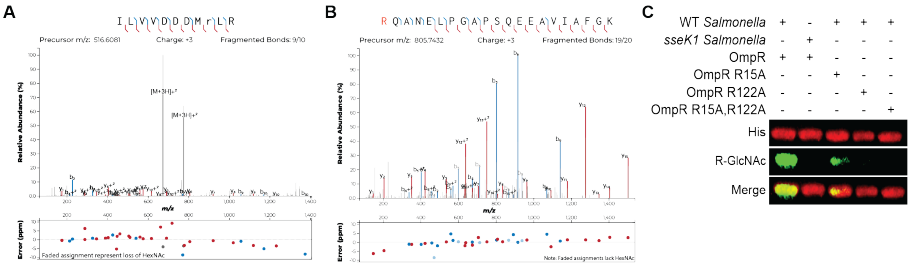

Figure 17 Identification of OmpR glycosylated residues.
(a) HCD spectra of the in vivo glycosylated OmpR tryptic peptides containing glycosylated R15; (b) R122; (c) Western blot verification of Arg-glycosylation of WT and R-to-A point mutations of OmpR..

Glycosylation of OmpR results in altered expression of *ompF*:

OmpR is a transcriptional regulator. To understand the consequence of OmpR glycosylation by SseK1, we measured the transcription of an important OmpR-regulated gene, *ompF*. OmpF is a key outer membrane porin protein that is essential for *Salmonella* adaptability to pH and osmolarity stress [28,45,46]. We used a mRFP (monomeric red fluorescent protein) transcriptional fusion assay where *mrfp* gene was inserted upstream of *ompF* promoter region, and the RFP level was measured in either WT, *ΔsseK1*, *ΔompR*, or *ΔsseK1/ompR* double mutant

strains. Our mRFP transcriptional reporter assay data showed decreased mRFP signal for *ompF* promoter fusions in WT *Salmonella* compared to the *ΔsseK1* strain (Fig 18A). As expected, *ΔompR* and the *ΔsseK1/ompR* double mutant showed significantly reduced activity of the *ompF* promoter (Fig. 18A). There was no significant growth difference among the strains (Fig. 18B).

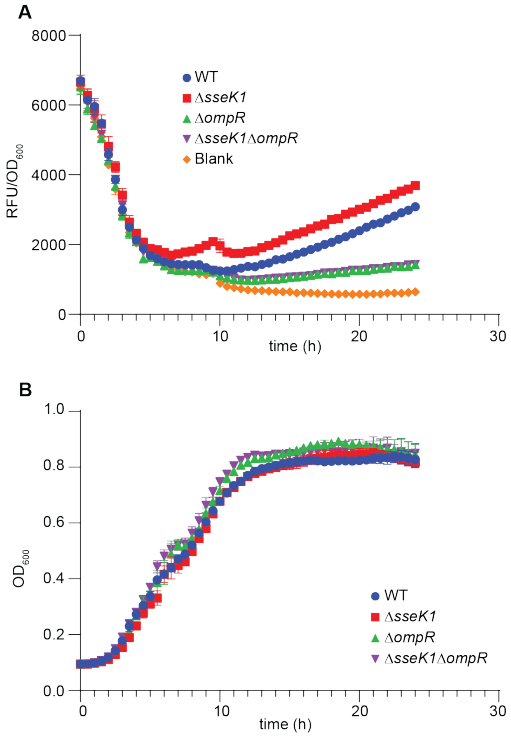

Figure 18 *ompF::mrfp* transcriptional reporter assay.

(a) Measurement of mRFP expression levels of *ompF::rfp* transcriptional fusions in WT *Salmonella enterica* and its *ΔsseK1* or *ΔompR* or *ΔsseK1/ ΔompR* derivatives. A *salmonella typhimurium* strain with empty pHG156a plasmid was used as a negative control (blank). mRFP levels are expressed as RFU (relative fluorescence units)/OD600 ratio.; (b) Growth comparison of different *Salmonella* strains used in the mRFP fusion assay.

To further validate our findings, we complemented *ΔsseK1 Salmonella* with either *sseK1* or *sseK1* HEN (inactive) and repeated the mRFP transcriptional assay. Our assay showed a

significant reduction of *ompF* promoter activity with *sseK1* complemented strain compared to *sseK1* HEN (inactive) complemented strain (Fig 19A). As expected, *Salmonella ΔompR* and *ΔsseK1/ompR* mutant strains failed to display any significant mRFP signal (Figs. 18A and 19A). No significant growth difference between the strains were observed (Fig 19B).

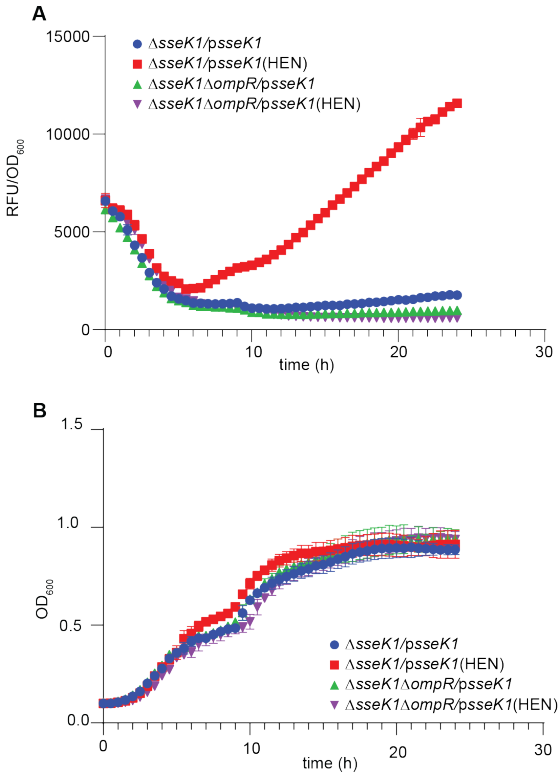

Figure 19 *ompF::mrfp* transcriptional reporter assay of DsseK1 complemented strains.

(**a**) Measurement of mRFP expression levels of *ompF::mrfp* transcriptional fusions in *ΔsseK1* or *ΔsseK1/ ompR Salmonella* complemented with either *sseK1* or *sseK1* HEN. mRFP levels are expressed as RFU (relative fluorescence units)/OD_{600} ratio.; (**b**) Growth comparison of different *Salmonella* strains used in the mRFP fusion assay.

To understand the molecular mechanism of SseK1-mediated reduced promoter activity of OmpR target genes, we conducted an EMSA assay in which glycosylated or unglycosylated OmpR (Fig 20A) was incubated with fluorescently labelled *ompF* promoter region DNA. Our EMSA experiment shows reduced affinity of glycosylated OmpR to its target DNA compared to unglycosylated OmpR (Fig 20B) which agrees with the decreased *ompF* promoter activity in wild type *Salmonella* compared to the *ΔsseK1 Salmonella* strain where OmpR glycosylation is not taking place.

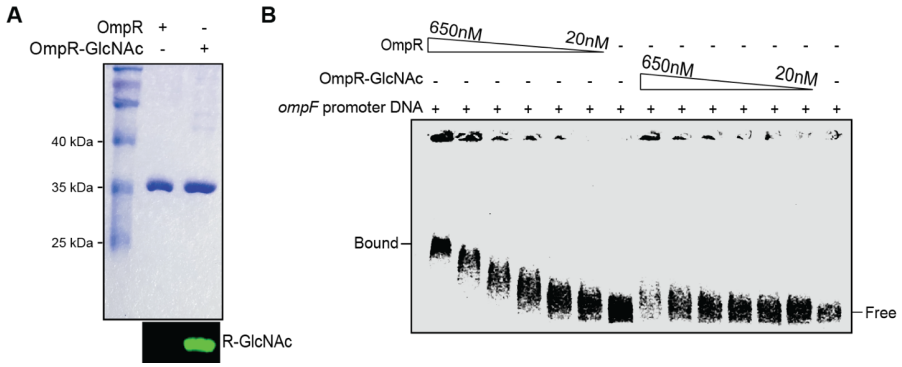

Figure 20 Glycosylation reduces OmpR DNA binding affinity.
(**a**) Purification of native and Arg-glycosylated OmpR combined with validation of glycosylation by Western blotting; (**b**). EMSA comparing the DNA-binding activity of OmpR and OmpR-GlcNAc towards *ompF* promoter DNA.

***Salmonella ΔsseK1* strain has increased Bile tolerance and Biofilm formation capacity:**

OmpR modulates several critical *Salmonella* pathways [32,33,47,48], one of which is bile salt tolerance [45]. Bile salts are natural antimicrobial compounds produced by the host to reduce pathogen proliferation [49]. Since OmpR is a positive regulator of bile salt tolerance, we investigated whether glycosylation of OmpR by SseK1 has any effect on bile salt tolerance. We measured growth of WT*, ΔsseK1, ΔompR* and *ΔsseK1/ompR* double mutant *Salmonella* strains for

their capacity to grow in the presence of bile salts. Our data shows that in the presence of 0.6% sodium deoxycholate, *ΔsseK1 Salmonella* strains grew faster than the WT *Salmonella* (Figs. 21A and 21B). In the presence of 0.3% sodium deoxycholate, the difference in growth is lower (Fig 21A). In contrast, and as expected, the *Salmonella ΔompR* mutant grew poorly in bile salts (Fig 21A and 21B). The *ΔsseK1/ompR* double mutant also grew poorly in the presence of bile salt, indicating that the observed increased bile salt tolerance of *ΔsseK1* mutant *Salmonella* is exerted through OmpR (Figs. 21A and 21B). No significant growth difference between these strains was observed when grown in the absence sodium deoxycholate (Fig. 21A)

Another key virulence factor of *Salmonella* is the biofilm formation process [50,51,52]. OmpR is a positive regulator of biofilm formation in several pathogens including *E. coli*, *Klebsiella*, and *Salmonella* [53,54,55]. We hypothesized that SseK1-mediated modification of OmpR might have an impact on *Salmonella* biofilm formation capacity. To address this question, we compared the biofilm formation ability of wild type *Salmonella*, *ΔsseK1*, *ΔompR* and *ΔsseK1/ompR*. Our results show that, compared to the WT *Salmonella*, *ΔsseK1* produces significantly more biofilm (Fig 21C). As expected, the *ΔompR* strain makes significantly less biofilm compared to the WT strain (Fig 21C). Additionally, we observed no statistically significant difference in biofilm production between *ΔompR* and *ΔompR/sseK1* double mutant signifying the epistatic effect of the *ompR* mutation on *sseK1* for this phenotype (Fig. 21C).

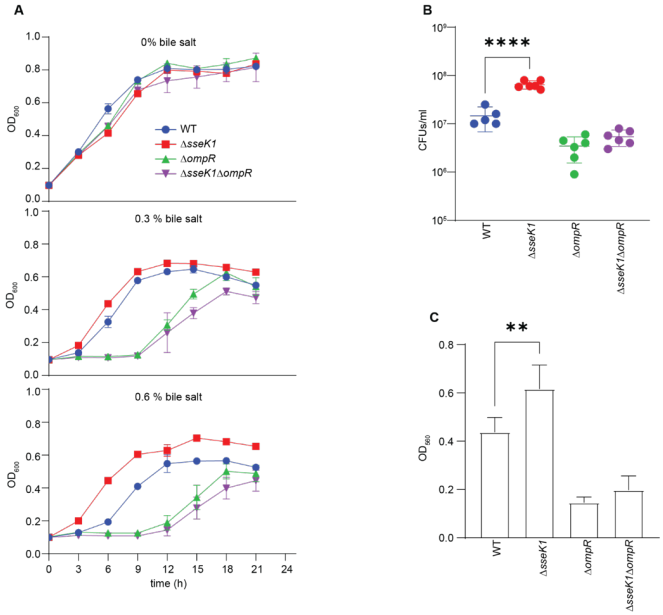

Figure 21 OmpR glycosylation results in altered bile salt tolerance and biofilm formation capacity of *Salmonella*.

(**a**) Growth curve analysis of WT *Salmonella enterica* and its *ΔsseK1*, *ΔompR*, and *ΔsseK1/ ompR* derivatives in 0% bile salt (sodium deoxycholate),0.3% bile salt, and 0.6% bile salt; (**b**) CFU counts of *Salmonella* strains after 16 hours growth in 0.6% bile salts in LB; (**c**) Biofilm formation capacity of WT *Salmonella enterica* and its *ΔsseK1*, *ΔompR*, and *ΔsseK1/ ompR* derivatives.

Discussion

The *Salmonella* T3SS effector SseK1 glycosylates host proteins to reduce host immune response during infection [10]. Breaking the dogma that T3S effectors are inactive inside the pathogen because they are kept under unfolded state within the bacterium due to their specific interaction with cognate chaperones, we and others have demonstrated that such theory is not always the case. Inside the pathogen, Ssek1 and its ortholog NleB targets several proteins from diverse pathways to fine-tune different stress response pathways. Here we demonstrate that among the SseK paralogs, only Ssek1 targets the two-component response regulator OmpR indicating a highly specific interaction. The effect of which could be observed through the differential expression of an OmpR target gene- *ompF*.

The regulation of *ompF* by OmpR is a complex multiunit affair involving several other transcription factors that work in concert to either up or downregulate *ompF* expression [22,56]. Even though the total amount of outer membrane porins (OMPs) of a cell generally does not change significantly [57], the combination of different OMPs are altered in response to different environmental stimuli [58] These alternation of OMP is orchestrated largely by the ratio of phosphorylated and unphosphorylated OmpR along with several other regulators of OMP expression [55,59-61]. While conducting the first set of mRFP fusion assays we used either wild type or mutant *Salmonella* strains to assess the effect of endogenously expressed SseK1 on OmpR transcriptional activity. A significant differential expression level of *ompF* promoter activity was detected under these conditions, signifying that OmpR glycosylation by SseK1 is more likely to naturally occur as an evolutionary bacterial adaptation to specific stress conditions.

Since *ompF* is part of OmpR regulon [62], we investigated whether OmpR glycosylation alters its binding affinity to *ompF* promoter. Our results show a loss in DNA binding affinity of

glycosylated OmpR compared to the native OmpR. Interestingly, the OmpR glycosylation sites, which were detected by mass spec analysis and validated by site directed mutagenesis experiments, are located outside the DNA binding motif of OmpR [63,64], rather in the N-terminal response regulatory region. Moreover, the OmpR glycosylation sites R15 and R122 are distant from the phosphorylation site, D55 [20,59,65]. One hypothesis is that glycosylation could alter DNA binding domains conformation to prevent its DNA binding to target promoters. The silenced affinity of OmpR to its target promoters upon glycosylation by SseK1 could be assimilated to the reverse image of the allosteric enhanced affinity of this transcriptional factor to DNA upon phosphorylation by EnvZ at the residue D55. Thus, this study illustrates an original example of bacterial transcription regulation where the same transcription factor can undergo two different post-transcriptional modifications (phosphorylation and glycosylation) with opposite effects on target genes expression.

Another possible mechanism could be glycosylation of these residues in the response regulator domain leads to altered interaction between OmpR and EnvZ, leading to reduced phosphorylation of D55 residue resulting in reduced DNA binding of OmpR. However, the fact that EMSA experiment, which was conducted in the absence of OmpR phosphorylation, show reduced affinity of OmpR-GlcNAc to DNA suggest that observed phenotype is more likely to occur, at least *in vitro*, independently of phosphorylation. Nevertheless, how glycosylation of OmpR arginine residues could reduce OmpR DNA binding capacity warrants future exploration.

Two-component response regulators are key players in bacterial adaptation to dynamic environmental conditions [16,17,66]. OmpR is vital for *Salmonella* adaptation in changing environments. As such, we investigated the significance of OmpR glycosylation by SseK1. Even though our finding that *Salmonella ΔsseK1* strain has increased bile salt and biofilm production

capacity seems counterintuitive at the first glance, we believe it can be rationally explained. Our experiments were done in laboratory conditions with rich media which are not always the best replication of the natural lifestyle the pathogen maintains. In *Salmonella*, SseK1 is transcribed from SPI-2, which is upregulated during its intracellular lifestyle [4,31-33,67-69]. While inside the host cell, increased SseK1 expression could lead to increased glycosylation of OmpR, leading to downregulation of pathways that are of less significance in the intracellular environment, such as bile salt tolerance and biofilm formation. Conversely, outside of host cells, SPI-2 is not induced [70,71], leading to less SseK1 which then leads to lower inhibition of OmpR activity. This would allow WT *Salmonella* to exhibit more of a *ΔsseK1* mutant-like phenotype during its extracellular lifestyle, with increased bile salt tolerance and surface adherence.

In contrast to our recent work on NagC whose glycosylation by SseK1 leads to increased DNA binding affinity, [14] here we saw decreased DNA binding affinity of Glycosylated OmpR. One significant difference between glycosylated NagC and glycosylated OmpR is that the glycosylated Arg residues of NagC resides within the Helix-Turn-Helix (HTH) DNA binding motif while OmpR glycosylation sites are located outside the hTH motif. Combined with work on PhoP glycosylation leading to altered DNA affinity, this work reaffirms the phenomenon of T3SS effector glycosyltransferases altering transcription factors to modulate gene expression. In addition to identifying additional glycosyltransferase targets, understanding the biochemical fundamentals of how glycosylation of different transcription factors can alter their DNA binding capacity and how it could differ from a transcription factor to another is also going to be an exciting field of research going forward. In conclusion, in this chapter, we demonstrate yet another example of intrabacterial activity of T3SS effector playing significant role in pathogens physiology and thus further highlighting the importance of studying T3SS effectors intrabacterial activity. The

following chapter we will explore how T3SS effectors with intrabacterial activity could be a great target for development of novel antivirulence therapeutics against gram-negative enteric pathogens.

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Chapter 4 - Repurposing Avasimibe to Inhibit Bacterial Glycosyltransferases.

I contributed to all the figures and tables in Chapter 4. A modified version of this chapter is published in Pathogens (Hasan MK, El Qaidi S, McDonald P, Roy A, Hardwidge PR. (2022).

Hasan MK, El Qaidi S, McDonald P, Roy A, Hardwidge PR. Repurposing Avasimibe to Inhibit Bacterial Glycosyltransferases. Pathogens. 2022 Mar 17;11(3):370. doi: 10.3390/pathogens11030370. PMID: 35335693; PMCID: PMC8953086.

Introduction:

We have characterized a conserved group of type III secretion system (T3SS) effector proteins that inhibit innate immune responses to infection [1-5]. These proteins (named NleB in enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC & EPEC) and SseK in *Salmonella enterica*) are glycosyltransferases that are important for bacterial virulence. These enzymes glycosylate host protein substrates with β-*D*-N-acetylglucosamine (GlcNAc) on arginine residues. Besides our discovery of intrabacterial activity of these effectors demonstrate that these effectors are not only necessary for host immune subversion, but also to the general physiology of the bacteria. Since the T3SS effector NleB and SseK1 are essentially fulfills dual purpose in the pathogen, we believe they are excellent targets for development of antivirulence therapeutics.

Several 'death domain'-containing proteins such as the Fas-Associated *protein* with Death Domain (FADD), tumor necrosis factor receptor type 1-associated death domain protein (TRADD), and RIPK1 are substrates of the NleB/SseK glycosyltransferases [6]. NleB1 disrupts tumor necrosis factor receptor (TNFR)-associated factor (TRAF) signaling, leading to inhibition of the pro-inflammatory NF-kB pathway [7]. Another target of NleB1 is glyceraldehyde 3-

phosphate dehydrogenase (GAPDH). In addition to its role in glycolysis, GAPDH binds to TRAF proteins and stimulates TRAF polyubiquitination [4].

Arginine glycosylation is biologically important because the glycosylation of arginines on host protein substrates leads to their irreversible inactivation and disrupts the normal functioning of the innate immune response. Mammals do not have the enzymatic machinery to add GlcNAc residues to arginine (N-GlcNAc), while this modification is critical for *E. coli* and *Salmonella* virulence. Inhibitors that prevent the formation of this unusual post-translational modification represent a potentially novel way to combat these infections. Non-traditional antibacterial therapeutic strategies have recently been reviewed and are of emerging interest [8].

We previously developed a high-throughput screening (HTS) assay to identify NleB/SseK inhibitors [9, 10]. Here we show that Avasimibe (Fig. 22A), an acyl-coenzyme A:cholesterol acyltransferase inhibitor [11], also inhibits NleB and SseK enzyme activity, leading to reduced pathogen colonization in macrophage and mouse model of *Salmonella* and *Citrobacter rodentium* infection, respectively.

Materials and Methods:

Cell lines:

Abelson-murine-leukemia-virus-induced, macrophage-like cells from BALB/c mice (RAW264.7) were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) (Atlanta Biologicals, Minneapolis, MN, USA) and 100 µg/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA) in 5 % CO_2.

Protein Purification:

Protein purification was conducted as previously described [1]. *E. coli* BL21 (DE3) strains were cultured in LB at 37 °C until an OD600 of 0.4, after which 0.5 mM IPTG was added for 4 h. The pellet was resuspended in 50 mM sodium phosphate buffer pH 8.0, 0.5 mg/mL lysozyme) after centrifugation. After 30 minutes on ice with periodic shaking, the suspension was treated with 50 mM sodium phosphate buffer pH 8.0, 2 M NaCl, 8 mM imidazole, 20 % glycerol, and 2 % Triton X-100 for 30 minutes. After sonicating and centrifuging the cell lysates, the supernatant was added to 2 mL Ni-NTA resin (Qiagen, Germantown, MD, USA) for 1 h of rotation at 4 °C. The mixture was added to a Poly-Prep Chromatography Column (Bio-Rad Hercules, CA, USA) and washed with 10 mL of 50 mM sodium phosphate buffer pH 8.0, 600 mM NaCl, 60 mM imidazole, and 10 % glycerol. Proteins were eluted in 2 mL 50 mM sodium phosphate buffer pH 8.0, 600 mM NaCl, 250 mM imidazole, and 10% glycerol, then dialyzed into the same buffer lacking imidazole.

Glycosylation Assays:

In vitro glycosylation assays were conducted as previously described [1]. Enzymes (200 nM of NleB1, NleB, or SseK1) were incubated in 50 mM Tris-HCl buffer pH 7.4, 1 mM UDP-GlcNAc, 10 mM $MnCl_2$, and 1 mM DTT with 1 mM GAPDH in the presence or absence of serial dilutions of avasimibe. After a 2 h incubation at room temperature, samples were blotted with anti-R-GlcNAc and anti-His tag monoclonal antibodies (Abcam, Cambridge, MA, USA). LI-COR Image Studio software (LI-COR Biosciences, Lincoln, NY, USA) was used to measure signal intensities and inhibition was estimated by measuring the relative reduction in substrate glycosylation.

OGT assays:

The UDP-GloTM Glycosyltransferase Assay Kit was used as specified by the manufacturer (Promega). OGT (200 nM) in 25 mM Tris-HCL buffer pH 7.5, 12.5 mM $MgCl_2$, 0.06 mg/mL BSA, 1 mM DTT, 50 µM OGT peptide substrate, and 100 µM UDP-GlcNAc were used in the reactions, which also included Avasimibe at concentrations ranging from 6 to 200 µM. The reactions were incubated at 22 °C for 1 h, and the luminescence signals were measured by using a FLUO star microplate reader (BMG Labtech, Cary, NC, USA).

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MTT Assays:

MTT tests utilizing RAW264.7 cells in the presence of 2-fold serial dilutions of avasimibe from 3 to 200 µM concentration were performed as specified by Millipore. Formazan absorbance was measured at 570 nm using a BioTek Microplate reader (BioTek, Winooski, VT, USA).

Bacterial growth assays:

Bacterial cultures were grown overnight, diluted 1:200 in LB, and then grown at 37 °C for 18 h in the presence of 2-fold serial dilutions of avasimibe (0–256 µM). The absorbance of the culture medium at OD_{600} was used to monitor bacterial growth.

Macrophage Infections:

RAW264.7 cells were seeded at 1×10^5 cells/well in TCP 24-well tissue culture plates, and avasimibe (6-100 µM) was added 1 h before infection. *Salmonella* cultures were grown overnight, and 10^6 CFUs were added to each well for 30 min. Cells were treated with 100 µg/mL gentamicin

for 1 h and then with 10 µg/mL gentamicin for an additional 23 h. Bacteria were released from RAW264.7 cells using 1 % saponin (Sigma), diluted in PBS, and plated to enumerate the number of intracellular *Salmonella*.

Mouse infections:

Five-week-old C57BL/6 mice (Jackson Laboratory) were housed at Kansas State University. *C. rodentium* DBS100 was cultivated in LB broth with shaking at 200 rpm at 37 ˚C overnight. Mice were infected via oral gavage with 10^9 CFUs of *C. rodentium* in 100 µL PBS. Avasimibe was administered to one group of mice via intraperitoneal (IP) injection immediately before oral gavage. Avasimibe was provided to another group of mice via IP injection at 24 and 48 h after oral gavage. Mice were euthanized 7 days after infection, colons were homogenized, serially diluted, and plated on MacConkey agar. The following day, colonies were enumerated and plotted to compare *C. rodentium* loads between experimental groups.

Results:

Avasimibe inhibits NleB1 and its orthologs:

We previously published the results of HTS assays in which we identified several small molecules (100066N, 102644N, and YM155) capable of inhibiting NleB/SseK enzyme activity *in vitro* [9, 10]. In those previous studies, we also discovered a potential activity for avasimibe in inhibiting EHEC NleB1 activity. We first validated the HTS data by quantifying the extent to which avasimibe could prevent Arg-glycosylation of the GAPDH substrate by the EHEC NleB1, *Citrobacter* NleB, and *Salmonella* SseK1 enzymes *in vitro*. We conducted the glycosylation assays in the presence of 2-fold serial dilutions of avasimibe. Avasimbibe inhibited each enzyme in a concentration-dependent manner, with apparent IC_{50s} of ~10 µM (Figs. 22 B-C).

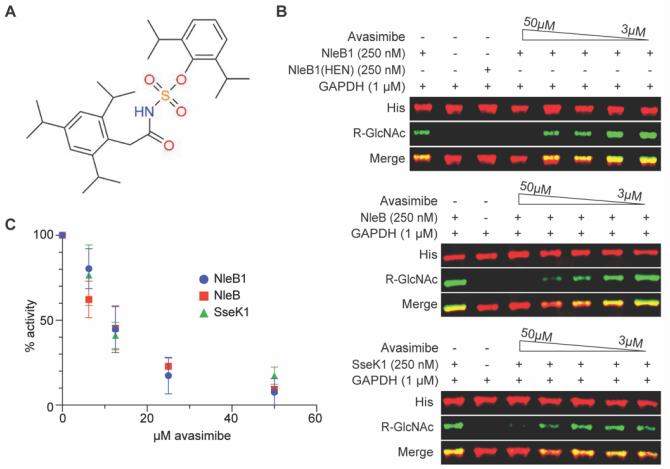

Figure 22 In vitro glycosylation assays.
In vitro glycosylation assays. (**A**) Avasimibe structure. (**B**) Western blot results for avasimibe inhibition of NleB1, NleB, and SseK1 (250 nM) glycosylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1 µM). Avasimibe was added at concentrations from 3 to 50 µM. The NleB1(HEN) enzyme is an inactive negative control [5]. (**C**) Quantification of Western blot signal intensities from replicates of in vitro glycosylation assays, $n = 3$ independent experimental replicates.

Avasimibe does not inhibit the mammalian OGT enzyme, is not toxic to mammalian cells, and is neither bacteriostatic nor bacteriocidal:

We next examined whether avasimibe affected the activity of the human serine/threonine N-acetylglucosamine (O-GlcNAc) transferase (OGT) that regulates protein glycosylation [12]. To assess OGT activity as a function of avasimibe concentration, we used a bioluminescence-based UDP-Glo glycosyltransferase assay. Avasimbe had no effect on human OGT activity *in vitro* at concentrations of up to 200 µM (Fig. 23A). Avasimibe was also found to be non-toxic to mammalian cells at concentrations of up to 50 µM, as determined by conducting an 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 23B). Avasimibe had no effect on the growth rates of *C. rodentium* or *Salmonella enterica* at concentrations of up to 125

μM when these bacteria were grown in LB broth (Figs. 23C-D). Thus, avasimibe does not appear to act as a general bacteriostatic or bactericidal agent.

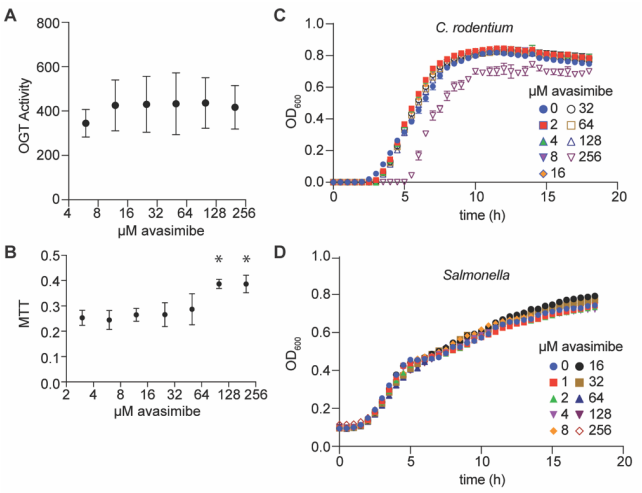

Figure 23 OGT activity, cytotoxicity, and bacterial growth assays.
(A) OGT activity assay. OGT activity was measured by using a UDP-Glo assay in the presence of avasimibe, n = 3. (B) Cell cytotoxicity as measured by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Avasimibe was added to RAW264.7 cells for 24 h and cell viability was assayed by monitoring MTT signal intensity, n = 3. Asterisks indicate significantly different MTT signals as compared to untreated cells, $p < 0.05$, Dunn's multiple comparisons test. (C-D) Bacterial growth assays. *C. rodentium* and *S. enterica* were cultured in LB media at 37 ˚C in the presence of the indicated concentrations of avasimibe. Bacterial growth was monitored as a function of time, n = 3.

Avasimibe inhibits *Salmonella* and *C. rodentium* survival in vivo:

We quantified the impact of avasimibe on *Salmonella* survival in cell culture to determine whether it can be used to reduce pathogen loads in mammalian cells. When avasimibe was added to RAW264.7 cells prior to *Salmonella* infection at concentrations greater than 10 μM, the amount of intracellular *Salmonella* was significantly reduced 24 h later (Fig. 24A).

We performed a mouse infection experiment to determine whether avasimibe inhibition of NleB had any effect *in vivo*. We administered avasimibe via intraperitoneal injection at doses of

either 25 mg/kg immediately prior to infecting mice with *C. rodentium*, or at 5 mg/kg 24h and 48 h post-infection. After 7 days, we euthanized the mice and quantified the amount of *C. rodentium* in the colon. We observed a significant reduction in *C. rodentium* in mice treated with avasimibe, as compared to untreated mice, regardless of whether the avasimibe was administered pre- or post-infection (Fig. 24B). These findings suggest that avasimibe could be used as an anti-virulence compound.

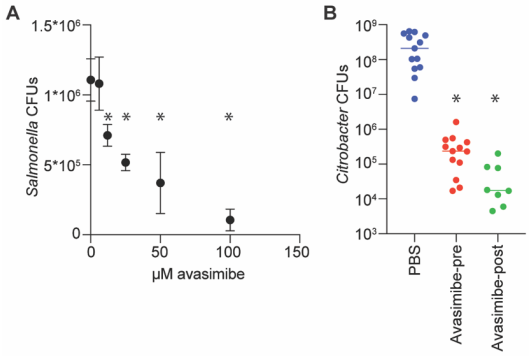

Figure 24 Infection assays.
(A) *Salmonella* infection assays. RAW264.7 cells were seeded at 1×10^5 cells/well in 24-well plates and avasimibe was added 1 h before infection with 10^6 CFUs of *Salmonella* for 30 min. Cells were incubated in medium containing 100 μg/mL gentamicin for 1 h, and then in 10 μg/mL gentamicin for an additional 23 h. Bacteria were released from RAW264.7 cells using 1 % saponin, diluted in PBS, and plated for colony counts, n = 3. Asterisks indicate significantly different *Salmonella* CFUs as compared to untreated macrophages, $p < 0.05$, Dunn's multiple comparisons test. (B) *C. rodentium* infections of mice. Mice were infected via oral gavage with 10^9 CFUs of *C. rodentium* in 100 μL PBS. Avasimibe (25 mg/kg) was administered to one group of mice via intraperitoneal (IP) injection immediately before oral gavage. Avasimibe (5 mg/kg) was provided to another group of mice via IP injection at 24 and 48 h after oral gavage. Asterisks indicate significantly different CFUs as compared to untreated mice, $p < 0.05$, Dunn's, n = 8-13.

Discussion

We discovered here that Avasimibe, a previously characterized ACAT inhibitor, also inhibits the NleB and SseK arginine glycosyltransferases. The key difference between our previous inhibitor studies and this study is that here we identified an inhibitor that is already safety and efficacy tested. Besides, this is also the first time we were able to demonstrate the effectiveness of

our approach to use inhibitor in animal models where we demonstrate promising results. Avasimibe has a well-documented solubility and safety profile [13], although it caused a potential reduction in the potency of Lipitor [11] and was thus not ultimately used to treat hyperlipidemia or atherosclerosis. Avasimibe also suppresses tumor proliferation and metastasis via the E2F-1 signaling pathway and has potential utility in treating prostate cancer [14]. Avasimibe alleviates insulin resistance in diet-induced obese mice [15]. Avasimibe impedes tick embryo development by interfering with tick lipid metabolism, making ticks more susceptible to bacterial infection [16]. Avasimibe has also shown encouraging results in inhibiting glioma cell proliferation [17]. Additionally, avasimibe was identified as a potential hepatitis C virus inhibitor where it targets the assembly of infectious viral particles [18].

Our glycosylation assay results suggest that avasimibe has an IC_{50} of ~10 μM against the NleB/SseK enzymes. At these concentrations, avasimibe has no substantial inhibitory effect on pathogen growth, no cytotoxicity to mammalian cells, and does not inhibit the human OGT enzyme. Finally, our *in vivo* findings suggest that avasimibe is well tolerated in mice and significantly reduces *C. rodentium* loads in the intestine, regardless of whether it is administered pre- or post-infection. Several other studies where Avasimibe has been administered to mice with similar dose and administration route for significantly longer duration, reported no adverse effect on mice, corroborating with our observation.

We have now identified and studied several inhibitors that have the potential to function as anti-virulence therapeutics against enteric pathogens. Our goal is to combine our understanding of the molecular mechanisms of action of these inhibitors to create new compounds with improved solubility, lower production costs, and good safety profiles. For example, using synthetic chemistry to modify avasimibe and reduce its interaction with the liver enzyme CYP3A4 [19]

could drastically lower its side effects while maintaining its safety and efficacy profile. Indeed, effort to better understand the molecular basis of these small molecules inhibiting the glycosyltransferase effector are already under works. We used computational approaches to conduct molecular docking and interaction simulations to identify essential amino acid residues that interact with inhibitors. Site directed mutagenesis techniques could be employed to validate the predicted in silico study data. Besides, we along with our collaborators are investigating the potential amino acid residues of the effectors that interact with the inhibitor molecule by co-crystalizing the effectors bound with inhibitors. The results from these studies would provide us with critical insights to be able to develop better inhibitors.

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Chapter 5 - Conclusions

Enteric pathogens pose a serious threat to both human and animal health around the world. These pathogens can cause a variety of diseases, ranging from mild gastroenteritis to life-threatening infections. Enteric pathogens can also cause long-term health problems, such as chronic inflammatory conditions and cancer, in some cases. Given the importance of these pathogens to human and animal health, it is critical to investigate their infection and virulence mechanisms. The study of type III secretion system (T3SS) effectors, which are bacterial proteins that are injected directly into host cells to subvert the host immune response and promote bacterial survival, is one area of particular interest.

T3SS effectors have intrabacterial activity, which means they can modify or interact with other bacterial proteins and processes. Intrabacterial activity of T3SS effectors is an exciting new phenomenon in the gram-negative pathogenesis research. These so far unknown activities and the vital role that they play in the survival of pathogens in adverse conditions are of great significance to better understand the evolutionary chain of events that lead to acquiring, maintaining, and tailoring the T3SS system and its effectors to better serve two different aspect of pathogens lifecycle: infectious and non-infectious.

The studies presented in this dissertation makes an effort to illustrate a comprehensive approach for identifying new T3SS effectors that are active inside the pathogen, finding out targets of that particular effector- if any present, and evaluating the significance of the particular intrabacterial activity/activities in terms of the pathogen's physiology and pathogenesis. Finally, we illustrated how this newfound knowledge about T3SS effectors makes them an even more lucrative target to develop novel antimicrobial therapeutics designed against them.

In chapter 2 we investigated a new group of effectors for their intrabacterial activity. The T3SS effector proteases primarily targets the host proinflammatory pathway proteins to dampen immune response during infection. In *Citrobacter rodentium*, NleC, NleD, and EspL are the protease effectors. We used a recombinant target protein cleavage assay to initially test the activity of each of these effectors. We used the best-known targets of these effectors to use as a model and identified which of these effectors can cleave their targets. We found that out of the three tested effectors, NleC and EspL is active whereas NleD is not. Our findings indicate that, just as with the T3SS effector NleB and its *Salmonella* orthologs, NleC and EspL might have intrabacterial targets too. When we investigated the difference between NleD and the other tested effectors, we found that only NleD has a putative Sec/SPI signal peptide sequence in its N terminal. Sec/SPI signal peptides are known to carry their cargo in unfolded configuration. Which might indicate that out of the tested T3SS effectors only NleD is unfolded in the pathogen and thus inactive. Other explanation could be that- the condition we used to test for intrabacterial activity of NleD, NleD might not be expressed or active. Indeed, we observed during our NleC intrabacterial activity assay that, NleC is more catalytically active at 30ºC compared to 37ºC. So, if a specific condition is required for NleD activity inside the pathogen, we might have not observed the cleavage.

We identified that NleC is active inside the pathogen, and such wanted to identify the bacterial target of NleC. Since NleC cleaves p65 which is a transcription factor itself and the known mechanism for substrate recognition by NleC is NleC mimics dsDNA to bind with its target protein, we hypothesized that NleC target has a high probability of being either a transcription factor or a DNA binding protein. If so, RNA seq analysis would provide us with some hints as to what could be a potential target of NleC, if there is any.

Our RNA seq results were quiet intriguing. We found that, majority of the protein that are differentially expressed are of phage origin. Not only that, NleB pseudogene, which is located next to NleC in the Citrobacter genome was also downregulated in NleC mutant strain. We rationalize this finding by recognizing that since NleC is a phage gene in origin itself, it has a high chance of modulating other phage genes. We would need to test the RNA seq hits by RT-PCR and reporter fusion assays to further validate these finding.

As NleC is a protease, we believe that if it does have bacterial targets, new cleavage products would be present in sample collected from *C. rodentium* strain that expresses active NleC vs a strain that carries an inactive NleC. We used an advanced proteomics approach to identify the differentially available N terminal peptides coming from NleC mediated cleavage in the sample expressing active NleC. HUNTER proteomics approach is used to enrich N terminal peptides from a sample during mass spec analysis to get a set of results which could be called "N-terminome". This approach allows for detection of newly formed N terminal as a result of proteolytic cleavage that is independent of trypsin treatment in a sample.

Our HUNTER analysis results data were valuable in two different ways. First, as a part of a HUNTER analysis, a standard mass-spec analysis is done to validate the initial quality of the sample and establish baseline to which the HUNTER enrichment data will be later corrected to. This "pre-HUNTER" data is useful to identify if there is any difference in proteomics between the samples being compared before the HUNTER analysis is done. Interestingly, we found two key proteins in *C. rodentium* to be differentially expressed in the samples of *C. rodentium* strains expressing either active or inactive NleC. One of which is the outer membrane protein OmpF and the RNA chaperone StpA. Our pre-HUNTER data shows that OmpF is upregulated in the active NleC expressing samples whereas StpA is downregulated. One potential explanation for this is

that StpA might be cleaved by NleC. Which would lead to its reduced availability in the active NleC expressing samples. StpA is already known to be target of proteolytic cleavage by Lon protease. Generally, StpA is protected from Lon protease since it forms heterodimer with H-NS. But The bacteria could utilize NleC to circumvent lon proteases limitation to gain an alternative strategy for StpA cleavage. Besides StpA is an RNA chaperone and also known to bind to *micF* small RNA. *micF* is well documented to bind with *ompF* mRNA and reduce its translation. If StpA is cleaved by NleC, it would then lead to reduced suppression of OmpF translation by *micF* RNA. Further studies to test this hypothesis is needed to be conducted. If NleC does contribute to increased expression of OmpF via StpA cleavage, this could be significant since OmpF is a major porin protein that is essential for bacteria response to changes in pH or osmolarity in the outside environment. Results from the 3rd Chapter of this studies further signifies how, OmpF expression is already transcriptionally regulated by another T3SS effector SseK1 and the implication of these regulations are quite significant.

Our HUNTER analysis data demonstrated that significant difference in N-terminome between Active NleC expressing *C. rodentium* and inactive NleC expressing *C. rodentium* exists. Primarily we saw a large number of cleavage peptide to be belonging to the protein translation and translocation pathways. The second group of the highly abundant cleavage peptides are from cellular nitrogen compounds metabolism pathway. Further validation of these results could be accomplished by using our recombinant target cleavage assay as demonstrated in the beginning of the chapter 2 results.

In chapter 3 we identified a novel target for T3SS glycosyltransferase effector SseK. We demonstrate yet another example of intrabacterial activity of SseK1 where it glycosylates two component response regulator OmpR. OmpR is the response regulator protein of EnvZ-OmpR two

component system, a ubiquitous TCS in gram negative pathogens. Out of the 3 three *Salmonella* SseK paralogs we found only SseK1 to be labelling OmpR. OmpR is significant in many cellular aspects of *Salmonella* including stress response, nutrient uptake, antimicrobial resistance, pathogenesis, and virulence factor regulation. To know the implication of OmpR labelling by SseK1 we looked at the transcription of *ompF* in strains where OmpR was being labelled versus the strain where it was not. *ompF* is a part of OmpR regulon and codes for a significant outer membrane porin protein relevant to physiological adaptation of the pathogen in changing environmental condition. We demonstrated that SseK1 labelling of OmpR results in reduced binding affinity towards is target genes promoter DNA.

EnvZ-OmpR Two Component System is responsible for regulation of a large number of gene in response to environmental changes. Depending on the changing environment, sensor kinase EnvZ phosphorylates response regulator OmpR. Phosphorylated OmpR has increased affinity towards its target genes promoters, and through the interaction with other Transcription Factors could up/down regulate specific genes of its regulon. Our results demonstrate for the first time that besides phosphorylation, *Salmonella* uses glycosylation as an additional way to biochemically modify OmpR activity. By employing Glycosylation besides phosphorylation modification of OmpR, *Salmonella* can "fine tune" OmpR activity beyond what is possible through phosphorylation only.

Salmonella glycosylates OmpR primarily in R122 residue. 3D structure analysis of OmpR reveals that this residue is not in the DNA binding domain of OmpR. Neither is R15. So, direct interruption of OmpR DNA interaction by glycosylation in this residue seems unreasonable. But given how the primary phosphorylation site D55 of OmpR is also not in the DNA binding domain but still results in significant alteration of OmpR DNA affinity (~10X) when phosphorylated,

similar but opposite result could very well be achieved by glycosylation at a residue far from the DNA binding domain. Further 3D structure studies are needed for deciphering the structural mechanism behind the reduction of OmpR affinity towards its target DNA while its glycosylated. Another interesting aspect to investigate is to determine how glycosylation and phosphorylation modification interplays with each other. Given both of this modification occurs at the OmpR receiver domain, whether preexistence of one block the domain from being modified by the second mechanism is intriguing to know. EnvZ also can also work as phosphatase for OmpR. It could also be possible that OmpR glycosylation could lead increased capacity of EnvZ to dephosphorylate OmpR, leading to reduced affinity of OmpR to its target DNA. This set of results also adds to our previous observation that NagC glycosylation leads to increased affinity towards it target gene *nagB* promoter. Which is opposite of what we have observed here with OmpR glycosylation. These results indicate that, glycosylation modification of a transcription factor can result in either increased or decreased affinity towards the target gene promoter depending on the specific transcription factor.

Our results on SseK1's role in biofilm formation and bile salt tolerance seems counterintuitive at the first glance and requires further explanation. We observed that, in absence of SseK1, the pathogen is more bile salt tolerance and produces more biofilm. Whereas in our previous studies, T3SS effector glycosyltransferases played a positive role in overall bacterial fitness by increasing its capacity to withstand certain stress conditions. Our results of OmpR glycosylation by SseK1 leading to negative virulence attributes of *Salmonella* is opposite of what one would expect. Even though the phenotypic observation of increased bile salt and biofilm production in absence of SseK1 are coherent with the molecular experiments results showing how SseK1 reduces OmpR affinity towards target DNA, we hypothesize that, in a natural environment

Salmonella uses SseK1 to modulate OmpR activity so that it can up or downregulate its virulence attributed depending on its habitat. For example, one key target for OmpR in *Salmonella* is the SPI-2 transcriptional regulator SsrA/B. It has been demonstrated by a study done by Choi *et al*. that SPI-2 induction should neither be too high nor too low for *Salmonella* to thrive in SCV (*Salmonella* Containing Vesicle). In theory, *Salmonella* could use SseK1 to glycosylate OmpR and dampen the SPI-2 induction. This way, it has an additional mechanism to modulate SPI-2 induction besides phosphorylation that is also directly linked with SPI-2 gene expression. Besides, since SseK1 is transcribed from SPI-2 region, it can regulate its own transcription via OmpR to fine tune SPI-2 induction. Additional evidence of TCS response regulator glycosylation leading to reduced virulence is also supported by Xue *et al.*'s study that shows PhoP glycosylation leads to reduction of *ssrB* transcription.

Another hypothesis is that the virulence properties tested here (bile salt tolerance and biofilm formation) are more relevant to *Salmonella* extracellular lifestyle than to its intracellular lifestyle in Macrophage and thus dampening these pathways while SPI-2 is induced would allow the pathogen to conserve resources when it's not needed. Indeed, SPI-2 induction is mostly relevant to *Salmonella* intracellular lifestyle and increased SseK1 expression during *Salmonella* intracellular condition could essentially downregulate these phenotypes that are not relevant in intracellular lifestyle. Future studies with in vitro cell culture and in vivo animal models could further validate these hypotheses.

The discovery of the phenomenon of T3SS intrabacterial activity is also therapeutically significant. Now that we know they are involved in many critical pathways of pathogen these previously not so significant proteins (in case of intrabacterial activity) becomes more viable options as potential targets for development of novel antimicrobials. Targeting bacterial virulence factors for

development of antimicrobial therapeutics are not new. But, knowing that they not only work inside the host but also inside the pathogen to regulate critical pathogenic physiology- makes them more attractive as alternatives to traditional antibiotics. Indeed, our results shows that administration of the small molecule inhibitors for NleB had no real effect on pathogens growth indicating a chance of development of resistance is low. In chapter 4 we, investigated a new inhibitor Avasimibe to test its efficacy as a potential anti virulence drug against *Salmonella* and *Citrobacter rodentium*. Our High Throughput Screening (HTS) assay has provided several potential inhibitors against T3SS effector glycosyltransferases. We have tested a few of those compounds but even though they showed promising results in vitro, they had several limitations. First, the compounds had issues with solubility that would limit them from being solubilized in neutral buffers that are safe to be administered in animals in a physiologically appropriate amount of dosage. Additionally, synthesis of these compounds in large quantity was quite expensive. Besides these compounds have no prior data on their safety and efficacy or any other pharmacodynamics attributes. Additionally, we also did not conduct any animal model studies with these compounds because of the prior mentioned limitations of the compounds. So, we decided to pick a compound from the HTS hit list that already has good safety, efficacy, and pharmacodynamics attributes. According to our criteria Avasimibe was chosen to investigate as a potential inhibitor for T3SS glycosyltransferase activity. Avasimibe was developed by Pfizer and made it through all three stages of clinical trials. Recently it has garnered increased attention from antitumor drug development community because of its potential antitumor activities. Our results indicated that, Avasimibe has potential against all three major glycosyltransferases tested -NleB, NleB1, and SseK1. Mammalian OGT is a critical enzyme that adds O-GlcNac residues to many important proteins in eukaryotic cells. For Avasimibe to be used as a potential antivirulence drug,

it needs to have little to no inhibitory effect on the mammalian OGT glycosyltransferase activity. Our results show that it had no significant inhibitory effect on OGT activity. We then tested whether Avasimibe is bactericidal or not. Since, bactericidal compounds used as antimicrobial has a higher chance of leading to development of antimicrobial resistance, any compound that works through this mode of action would be deemed to have an increased chance of resistance development when administered as therapeutics. We found that, up to a very high concentration, Avasimibe was not bactericidal. Which indicates it has lower chance of antimicrobial resistance development against it. Besides, it also indicates that when administered orally, avasimibe might also have little to no effect on gut microbiota. Future studies on Gut microbiomes of animal models administered with avasimibe and comparing it with control could be useful to test this hypothesis. Assays such as LLMDA (Lawrence Livermore Microbial Detection Array) could be successfully employed to test whether Avasimibe administration has any significant detrimental effect on gut microbiota or not. To further fine tune the Avasimibe activity and reduce any side effects, if any found, we along with our collaborators are employing X-ray co-crystallography experiments of Avasimibe bound to NleB to identify NleB residues that are significant for interaction with the drug. Once these key data are available, we can then use this knowledge to better design derivatives of Avasimibe that are even more efficient and has little to no side effects. We also think that an alternative method of avasimibe admistration would be to use it in combination with traditional antimicrobial drugs. Specially in the case of organisms that already has developed significant amount of multidrug resistance, co-administration of the drugs with Avasimibe could help reduce pathogen burden to a level where the hosts natural immune system could fight back. Another special use case scenario is during infection with Shiga-like toxin producing *E. coli* strains. It is known that administration of traditional antimicrobials during a Stx harboring pathogens infection

might lead to increased production of Shiga like toxins and ultimately lead to life threatening HUS (Hemolytic Uremic Syndrome). In such infections the patients are sometimes out of option for treatments. Since Avasimibe is not bactericidal, it might not induce Shiga toxin production in EPEC/EHEC infection, and such could be a live saving option. Regardless, whether Avasimibe administration leads to Shiga toxin production or not remains untested and could be an important future study.

In conclusion, these group of studies have successfully demonstrated that, intrabacterial activity of T3SS effectors are an exciting new phenomenon in gram negative pathogen research field. Intrabacterial activity of effectors are significant in several key aspects of gram-negative pathogens lifestyle and targeting effectors with significant host and intrabacterial activity could be a useful strategy to fight these life-threatening infectious diseases.

Strains and Plasmids

Chapter 2:

Construct	Plasmid	Source
Flag GST p65 His	pET16-*p65*	This study
Flag GST JNK His	pET16-*JNK*	This study
RIPK1 His	pET28a-*RIPK1*	This study
RIPK3 His	pET28a-*RIPK3*	This study
His-NleC	pET28-*nleC*	This study
His-NleC E184A	pET28-*nleC* E184A	This study
His-NleD	pET28-*nleD*	This study
His-NleD w/o SP	pET28-$nleD_{\Delta SP}$	This study
His-NleC w SP	pET28-$nleC_{SP}$	This study
Flag GST p65 C38A, E39A His	pET16-*p65* C38A,E39A	This study
Flag GST JNK P184A, Y185A His	pET16-*JNK* P184A,Y185A	This study

Strain	Source
Citrobacter rodentium	This study
Citrobacter rodentium × pET16-*p65*	This study
Citrobacter rodentium × pET16-*JNK*	This study
Citrobacter rodentium × pET28-*RIPK1*	This study
Citrobacter rodentium × pET28-*RIPK3*	This study
Citrobacter rodentium ΔnleC	This study
Citrobacter rodentium ΔnleD	This study
Citrobacter rodentium ΔnleC × pET16-*65*	This study
Citrobacter rodentium ΔnleD × pET16-*p65*	This study
Citrobacter rodentium × pET28-*nleD*$_{\Delta SP}$ × pET16-*JNK*	This study
Citrobacter rodentium × pET28-*nleC*$_{SP}$ × pET16-*p65*	This study
S. Typhimurium × pET16-*65*	This study

Chapter 3 :

Construct	Plasmid	Source
Flag-SseK1	pFLAG-CTC-*sseK1*	[38]
GST-SseK1	pET42a-*sseK1*	[7]

GST-SseK1 (HEN)	pET42a-*sseK1* H244A E255A N256A	[38]
His-OmpR	pTac-*ompR*	This study
His-OmpR R15A	pTac-*ompR* R15A	This study
His-OmpR R122A	pTac-*ompR* R122A	This study
His-OmpR R15,122A	pTac-*ompR* R15,122A	This study
ompF::mrfp	pHG156a- *ompF::mrfp*	This study

Strain	**Source**
S. Typhimurium ATCC 1402 ×pTac *ompR*	This study
S. Typhimurium ATCC 14028 ×pTac *ompR* R15A	This study
S. Typhimurium ATCC 14028 ×pTac *ompR* R122A	This study
S. Typhimurium ATCC 14028 ×pTac *ompR* R15,122A	This study
S. Typhimurium ATCC 14028 ×pTac *qseF*	This study
S. Typhimurium ΔsseK1×pTac *ompR*	This study
S. Typhimurium ΔsseK2×pTac *ompR*	This study
S. Typhimurium ΔsseK3×pTac *ompR*	This study
S. Typhimurium ΔsseK1/sseK2×pTac *ompR*	This study

S. Typhimurium ΔsseK1/sseK3×pTac *ompR*	This study
S. Typhimurium ΔsseK2/sseK3×pTac *ompR*	This study
S. Typhimurium ΔsseK1/sseK2/sseK3×pTac *ompR*	This study
S. Typhimurium ΔompR	This study
S. Typhimurium ΔsseK1/ompR	This study
S. Typhimurium × p*ompF* promoter::*mrfp*	This study
S. Typhimurium ΔsseK1 × p*ompF* promoter::*mrfp*	This study
S. Typhimurium ΔompR × p*ompF* promoter::*mrfp*	This study
S. Typhimurium ΔsseK1/ompR × p*ompF* promoter::*mrfp*	This study
S. Typhimurium ΔsseK1 × p*ompF* promoter::*mrfp* × *psseK1*	This study
S. Typhimurium ΔsseK1 × p*ompF* promoter::*mrfp* × *pHEN*	This study
S. Typhimurium ΔsseK1/ompR × p*ompF* promoter::*mrfp* × *psseK1*	This study
S. Typhimurium ΔsseK1/ompR × p*ompF* promoter::*mrfp* × *pHEN*	This study
Salmonella typhimurium ATCC 14028	[39]
S. typhimurium ΔsseK1	[39]

S. typhimurium ΔsseK2	[39]
S. typhimurium ΔsseK3	[39]
S. typhimurium ΔsseK1ΔsseK2	[39]
S. typhimurium ΔsseK1ΔsseK3	[39]
S. typhimurium ΔsseK2ΔsseK3	[39]
S. typhimurium ΔsseK1ΔsseK2ΔsseK3	[39]