

FUNCTIONAL ANALYSIS OF *CLOSTRIDIUM SORDELLII* LETHAL AND  
HEMORRHAGIC TOXINS

By

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To my wife Lauren,

Without your support this would not have been possible

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## TABLE OF CONTENTS

DEDICATION .....	ii
ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES .....	vii
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	x
Chapter	
I. INTRODUCTION .....	1
<i>Clostridium sordellii</i> .....	1
<i>Clostridium sordellii</i> infection .....	1
Epidemiology .....	1
Symptoms.....	5
Treatment .....	6
Disease in livestock .....	7
<i>Clostridium sordellii</i> virulence factors.....	10
Pathogenicity locus and genetics .....	10
Role of toxins in disease and animal models.....	13
TcsH and TcsL.....	16
Large clostridial toxins .....	16
Host cell binding .....	19
Pore formation .....	23
Autoprocessing.....	25
Glucosyltransferase.....	28
Host GTPases.....	32
Role of Rac in cells.....	34
Role of Ras in cells.....	35
Localization of Rac and Ras GTPases .....	36
Research Objectives.....	37
II. COMPARATIVE STUDY OF TcsH AND TcsL WITH THEIR LARGE CLOSTRIDIAL TOXIN HOMOLOGUES FROM <i>CLOSTRIDIUM DIFFICILE</i> .....	40
Introduction .....	40
Methods .....	41
Results.....	45
TcsH and TcsL induce cytotoxicity in endothelial cells .....	45
Apoptosis or necrosis induced by TcsH and TcsL .....	46

Signaling events induced by TcsH and TcsL intoxication .....	49
Discussion .....	52
<b>III. CLOSTRIDIUM SORDELLII LETHAL TOXIN AUTOPROCESSING AND MEMBRANE LOCALIZATION ACTIVITIES DRIVE GTPASE GLUCOSYLATION PROFILES IN ENDOTHELIAL STUDIES .....</b>	<b>55</b>
Introduction .....	55
Methods .....	57
Results .....	61
TcsL induces cytotoxicity in mPMVEC cells .....	61
TcsL autoprocessing and glucosyltransferase activities are important for cytotoxicity .....	63
Mutations in the GTD membrane localization domain inhibit TcsL cytotoxicity...	67
Discussion .....	71
<b>IV. CONCLUSIONS AND FUTURE DIRECTIONS.....</b>	<b>75</b>
Conclusions .....	75
Future Directions.....	77
Determine mechanism of TcsH-induced necrosis .....	77
Determine the importance of TcsH enzymatic activities .....	79
Identify TcsL and TcsH surface binding receptors.....	81
Identify compounds that inhibit TcsH and TcsL cytotoxicity.....	83
<b>APPENDIX .....</b>	<b>85</b>
List of publications .....	85
<b>BIBLIOGRAPHY .....</b>	<b>86</b>

## LIST OF TABLES

Table		Page
1-1	Protein sequence identity between large clostridial toxins.....	18
1-2	Glucosylation profiles for the large clostridial toxins .....	30
2-1	Protein sequence identity between toxin domains.....	53
3-1	Plasmid information and primer sequences for TcsL and TcsL mutations.....	64

## LIST OF FIGURES

Figure	Page
1-1 <i>C. sordellii</i> pathogenicity locus .....	11
1-2 Domain structure of TcsH and TcsL .....	17
1-3 Model of TcsH and TcsL Intoxication.....	19
1-4 Structure of CROPs domain .....	21
1-5 Structure of GTPase switch region conformations.....	32
1-6 GTPase activation and inactivation .....	33
2-1 Cytotoxicity in HeLa cells.....	45
2-2 Cytotoxicity in mPMVECs .....	47
2-3 TcsH induces LDH release in endothelial cells.....	48
2-4 TcsL induces caspase activation .....	49
2-5 TcsH induces intracellular calcium release.....	50
2-6 ROS induction by TcsH and TcsL.....	51
3-1 mPMVECs as a model to study cytotoxicity .....	62
3-2 TcsL C698A is deficient in autoprocessing ability.....	63
3-3 TcsL autoprocessing and glucosyltransferase mutants are impaired in cytotoxicity .....	65
3-4 Kinetics of Rac1 versus Ras glucosylation differ when cells are treated with TcsL autoprocessing mutant .....	66
3-5 Autoprocessing mutation does not alter glucosylation.....	67
3-6 TcsL MLD location and mutations .....	68



3-7	MLD mutants disrupt lipid binding.....	69
3-8	TcsL membrane localization domain is important for cytotoxicity .....	70
3-9	Membrane localization domain is required for efficient Rac modification .....	71

## LIST OF ABBREVIATIONS

3T3	3-day transfer, inoculum $3 \times 10^5$ cells
BLAST	basic local alignment search tool
CHO	Chinese hamster ovary
CROPs	combined repetitive oligopeptides
GAP	GTPase-activating protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GTD	glucosyltransferase domain
GTP	guanosine triphosphate
IP6	inositol hexakisphosphate
kDa	kilodaltons
LCT	large clostridial cytotoxin
LDH	lactate dehydrogenase
MLD	membrane localization domain
mPMVEC	murine primary microvascular endothelial cells
NAC	N-acetylcysteine
PCR	polymerase chain reaction
ROS	reactive oxygen species
TcdA	<i>Clostridium difficile</i> toxin A
TcdB	<i>Clostridium difficile</i> toxin B

TcdC	<i>Clostridium difficile</i> toxin C
TcdE	<i>Clostridium difficile</i> toxin E
TcdR	<i>Clostridium difficile</i> toxin regulator
Tcna	<i>Clostridium novyi</i> alpha toxin
TcsE	<i>Clostridium sordellii</i> toxin E
TcsH	<i>Clostridium sordellii</i> hemorrhagic toxin
TcsL	<i>Clostridium sordellii</i> lethal toxin
TcsR	<i>Clostridium sordellii</i> toxin regulator
TpeL	<i>Clostridium perfringens</i> large cytotoxin
UDP	uridine diphosphate
USFDA	United States Food and Drug Administration
YAMC	young adult mouse colonic cell

## CHAPTER I

### INTRODUCTION

#### ***Clostridium sordellii***

*Clostridium sordellii* is a Gram positive, spore-forming anaerobic bacterium that is present in the environment, commonly found in the soil, and capable of causing infections in humans and livestock (1). *C. sordellii* was first characterized in 1922 by Alfredo Sordelli and was named *Bacillus oedematis sporogens* (2). Other early work identified it separately as *C. oedematoides* (1) before it was realized they were the same bacterium and the name was changed to *C. sordellii*. Some initially considered it to be a pathogenic form of *C. bifermentans* (3); there is high similarity between the bacteria except for the additional virulence factors expressed by *C. sordellii*. The bacteria produce two large exotoxins, TcsH and TcsL, which are the major virulence factors of infection (4–7).

#### ***Clostridium sordellii* infection**

##### *Epidemiology*

When *C. sordellii* was originally isolated and identified, it was seen in patients who suffered from gas gangrene and was thought to be a disease of war (3). During the world wars, a higher incidence of gas gangrene was seen in wounded or post-operative

soldiers, and it was noted that the longer it took for a wound to be dressed in the field, the higher the risk of infection due to increased exposure to the environment (8). Gas gangrenous infections were also seen at the site of surgeries during post-operative recovery of patients (1). Although the incidence of *C. sordellii* infections is relatively low, the severity of infection and high rate of mortality makes infection important (2).

Another frequent source of *C. sordellii* infection is in post-partum females. Women who have natural childbirth and those who undergo spontaneous abortions can develop *C. sordellii* infection. Several case studies report women experiencing abdominal pain and swelling that can progress to weakness, nausea, vomiting, and diarrhea (9, 10). Often the cases are thought to be a result of giving birth or operative birth so the doctors do not look for *C. sordellii* infection specifically when addressing the symptoms. By the time bloodwork positively identifies *C. sordellii*, the infection is often severe and the patient can quickly succumb to toxic shock syndrome-like conditions in a matter of a few hours or several days (2, 9, 10). It is not fully understood how the bacteria are able to enter the patient and cause infection, although some reports indicate clostridial species present in up to 18% of normal vaginal flora, and *C. sordellii* may be present long-term in the vaginal tract of up to 10% of women (2, 11, 12). Fecal contamination that enters the vaginal tract during childbirth is another source of possible infection. It is thought that the bacteria are able to enter the host through tears in the vaginal tissue or by directly accessing the cervix (2).

A major outbreak of *C. sordellii* infection was seen in women who underwent medically-induced abortions. Two drugs, mifepristone and misoprostol, are given orally to induce abortion, however many providers gave the drugs directly into the uterus. In

these patients, an increased occurrence of *C. sordellii* was seen (2, 13, 14). The symptoms reported in case studies are similar to those for postpartum females, with patients complaining of abdominal pain, weakness, and swelling, which despite treatment with antibiotics often leads to death (13, 14). As a result of the increase in infections, the United States Food and Drug Administration (USFDA) released a public health advisory in 2005 suggesting a possible link between the use of these two drugs and *C. sordellii* infections (15), and literature provided to health care providers offering abortions discusses the role of the drugs in deaths associated with *C. sordellii* (11). It was later shown that intrauterine injections of misoprostol in rats leads to increased mortality from *C. sordellii* due to a decreased macrophage and TNF- $\alpha$  response (16). After the USFDA advisory, the prevalence of mifepristone and misoprostol use has declined and so has the incidence of infection associated with medically induced abortions.

*C. sordellii* infections are also seen among injection drug users (17–20). Injection drug users, such as black tar heroin users, have been seen to develop infections in soft tissue at the site of drug injection. Drugs, particularly those cut with dirt as is seen in black tar heroin, are thought to become contaminated with *C. sordellii* spores that are then directly injected into the patients. The presence of *C. sordellii* on drug paraphernalia gathered from the homes of several patients has been observed (19). It is thought that the spores are able to germinate in the anaerobic conditions present at the site of injection and lead to infection. This was evident in one outbreak of *C. sordellii* infection that was documented in the San Francisco Bay area in the late 1990s and early 2000s resulting in a cluster of seven cases (18, 19). Around that same time,

Kimura documented the presentation of nine particular cases in Ventura County, near Los Angeles, CA that was also associated with black tar heroin use (20). The patients were noted to have used “skin popping” to inject the heroin subcutaneously, and the infections and necrosis were seen at these sites.

Infants have also been found to have *C. sordellii* infections. Shortly after birth, six neonatal patients were identified as having *C. sordellii*-associated necrotizing omphalitis, an infection of the remnant umbilical cord at the navel (21). The babies were seen to have gastrointestinal issues and were irritable at the early stages of infection, which then progressed to edema, tissue necrosis and thickening of the abdominal wall. While one infant survived following rapid and aggressive surgery to remove the infected tissue, the remaining five died within four days of initial onset and diagnosis. In each case it was suspected that the infection was a result of direct transmission from the mother based upon the presence of bacteria in umbilical discharge and the mother’s vagina (21).

*C. sordellii* infections may also occur, although extremely rare, due to general trauma or injuries which break the skin and allow for bacteria and spores to enter the tissue and grow in an anaerobic environment. One report is made of a 4.5 year old girl who developed swelling and pain in her big toe after jamming it on a door (22). The initial injury had caused minor bleeding at the toe nail and it is believed that the injury allowed *C. sordellii* entrance into the patient where the infection progressed. The patient suffered edema in her leg and lost the toenail at the site of infection but was able to survive after debridement and intensive use of antibiotics. This case was the first noted survival of a patient after soft tissue infection, and is most likely due to aggressive

treatment as well as the patient's ability to develop antibodies for the toxins produced (22). Another case was reported of an 81-year old woman who was found to have *C. sordellii* infection (23). This particular patient was seen to have necrosis which was focused around the anus and erythema extending from the genitals to the buttocks and lower back after a self-induced break in the skin lead to development of *C. sordellii* infection and sepsis. The patient died after deterioration of her symptoms despite aggressive treatment with antibiotics (23).

Although extremely rare, there has been one report in the literature of a patient becoming infected with *C. sordellii* following a tissue allograft. The 23-year old patient had undergone knee surgery and musculoskeletal tissue was obtained from a cadaver. The patient presented with symptoms similar to *C. sordellii* infection and blood culture tested positive. The patient rapidly succumbed to the infection and spurred the investigation of *Clostridium* infections in tissue allografts (24).

### *Symptoms*

*C. sordellii* infections are often difficult to diagnose due to the fact that the symptoms are often very non-specific, can be similar to symptoms of other insults, and are varied across different infection sites. Because *C. sordellii* infections have a low prevalence, doctors do not initially suspect it as the cause of patient symptoms. Often, initial infection will present itself as a mild rash or sensitivity at the site of infection and can be accompanied by weakness and leukocytosis, an elevated white blood cell count. One of the hallmarks of *C. sordellii* infection is the lack of an elevated body temperature, which masks the presence of a bacterial infection to health care providers (2).



As the infection persists and worsens, the patient's symptoms will become more severe and mimic those of toxic shock syndrome. The patient will begin to display hypotension and tachycardia, an elevated heart rate (2). Typically the development of more severe symptoms prompt health care providers to become more aggressive with treatment and the use of antibiotics. Further investigation can reveal the presence of necrotizing fasciitis and edema. Bloodwork may also reveal the patient to have reduced serum protein levels and elevated hematocrit. The infection will progress rapidly into fluid secretion as the capillaries become leaky and multi-organ failure occurs.

The lack of effective treatment means that the infection often ends in rapid death in a matter of hours or days after patients initially display symptoms and begin treatment at the hospital. Those patients who develop a fever early in the infection have been shown to have a higher rate of survival (2), likely because doctors will pursue a specific diagnosis and treatment earlier preventing as much damage to the infected patient. Those patients who are identified as injection drug users are also more likely to survive infection because the doctors are quicker to suspect *C. sordellii* as the cause of infection. In contrast, a post-partum female or those who have undergone trauma may experience symptoms such as tenderness and weakness due to a variety of reasons associated with childbirth or surgery related to the trauma.

### *Treatment*

Unfortunately, there is not an established effective treatment course for *C. sordellii* infection. In-depth studies and analysis of successful therapeutics have not been done due to the low frequency of infections as well as the quick onset of a high

rate of mortality (2). Early work to understand the gas gangrene infections seen on the battlefield indicates that these infections were given the highest priority upon diagnosis and that the best course of treatment was immediate debridement at the site of infection, treatment for septic shock, and supportive care (8, 25). To date, the best course of treatment is to start the patient on intravenous antibiotics as soon as infection is identified. Original studies suggest that *C. sordellii* is susceptible to penicillin, tetracycline, erythromycin, clindamycin, chloramphenicol, augmentin, and metronidazole (8, 26). Although more recent research indicates some antibiotic resistance may be present among various strains of *C. sordellii* (27), the use of antibiotics is still one of the only methods of treatment.

Along with antibiotics, debridement can increase the survival rate of infected patients. Emergency surgery allows the health-care provider to identify the area of tissue necrosis and to remove the dead tissue that contains high levels of both the *C. sordellii* bacteria and the toxins produced during infection (20)(25). Because *C. sordellii* infection is a toxin driven disease, after the bacteria have produced toxin and those toxins have begun to disseminate through the body, very little can be done. The use of antitoxin to prevent intoxication in animal models has suggested that treating with an antitoxin may have a protective effect, although the use of antitoxin in patients was discontinued in 1943 due to a lack of value seen during treatment (4, 7, 25).

#### *Disease in livestock*

Not only is *C. sordellii* responsible for serious infections in humans, it can also cause disease during infections in livestock. *C. sordellii* has been implicated in causing

gastrointestinal disease and gas gangrene in sheep and cows (4). The bacteria are also seen in horses, and as a result, many horses are given antitoxin vaccines to prevent disease (28, 29).

Infections of *C. sordellii* have been identified in the umbilical remnants of young foals. The infections present as omphalitis of the infected internal umbilical remnant and leads to the death of the foals (30). The tissue surrounding the navel was red and swelling was present along with cloudy, bloody liquid. Examination of the animals showed damage of the vasculature present in the umbilical remnant and hemorrhaging was present in the abdominal organs surrounding the navel. It is believed that the foals became infected through tears in the umbilical cord during birth, and *C. sordellii* intoxication and septicemia led to a fatal outcome in each case (30).

There have also been reports of *C. sordellii* infections in sheep and lambs. One study from Norway showed that *C. sordellii* was responsible for some cases of abomasal bloat, swelling of the fourth stomach in ruminant livestock. Out of 41 lambs that were tested for abomasal bloat, 8 (20%) tested positive for the presence of *C. sordellii* while no lambs lacking symptoms of abomasal bloat tested positive (31). The sheep who tested positive for *C. sordellii* were also more likely to show symptoms of hemorrhaging and ulcers along with the gaseous distension of abomasal bloat. Another case out of Argentina supports the idea that contamination from environmental sources into needle puncture or wound sites can allow for infection (32). One sheep became infected after a blood sample was taken without prior sterilization of the puncture site and the bedding environment was found to be unsanitary. The resulting *C. sordellii* infection lead to death of the sheep the morning after the procedure, and the body was

found to contain necrotizing fasciitis, severe edema, and hemorrhaging caused by the release of toxins around the location where the blood was drawn (32).

Interestingly, not all cases in livestock are infections of soft tissue but can be gastrointestinal infections instead. One report tells of a horse who was experiencing abdominal discomfort and depression after importation from Holland (33). The symptoms progressed to increased abdominal discomfort, depression, agitation, and neurologic symptoms that seemed consistent with encephalopathy. Examination revealed a gas-distended colon and an increased ammonia level in the blood, among other symptoms. Aggressive treatment led to the lessening of severe symptoms after four days, and the horse experienced several cases of watery diarrhea. Fecal samples revealed large quantities of *C. sordellii* present, which was suspected as the cause of disease due to the fact that *C. sordellii* produces a urease which can break down proteins and cause a buildup of ammonia, as was seen in this incident. The veterinarians suspected that the disruption and stress of importation upon the gastrointestinal tract allowed the bacteria to overgrow and lead to the infection and symptoms (33).

Interestingly, there has also been a recent report of *C. sordellii* infection in captive lions. A zoo in Spain had five adult lions die after developing depression, lethargy and loss of appetite and treatment with intravenous fluids and antibiotics was unsuccessful (34). After testing for infectious diseases and parasites, only *C. sordellii* was found in the infected lions. The cause of the infection was traced to contaminated water available only to the infected lions, and no other animals were affected (34). This

case shows the importance of the environmental presence of *C. sordellii* and spores that can lead to infection in animals upon ingestion.

### ***Clostridium sordellii* virulence factors**

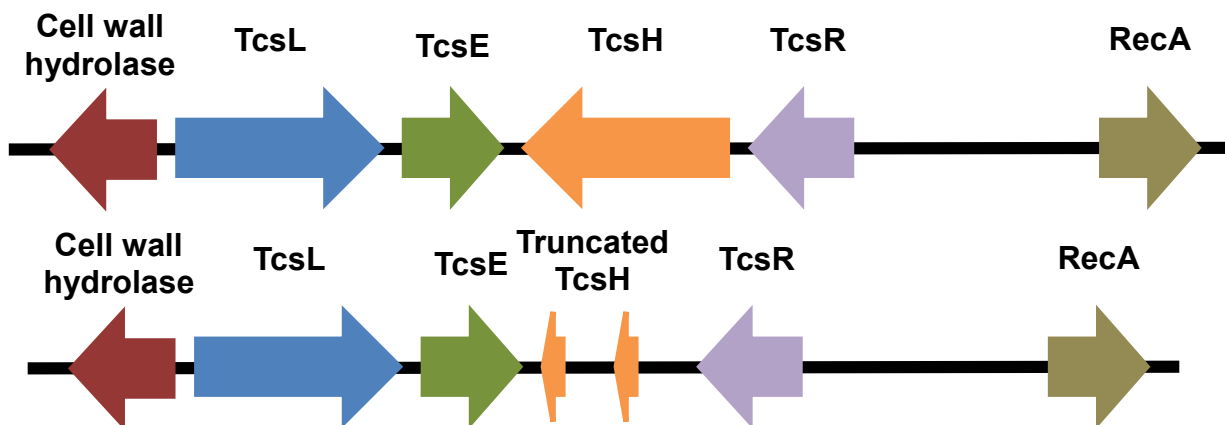
#### *Pathogenicity locus and genetics*

The gene for TcsL expression was first published in 1995 by making probes for the repetitive C terminus based on the sequence of the TcdB, a toxin from *C. difficile* which has been shown to be biologically and immunologically similar to TcsL (35). The sequence for TcsL supported the high homology to TcdB of *C. difficile* (76%) and also indicated the presence of a hydrophobic region between amino acids 980 and 1045 as well as the presence of four conserved cysteines between TcsL and TcdB, suggesting possible mechanistic roles for these amino acids (35).

The genomes of two strains of *C. sordellii* have been sequenced and analyzed (36). The work was able to identify a pathogenicity locus that shared many similarities to those seen in other *Clostridium* species, particularly *C. difficile* (36). The gene for TcsL was identified in the pathogenicity locus based on the previously identified sequence and was conserved between the two strains sequenced. Located just downstream of *tcsL* is the *tcsE* gene which is thought to encode a holin-like protein similar to TcdE of *C. difficile*. In *C. difficile*, TcdE has been suggested to be important in secretion of toxins TcdA and TcdB (37). Of the two *C. sordellii* strains sequenced, one contained a full-length gene for expression of TcsH while the other contained a truncated *tcsH* gene.

One of the unique characteristics of the *C. sordellii* pathogenicity locus is that *tcsH* and *tcsL* are expressed in opposite directions (36).

Another gene that has been identified on the *C. sordellii* pathogenicity locus is *tcsR* which is homologous to other sigma-factor genes (36). The gene is located upstream of *tcsH* and is transcribed in the same direction, opposite to *tcsL*. When *TcsR* was deleted, it was seen that the expression of *C. sordellii* cytotoxins *TcsH*, *TcsL*, and *TcdE* was decreased and the expression levels were recovered when *tcsR* was inserted back into the genome (36). This supports the understanding that *TcsR* is a regulatory sigma factor responsible for driving toxin expression. Interestingly, other clostridial pathogenicity loci contain an anti-sigma factor, such as *tcdC* in *C. difficile*, that is responsible for regulating the sigma factor (*TcdR*) activity and down-regulating toxin expression, but there is no homologous gene found on the pathogenicity locus of *C. sordellii* (36)(38).



**Figure 1-1: *C. sordellii* pathogenicity locus.** The major genes expressed in the pathogenicity locus are shown, including the direction of expression for a strain expressing *TcsH* (top) and one lacking *TcsH* expression (bottom). The genes shown are cell wall hydrolase (red), *tcsL* (blue), *tcsE* (green) *tcsH* and truncated *tcsH* (orange), *tcsR* (light purple), and *recA* (tan). Strains VPI 9048 (top) and ATCC 9714 (bottom) represented above. Adapted from Sirigi Reddy *et al.* (36).

The pathogenicity locus for *C. sordellii* contains characteristics that suggest it is a mobile element within the bacterial genome. Sequencing identified several genes, such as transposase elements and *recA*, that are important for gene mobility and homologous recombination (36). This supports the idea that is held for *C. sordellii* and other toxin-expressing *Clostridium* that the toxin genes and pathogenicity loci may have derived from one source that was then shared between the species (39). The understanding of the pathogenicity locus as a mobile element of the genome also explains why not all *C. sordellii* strains and isolates contain the pathogenicity locus or contain both *tcsH* and *tcsL* genes (40)(28).

Work done using PCR amplification has identified the presence of tetracycline resistance genes in several *C. sordellii* isolates from cattle (27). The amplified genes were identified as *tetA(P)* and *tetB(P)* which have been shown to be located on transposons within the bacterial genome (27). The study showed that both genes were present in each of three *C. sordellii* isolates studied and that each isolate was resistant to oxytetracycline while two of the isolates were resistant to lincomycin.

Until recently, genetic manipulation in *Clostridial* species has been extremely difficult. The bacteria are resistant to DNA transformation so the introduction of outside DNA and plasmids is difficult (41). In 2007, the Nigel Minton group created a ClosTron gene knockout strategy that relies on *E. coli* conjugation to introduce a plasmid that can recombine with the bacterial genome to introduce a targeted knockout (42). So far, this technique has only been used twice in *C. sordellii*, to introduce a deletion of *tcsL* and *tcsR* (5, 36). Because of the limited number of studies in *C. sordellii*, a lot of questions still remain regarding the importance and impact of specific genes.

### *Role of toxins in disease and animal models*

*C. sordellii* is known to produce a number of toxins that are implicated in driving the disease seen during infection. Published in 1962, MacLennan describes that *C. sordellii* produces a lecithinase (later known as phospholipase C), a hemolysin, a fibrinolysin, a urease, and lethal toxin (TcsL) (3). Evidence for expression of a hemorrhagic toxin, called TcsH, was identified in 1969 as well as a deoxyribonuclease a few years later (4).

Lecithinase, or phospholipase, production has been observed in *C. sordellii* as well as other *Clostridium* species: *C. perfringens*, *C. bifermentans*, and *C. novyi* (3, 43, 44). Although the lecithinase activity of *C. sordellii* was identified by the 1940s, the characterization was limited to comparisons to other clostridial lecithinases and the antigenic similarity to those same lecithinases (43, 44). A more detailed study published in 2003 cloned the *C. sordellii* phospholipase and tested its ability to hydrolyze egg phospholipids as well as test hemolysis activity. The authors found that *C. sordellii* phospholipase was active in both enzymatic assays, although at decreased rates (38.2% and 7.6%, respectively) when compared to the phospholipase of *C. perfringens*, with which it shares 53.4% amino acid identity (45).

*C. sordellii* has been shown to express a gene (*nanS*) that encodes for a neuraminidase protein (46). The neuraminidase is a cysteine dependent enzyme that is responsible for removing sialic acid moieties and is believed to be important in infection. Previous work has shown that the neuraminidase can increase the proliferation ability of a promyelocytic cell line. The increase in cell proliferation is believed to be important for



the leukemoid reaction, or increase in white blood cells in the bloodstream, that occurs during infection and correlates with mortality outcomes (46).

Another toxin that is produced by *C. sordellii* is a cholesterol-dependent cytolysin called sordellilysin (40). The sordellilysin gene was identified in *C. sordellii* isolates using the sequence for *Clostridium perfringens* perfringolysin O and expression was detected using antibodies to perfringolysin O (40). Experiments performed with different cell lines showed that sordellilysin is capable of inducing cytotoxicity in cells and that the concentration of sordellilysin required was ten-fold higher than when compared to the concentration needed for TcsL-induced cytotoxicity in mice (40).

The two main toxins of *C. sordellii* are the hemorrhagic toxin (TcsH) and lethal toxin (TcsL). Crude filtrates and culture broth were seen to induce edema and lesions that led to death when injected into guinea pigs (47). TcsL was shown to be an exotoxin based on the observation that rabbit antiserum provided protection when injected into mice alongside TcsL (1). It was not until several decades later that Arseculeratne, Panabokke, and Wijesundera presented data suggesting a second major toxin was produced by *C. sordellii* that was responsible for hemorrhaging when injected into guinea pig skin (4). The hemorrhaging caused by what would later be known as TcsH was also seen when crudely purified toxin was injected into the skin and tissue of rabbits, mice, and rats (4).

TcsH purification and characterization was first reported by Ramon Martinez and Tracy Wilkins in 1988. They found TcsH to be 300 kDa in size with a pI of 6.1 based on isoelectric focusing (7). When the toxin was injected into rabbit ileal loops, fluid accumulation and hemorrhaging occurred (7) and similar hemorrhaging and destruction

of the mucosal layer was seen when TcsH was injected into a guinea pig ileal loop (6). After initial characterization, further work on TcsH focused primarily on the cytotoxic effects in cell culture.

The purification and characterization of TcsL was published in 1987 by Michel Popoff. The original work suggested TcsL was a 250 kDa protein (6); we know now it is 270 kDa. TcsL was shown to be lethal when injected into mice and caused edema and erythema when injected into guinea pig skin. TcsL also induced fluid accumulation in an ileal loop of guinea pigs, but this was not accompanied with any histological changes (6). In 2007, it was shown that when TcsL was injected intraperitoneally into mice, TcsL killed the mice by causing vascular permeability in the lungs and massive fluid secretion (48). The mice initially displayed a loss of mobility and ataxia, darkened tails, and signs of dehydration 6 hours after toxin injection. The fluid build-up in the thoracic cavity occurred within hours of TcsL injection, and edema was seen around blood vessels in the lung. The hearts of intoxicated mice were also shown to exhibit edema (48), which suggests TcsL acts upon endothelial cells to disrupt tight junctions and make the vasculature leaky, something that has been reported in cell culture (49).

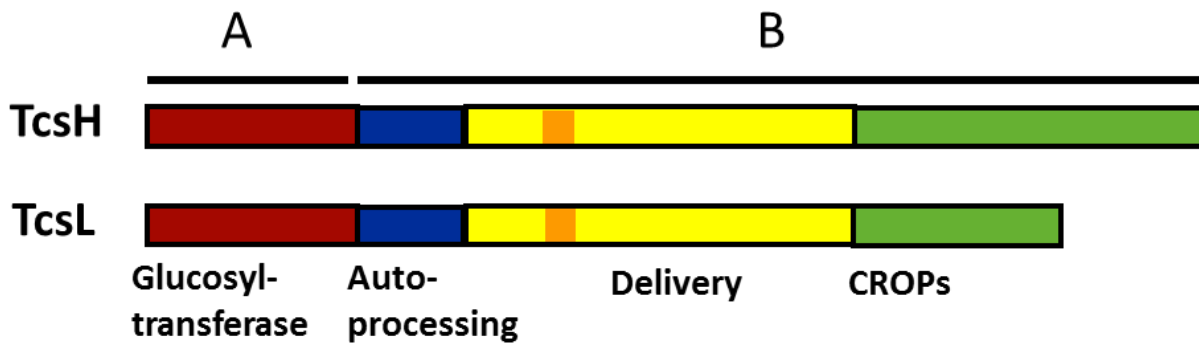
TcsL is thought to be the main virulence factor in *C. sordellii* based on several studies. A study was performed using a clinical isolate of *C. sordellii* lacking TcsL expression compared to a TcsL-positive strain (50). The authors saw that infection of mice and rats with the TcsL-negative strain did not lead to death, and the infected rodents also lacked symptoms often associated with *C. sordellii* infection, such as decreased mobilization and signs of dehydration. It was also seen that intoxication with cultures from the TcsL-negative strain did not lead to death in the animals but all the

animals intoxicated from the TcsL-positive strain died (50). Work published a year later also showed that TcsL is required for virulence in *C. sordellii* infections. Glen Carter and colleagues were able to introduce a mutation into *tcsL* of the *C. sordellii* genome which prevented expression of TcsL (5). The mice infected with the TcsL-deficient strain all survived while none of the mice infected with the wild-type strain did, and the TcsL-deficient strain did not induce symptoms of illness. Further infection of the uterus of mice with the two *C. sordellii* strains showed that the TcsL-positive strain induced edema of the uterine tissue, which was absent in mice infected with the mutant strain (5). This shows the importance of TcsL as the primary virulence factor for *C. sordellii*.

## **TcsH and TcsL**

### *Large clostridial toxins*

The large clostridial toxins (LCTs) are a group of toxin proteins expressed by species in the bacterial *Clostridium* family. The family consists of the lethal toxin (TcsL) and hemorrhagic toxin (TcsH) from *C. sordellii*, TcdA and TcdB of *C. difficile*,  $\alpha$ -toxin of *C. novyi* (Tcn $\alpha$ ), and *C. perfringens* large cytotoxin (TpeL). The toxins are large proteins ranging from 250 to 307 kDa in molecular weight and are expressed as a single protein. Each toxin is an AB toxin that consists of an enzymatic A portion and a B portion responsible for host cell binding and translocation. The toxin activity relies upon the enzymatic domain to transfer a glucose moiety from host cytosolic UDP-glucose, or N-acetylglucosamine for Tcn $\alpha$ , onto small GTPases in the host cell. Each toxin consists of four distinct subdomains: a CROPs (combined repetitive oligopeptides) domain, pore



**Figure 1-2: Domain structure of TcsH and TcsL.** TcsH and TcsL contain four distinct domains: glucosyltransferase domain (red), autoprocessing (blue), delivery or translocation domain (yellow), and a CROPs domain (green). The toxins are AB toxins consisting of an A enzymatic portion and a B region that is responsible for delivery of the toxin's enzymatic portion.

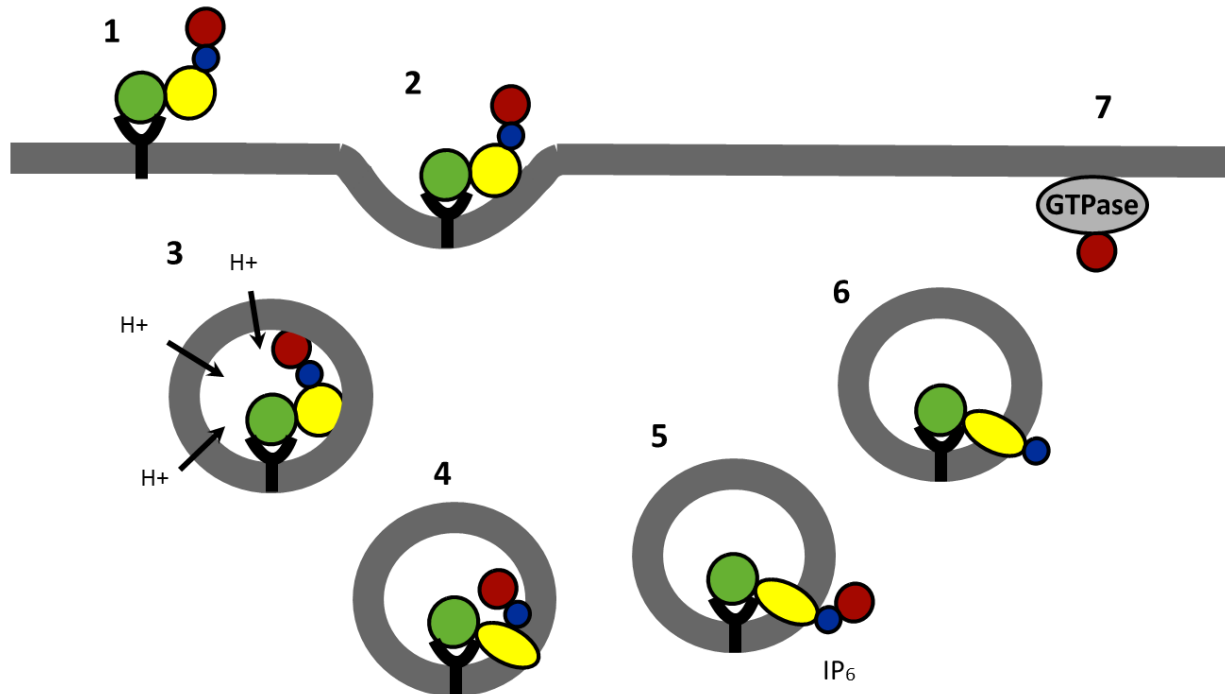
formation/translocation domain, autoprocessing domain, and enzymatic or glucosyltransferase domain (GTD) (Figure 1-2).

The LCTs are homologous and share a high level of sequence identity. The highest identity is shared between TcdA and TcsH and between TcdB and TcsL (Table 1-1). The high homology across species supports the idea that the toxins may have originated from a common ancestor and that a possible duplication event gave rise to TcdA and TcdB of *C. difficile* and TcsH and TcsL of *C. sordellii*. Much of the research performed on LCTs has been done on TcdA and TcdB, but because of the high sequence and structural homology, the model of intoxication and mechanisms of toxin action are thought to be extremely similar.

Holotoxin	TcdA	TcdB	TcsH	TcsL	Tcna
TcdA					
TcdB	48%				
TcsH	78%	48%			
TcsL	48%	76%	49%		
Tcna	31%	30%	31%	30%	

**Table 1-1: Protein sequence identity between large clostridial toxins.** Protein sequences for TcdA and TcdB from *C. difficile* strain 10643, TcsH and TcsL from *C. sordellii* strain JGS6382, and Tcna from *C. novyi* ATCC 19402 (168) were compared by Protein BLAST. The values shown indicate the amino acid sequence identity between each of the large clostridial toxins.

The large clostridial toxins of *C. sordellii* and *C. difficile* are not only structurally homologous but the sequence conservation resulted in similar epitopes that provide cross-reactivity between antibodies. When TcsL was first purified and characterized, it was shown that the antibody that neutralized TcsL was also able to recognize and bind TcdB (6). Purification of TcsH and studies using antiserum for TcsH also showed specificity for TcdA as well (7). The similarity seen between TcsH/TcdA and TcsL/TcdB was recognized before the toxins were fully characterized (51), and it was even thought that pseudomembranous colitis, caused by *C. difficile* infection, may have been caused by *C. sordellii* because antiserum toward TcsH and TcsL protected against colitis (52, 53). Even after *C. difficile* toxins were implicated as responsible for causing pseudomembranous colitis, antitoxin toward TcsH and TcsL was studied for its



**Figure 1-3: Model of TcsH and TcsL Intoxication.** (1) TcsH and TcsL enter cells by first binding to a cell receptor at the plasma membrane and (2) being taken up into the cell through clathrin-mediated endocytosis. (3) The toxin is then trafficked to the endosome which undergoes a drop in pH during maturation. (4) The pH drop facilitates conformational change of the translocation domain (yellow) and allows for the formation of a pore through which the autoprocessing domain (blue) and GTD (red) are shuttled to the host cytosol (5). In the cytosol, the autoprocessing domain encounters host IP<sub>6</sub> and cleaves the GTD (6) and the free GTD travels to and glucosylates host GTPases (7).

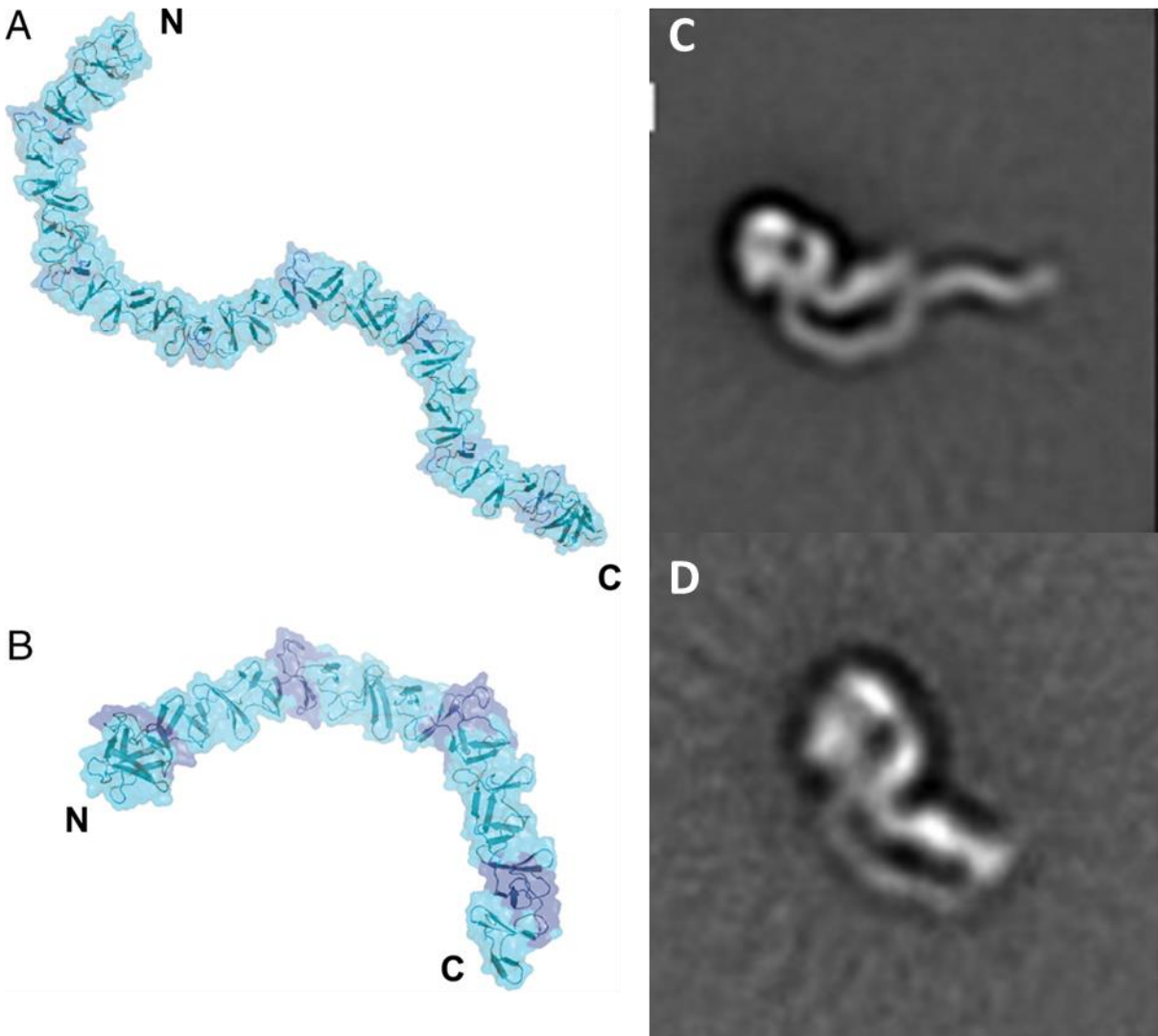
protective effects (54), supporting the significance of the sequence similarity shared between TcsH/TcdA and TcsL/TcdB.

#### *Host cell binding*

The C-terminus domain of both TcsH and TcsL contains amino acid repeats that have been described as the host cell binding domain in TcdA. The TcdA C-terminal sequence was shown to have a series of combined repetitive oligopeptides, or CROPs. TcdA contains seven long repeats of 90 amino acids and thirty-one shorter repeats of 60 and 63 amino acids (55). The short repetitive amino acid sequences were grouped

into five CROPs regions that are separated by long repeats (56). A crystal structure was obtained of a portion of the TcdA CROPs in 2005 and used to create a model for the entire domain of TcdA as well as a model of the shorter TcdB CROPs as well (57). The proposed structures for the TcdA and TcdB CROPs were supported by electron microscopy imaging that showed the extended CROPs in the context of the holotoxin (Figure 1-4) (58). The modular nature of the repetitive repeats in the binding domains, which has also been seen in the sequences of TcsH and TcsL, suggests a similar structure for the CROPs that can be easily modeled by adding on or subtracting the correct number of repeats from the structure.

Work implicating the CROPs in host cell binding was performed for TcdA. It was shown that the TcdA CROPs is able to recognize sugar moieties found on the surface of host epithelial cells *in vitro* (59). The importance of the C-terminal repeats of the CROPs for cell binding was also shown separately by Frey and Sauerborn. Frey was able to show that an antibody (PCG-4) which protects against TcdA enterotoxicity, binds to the CROPs region and inhibits cell binding (60, 61). Sauerborn and his colleagues



**Figure 1-4: Structure of CROPs domain.** Models based on the crystal structure of a single CROP repeat were created for TcdA (A) and TcdB (B) showing the structure of the entire domain by combining repeats of the solved structure. EM structures for TcdA (C) and TcdB (D) holotoxins confirm that the modeling is consistent with the CROPs regions seen in each toxin. Adapted from Ho et al. and Pruitt et al. (57, 58).

expressed short segments of the TcdA repeats and showed that treatment of cells with the repeats protected the cells from intoxication by TcdA (62). The work also shows that protection of cells was achieved by the use of antibodies specific to the CROPs region. Support for the importance of TcdA binding to sugars was shown when cells were treated with purified lectins, which decreased the cytotoxicity of TcdA.



The binding of TcdA to carbohydrate was shown in a co-crystal structure of a TcdA CROPs segment with  $\alpha$ -Gal-(1,3)- $\beta$ -Gal-(1,4)- $\beta$ -GlcNAcO(CH<sub>2</sub>)<sub>8</sub>CO<sub>2</sub>CH<sub>3</sub> (63). The crystal structure suggests that TcdA (and perhaps other LCTs) bind glycolipids and/or glycoproteins and that multiple binding sites increase the avidity of the toxin binding to the host cell, even if the affinity for individual carbohydrates is low. The ability of TcdB to bind carbohydrates was shown to be similar to TcdA, and both displayed tolerance for variation in the glycolipid receptors (64).

The role of the CROPs in TcdB binding is less clear. Bezlotoxumab is an antibody that had been developed by Merck for the treatment of *C. difficile* infections and has shown protection against recurrence of *C. difficile* infection in clinical trials (65). The antibody binds specifically to the CROPs region on TcdB and the complex has been crystallized. This antibody interaction protected cells from TcdB cytotoxicity (65). This work suggested that the CROPs of TcdB was also important for cell binding.

Interestingly, recent work has shown that the CROPs of large clostridial toxins may not be the only binding site for toxin receptors. Olling and colleagues used a truncated TcdA lacking the CROPs to show that binding and intoxication of cells was still possible (66). When truncated, CROPs-less TcdA was added to 3T3 and HT29 cells, the toxin still showed cytopathic effects, although at a 5 to 10-fold lower potency than full-length TcdA, but was just as potent as full-length TcdA on CHO cells. The cytopathicity of truncated TcdA was not inhibited by either full-length TcdA or CROPs domain peptides, suggesting that a separate binding event was responsible for the cytopathicity of truncated TcdA. Recent work from our lab identified PVRL3 as a receptor for TcdB. A truncated version of TcdB that does not contain the CROPs also

binds the receptor (67). Another report found that TcdB was able to bind to chondroitin sulfate proteoglycan 4 and be internalized in cells, and that the binding did not occur with the CROPs of TcdB alone (68). These works suggest a model of binding for large clostridial toxins where two binding events occur, one mediated by the CROPs that relies on binding to carbohydrates and a second binding event that relies on a receptor binding to the toxin outside of the CROPs.

After binding, the toxins are internalized into cells. The internalization of TcdA and TcdB has been reported to occur by clathrin-mediated endocytosis (69). TcdB-induced cell rounding was inhibited by dynasore, an inhibitor of dynamin which is important for pinching off endocytic vesicles, and by chlorpromazine, a chemical that prevents clathrin-coated pits from forming. Clathrin-mediated endocytosis of TcdB was also implicated by the siRNA depletion of clathrin heavy chain and the use of a dominant-negative dynamin. The same inhibition of cell rounding was seen for TcsL, TcdA, and Tcn $\alpha$  when cells were treated with dynasore and chlorpromazine (69). The work shows that TcdB internalization occurs by clathrin-mediated endocytosis and suggests the same mechanism of internalization is conserved for all the large clostridial toxins.

### *Pore formation*

Work was done to understand the mechanisms of cell entry for the LCTs. The authors showed that TcdB enters cells through endocytosis based on the inability of antitoxin to prevent cytopathicity after intoxication and due to a decrease in toxicity when cell lines that are deficient in endosomal acidification were intoxicated (70).

Qa'Dan then showed that the cytopathic effects of TcsL were blocked when cells were treated with endosomal and lysosomal acidification inhibitors such as bafilomycin and ammonium chloride (71). The work also showed that the cytopathic effects on cells occurred faster when the toxins were exposed to a low pH environment, under pH 5.5, than compared to toxins held at a neutral pH. They were also able to show that exposure of TcsL to acidic pH causes a conformational change in the toxin that allows for an increased exposure of hydrophobic regions, based on an increase in TNS fluorescence (71). Studies done previously by the same group showed the reliance on pH for cytopathic effects and conformational change to also be true for TcdB (72). This supported that delivery of the enzymatic GTD required a conformational change in the toxin that allowed delivery into the host cell, which occurs commonly in AB toxins to deliver the enzymatic A portion. Work was able to show this conformational change in the translocation domain by EM by exposing TcdA to neutral and acidic pH and comparing the structure (58).

Based on the knowledge that a conformational change, most likely occurring during endosomal maturation, was responsible for toxin entry and intoxication, Barth et al. looked at whether TcdB was capable of forming pores in the membrane. They measured the ability of TcdB to form pores in cells loaded with radioactive <sup>86</sup>rubidium (<sup>86</sup>Rb) (73). By adding TcdB and decreasing the extracellular pH, the cells (both Vero and CHO) released <sup>86</sup>Rb indicating the formation of a pore. Further, they showed that the pore formation was blocked by an antibody that prevented cell binding but not blocked by an antibody for the GTD, thus localizing the pore formation ability to the non-enzymatic portion of the toxin. The localization of the pore forming activity was

supported by the use of a TcdB construct lacking the GTD that was still able to cause  $^{86}\text{Rb}$  release. The authors also performed the  $^{86}\text{Rb}$  release assay with TcsL and showed that TcsL induces pore formation after the toxin environment is acidified (73). The ability of TcdA, which is highly homologous to TcsH, to form pores was shown as well by the release of  $^{86}\text{Rb}$  in HT-29 cells (74), supporting the conserved nature of pore formation for large clostridial toxins.

Further work published in 2003 was done focusing on the localization of the autoprocessing domain of TcdB during intoxication. The authors showed that the cleaved GTD was found in the cytosol after endocytosis by cell fractionation and they showed that the C-terminal portion of TcdB containing the binding and pore formation domains remained bound to the membrane (75). Although previous work suggested that the membrane bound portions of TcdB were located in endosomal membrane, Pfeifer showed that the TcdB C-terminus was in the endosome based on immunofluorescent co-localization with Rab5, an endosomal marker, while the TcdB GTD fluorescent signal became diffuse in the cytosol following intoxication.

### *Autoprocessing*

The cleavage site for the large clostridial toxins was not known until 2005. It was found to be located between the leucine 544 and glycine 545 in TcdB based on holotoxin cleavage in the context of cells (76). The location of cleavage corresponds to leucine 543 in TcsL and leucine 542 in TcsH from *C. sordellii* strain JGS6382. It was originally believed that the cleavage was performed by a host cell protease yet the autoprocessing was not inhibited even in the presence of a variety of cell protease

inhibitors (76). Work done by Reineke et al. to identify the host factor involved in cleavage of the holotoxin was surprising in that it was not a host protease but a small molecule, inositolphosphate, that was identified as required for toxin cleavage (77). They identified inositolhexakisphosphate (IP6) as the compound that, when combined with TcdB, had the highest cleavage activity. It was shown that IP6 was also necessary for cleavage of TcsL, as well as other LCTs TcdA and Tcn $\alpha$ .

Further work was able to identify the mechanism of action for autoprocessing in the large clostridial toxins. Using TcdB, Egerer et al. showed autocatalysis was inhibited by the addition of *N*-ethylmaleimide, an inhibitor of cysteine-dependent reactions (78). They were also able to identify a cysteine, C698, which is conserved among the large clostridial toxins and homologous to a cysteine present in another cysteine proteases, RTX toxin of *Vibrio cholera* (79). When the cysteine was mutated to an alanine, the autoprocessing ability of TcdB was inhibited (78).

In 2009, Rory Pruitt from our lab determined the crystal structure of the TcdA autoprocessing domain (80). In this paper, he defined the autoprocessing domain as amino acids 543-809 for TcdA. This corresponds to amino acids 544-807 in TcsL based on sequence homology. Using his crystal structure, Pruitt determined that IP6 bound one face of the toxin domain and that the IP6 binding site was separated from the autoprocessing active site by a  $\beta$ -flap structure. Pruitt also showed that when IP6 bound to the autoprocessing domain, it induced a conformational change, and proposed that the added stability of IP6 binding allows for the autoprocessing domain to be in the active conformation (80).

The first study of the TcsL autoprocessing domain was published in 2011 by Guttenberg and colleagues. In this paper, they expressed a fragment of TcsL that contained the glucosyltransferase domain and the autoprocessing domain, amino acids 1-807 (81). As was seen previously with TcdA and TcdB, TcsL autoprocessing was induced with addition of IP6 to the TcsL toxin fragment and the glucosyltransferase domain was released. This study showed that the autoprocessing of TcsL did not require any other toxin domains beyond the first 807 amino acids. Interestingly, when the cleavage of the GTD was performed with the TcsL holotoxin at pH 7.4, the autoprocessing efficiency was greatly diminished and the toxin was much more stable, even when compared to TcdB or Tcn $\alpha$ . When TcsL was shifted to a lower pH, more IP6 was able to bind and the autoprocessing activity increased (81).

Guttenberg also showed that when the catalytic cysteine (C698) was mutated to an alanine, cleavage of the toxin fragment and release of the GTD no longer occurred, even in the presence of 100-1000 fold more IP6 than needed for the original TcsL fragment (81). When Vero cells were treated with TcsL C698A, the cells did not show cytopathic effects after six hours compared to cells treated with wild-type TcsL. This supported the identification of the conserved cysteine, which had previously been identified for TcdA and TcdB, as important for toxin autoprocessing in the large clostridial toxins and suggested that autoprocessing and release of the GTD is important for toxin activity. Interestingly, recent work has shown that the loss of autoprocessing activity in TcdB does inhibit cytotoxicity (82).

## *Glucosyltransferase*

Studies to determine the localization of the enzymatic domain, which would become known as the glucosyltransferase domain (GTD), began to be published in 1997. It was known by then that the C-terminus of the toxin was involved in cell binding and that the middle portion of the toxin played a role in translocation, so it was believed that the N-terminus of the LCTs would be important for the enzymatic activity. Fred Hofmann first published data supporting this when he expressed truncation mutants of TcdB focusing on the N-terminus. He found that the fragment expressing amino acids 1-546 of TcdB was the shortest fragment that kept full glucosylation activity against targeted GTPases (83). He was able to confirm this when TcdB 1-546 was microinjected into NIH 3T3 cells and the toxin fragment induced cytopathic effects similar to full length TcdB.

The TcsL glucosyltransferase domain was defined as amino acids 1-546 through the creation of a truncated TcsL fragment that was capable of full glucosylation (84). This work showed that for TcsL, truncation to a fragment containing amino acids 1-546 did not lose glucosylation activity, but a fragment of amino acids 1-517 did show decreased glucosylation activity. The definition of the GTD and its role in GTPase specificity was supported when chimeras were made, exchanging fragments of TcdB and TcsL. Based on the glucosylation profiles (as described further below), Hofmann was able to show that amino acids 364-516 largely play a role in determining whether the GTD has a TcdB-like or TcsL-like glucosylation profile (84).

It has been observed that treatment of cells with TcsL causes cell rounding and disruption of the actin fibers of the cytoskeleton. The use of UDP-[<sup>14</sup>C]-glucose showed

that TcsL was able to glucosylate small 21 kDa proteins in HeLa cells and when individual GTPases were used, TcsL was able to glucosylate HRas, Rap2, and Rac1 but not Cdc42 or RhoA (85). TcsL was shown to glucosylate Rac1 and HRas in NIH 3T3 cells (86). The glucosylation required UDP-glucose, which was shown when cells lacking UDP-glucose did not show cytopathicity after TcsL treatment until UDP-glucose was microinjected into cells (85). This same work was able to localize the glucosylation of the GTPases by TcsL to the threonine at amino acid 35 based on the presence of radioactively labeled UDP-glucose on protein fragments of treated HRas. The authors were also able to show that the glucosylation occurred after internalization of TcsL by microinjecting TcsL into cells and seeing cytopathicity as well as the ability to protect cells by injecting anti-TcsL antibodies into cells to block the toxin activity.

The exact GTPases targeted by TcsL seem to be dependent on the strains of *C. sordellii* from which the toxin is isolated. TcsL can modify Rac, Ras, and Rap (as described above) but can also glucosylate Cdc42 or Ral, depending on the strain of origin (87). The same work also showed that Ral is glucosylated at threonine 41, the equivalent of threonine 35 in other targeted GTPases.

The first study looking at the specificity of glucosylation by TcsH was published by Harald Genth and colleagues in 1996. They found that TcsH glucosylated RhoA, Rac1, and Cdc42 but not HRas, Rap1, Ral or several other GTPases (88). The TcsH glucosylation of RhoA was shown to occur at threonine 37, equivalent to threonine 35 of other GTPases as previously reported for TcsL, and that the glucosylation also occurred *in vivo* in NIH 3T3 cells. In depth studies of the glucosylation profiles of the large clostridial toxins, including TcsH and TcsL, have been done using both a proteomics



approach of detecting glucosylation in cells (89) as well as *in vitro* glucosylation reactions using recombinant GTPases and GTDs from a variety of LCTs (90). A summary of glucosylation profiles is shown in Table 1-2. It is of note that the glucosylation profile of TcsH is like that of TcdA and TcdB of *C. difficile* and differs from the glucosylation profile of TcsL.

	Rho (A/B/C)	RhoG	Rac1	Cdc42	H/K/N Ras	RRas	Ral (A/B/C)	Rap1	Rap2
TcsH-9048	+	+	+	+	*	*	*		*
TcsL-9048	*	*	+	*	+	*	+	+	+
TcsL-82	*	*	+		+	+	+	+	+
TcsL-6018		+	+	+	+	+	+	+	+
TcdA-10463	+	+	+	+	*		*		*
TcdA-C34	+	+	+	+				+	+
TcdB-10463	+	+	+	+					
TcdB-C34	+	+	+	+			+	+	+
TcdB-CD196	+	+	+	+					
TcdB-1470		*	+	+	*	+	+	+	+
Tc $\alpha$	+	*	+	+					
TpeL			+		+	*	*	+	

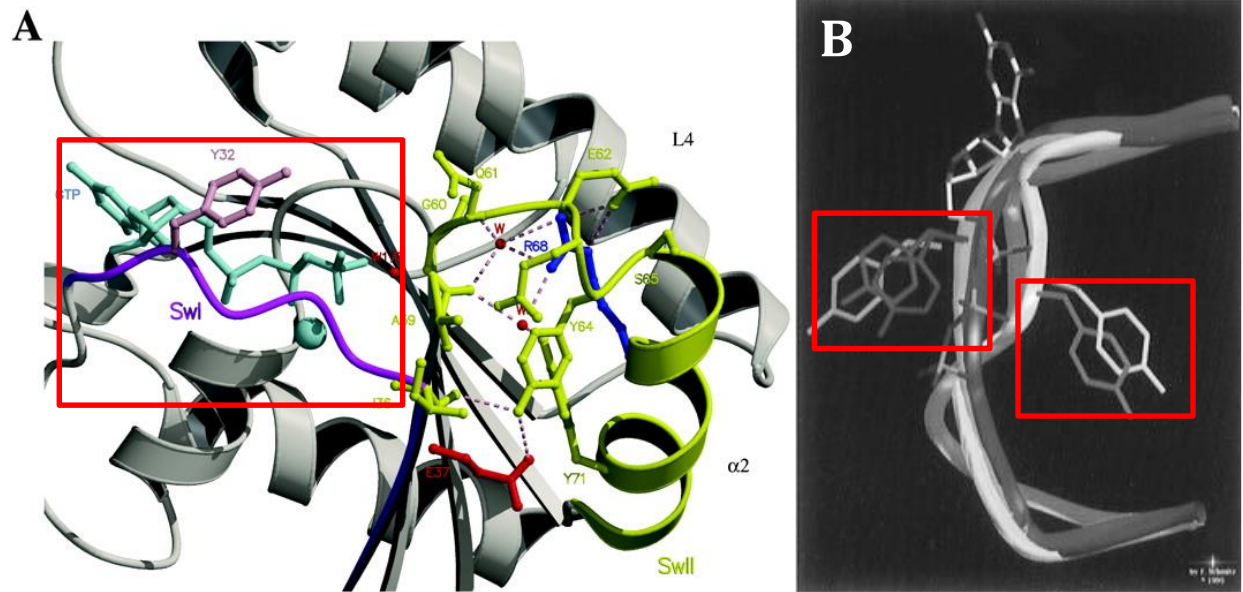
**Table 1-2: Glucosylation profiles for the large clostridial toxins.** Toxins from different strains were analyzed for their ability to modify Rho and Ras family GTPases and the profiles were summarized. + indicates a target for glucosylation and \* indicates glucosylation has been seen at low levels in some *in vitro* studies. Adapted from Zeiser et al. and Genth et al. (89, 90).

It has been shown that for the glucosyltransferase domain to perform its enzymatic function, it requires a divalent cation. Based on work showing that other glucosyltransferases are stimulated by a manganese ion, Just and his colleagues tested whether this was true for TcsL GTD. They found that TcsL glucosylation was stimulated with manganese ions ( $Mn^{2+}$ ) as well and that glucosylation was blocked by the addition of EDTA, a chelator of  $Mn^{2+}$ , to the reaction (86). The importance of  $Mn^{2+}$  for TcsH glucosylation activity was also shown by the addition of divalent ions to chelated toxin,

of which only  $Mn^{2+}$  and to a lesser extent cobalt ( $Co^{2+}$ ) were able to stimulate TcsH glucosylation (88).

To better understand the mechanism of glucosylation by large clostridial toxins, work was done to identify key amino acids in the toxin enzymatic activity. A conserved motif of two aspartates (DxD) was found in a yeast mannosyltransferase enzyme that was shown to be conserved across a number of glucosyltransferases (91). Based on this motif, Busch saw the presence of the DxD motif in the glucosyltransferase domain of TcsL, as well as the other large clostridial toxins (92). Busch and colleagues mutated each aspartate to alanine and saw that the ability to glucosylate GTPases was greatly inhibited; the introduction of a double mutant inhibited glucosylation below levels of detection. Other amino acid residues around the DxD motif were modified in the TcsL GTD but the mutants were not inhibited in glucosylation activity (92).

Structural work has also been done to understand the mechanism of glucosylation and how it affects the host GTPases targeted. GTPases cycle between a GDP-bound state and a GTP-bound active state and the cycling is mediated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In the GTP-bound state, the switch I region of Ras is found in two different conformations, one which facilitates the interaction with Ras GAPs (93). When the GTPases are glucosylated by LCTs, the ability of the GTPase to switch into the active conformation is blocked, as shown for HRas upon TcsL glucosylation (94). Interestingly, glucosylation does not occur on the loop of the GTPase that changes conformation but stabilizes the GTPase in the inactive conformation (95). It has been shown that by preventing GTPases to switch to the active conformation upon glucosylation, the GTPases have a



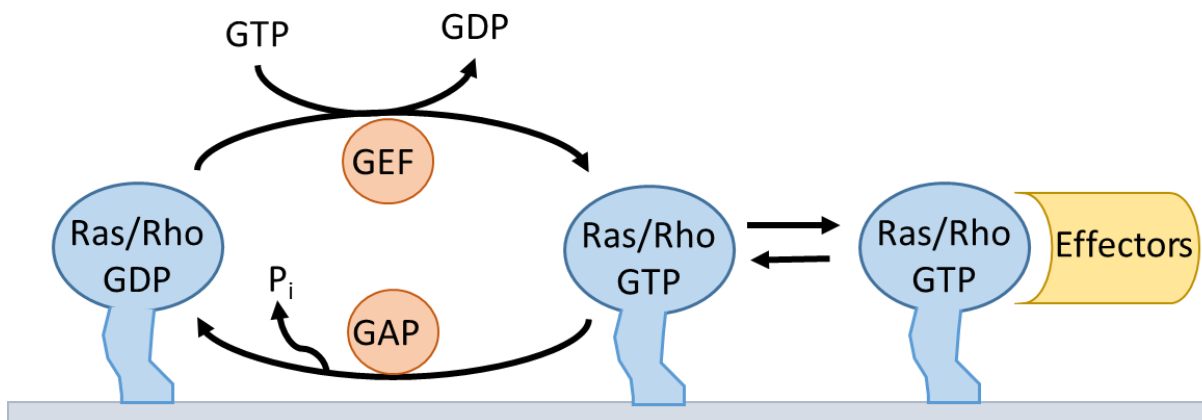
**Figure 1-5: Structure of GTPase switch region conformations.** The crystal structure of the two conformations for the switch 1 region in Ras GTPases is shown. The switch one region shown in purple shows the position of the tyrosine (A, red box) in the context of the Ras structure. (B) The active conformation of Ras (and Rap1) on the left shows the tyrosine pointing away from Ras and allowing for other molecules to bind and interact with Ras, while the tyrosine is pointed inward in the second conformation and prevents binding of effectors. Adapted from Hall et al. and Geyer et al. (93, 169).

decreased affinity toward their effectors, below physiologically relevant levels, and are rendered inactive (94–96).

### Host GTPases

GTPases are small proteins found in cells that act as molecular switches and are involved in many different cellular processes and signaling pathways. There are hundreds of GTPases expressed in mammalian cells, with over 60 in the Ras superfamily alone (97, 98). Within the Ras superfamily are five groups of GTPases: Ras, Rho, Rab, Arf, and Ran GTPases. TcsH and TcsL are known to glucosylate

GTPases from the Rho and Ras subfamilies, so these groups will be the focus moving forward. The GTPases are responsible for a wide variety of cell functions including cell morphology and motility (99–101), cell polarity (102–104), and cell cycle progression (105, 106).



**Figure 1-6: GTPase activation and inactivation.** Regulation of Rho and Rac GTPases is achieved by GEFs and GAPs. GAPs inactivate GTPases by hydrolyzing GTP to leave the GDP-bound GTPase. GEFs activate GTPases by facilitating exchange of GTP- instead of GDP-bound states. Once GTPases are in the active state, they can interact with their effectors to perform functions within the cell. Adapted from Jank et al. 2007 (170).

GTPases are constantly cycling between the active and inactive state. In the active state, the protein is bound to GTP and then switches to binding GDP in the inactive state. When in the active state, GTPases hydrolyze GTP into GDP and interact with other host protein effectors to perform a variety of functions and signaling events. The cycling between active and inactive states is mediated by GAPs and GEFs, which are also important for regulating the activity of GTPases. GAPs, GTPase-activating proteins, stimulate the hydrolysis of GTP to switch GTPases to the inactive state, while guanine nucleotide exchange factors (GEFs) facilitate the release of GDP and binding of GTP to return the GTPase to an active state. This is depicted in Figure 1-6.

The GAPs and GEFs that regulate GTPases are abundant in cells, over 60 GAPs and 70 GEFs have been identified that are specific to Rho GTPases alone (98). The multitude of regulators means that there is a redundancy for each GTPase and shows how complex and varied the roles of the GTPases are. There are also large numbers of effector proteins that the GTPases interact with that are only present in limited locations or times within the cell. This increases the specificity and the diversity of the functions each GTPase can perform through spatial and temporal regulation (97, 107). It has even been shown that Rho GTPases can be activated by Ras or Rho GTPases to increase the complexity of the signaling events that occur (99, 108, 109).

#### *Role of Rac in cells*

Rac GTPases are important for a number of cellular functions but one of the primary roles of Rac is the regulation of the host cytoskeleton. Rac is important in controlling the cytoskeleton, particularly the assembly and disassembly of actin filaments. The formation of lamellipodia and membrane ruffles are driven by Rac and are important for the motility of cells (99, 100, 108). Rac regulation of the cytoskeleton is also important in neuronal cells where it promotes axon growth, membrane protrusions, and an increase in the number of dendritic spines (110–112). The role of Rac in cytoskeleton regulation is very important to cells because of the many functions that rely on or interact with the cytoskeleton.

Other important cellular functions regulated by Rac GTPases include cell cycle progression, kinase signaling pathways, and cellular morphology. Rac regulation of cell cycle progression occurs in part through control of the cytoskeletal events important for

growth and duplication but also controls the expression of cyclins, such as D2 in B cells and D1 during the G1 phase (113, 114). There are many kinase signaling pathways and Rac is also important for activating some of these in cells as well, such as the JNK and p38 MAP kinase pathways (115, 116). Cell morphology, particularly in tissues, is dependent upon the establishment of cell adherens junctions and tight junctions that create a barrier between the apical and basolateral sides of polarized cells. Rac enables the production of these junctional interactions between cells through cadherin-dependent and integrin-based cellular contacts (117–119).

### *Role of Ras in cells*

The major role of Ras GTPases in cells is the control of cell cycle progression and proliferation. The majority of research that has been done on Ras GTPases and signaling relate to cancer biology due to the fact that many of the regulators have been indicated as or suspected to be oncogenes and anti-oncogenes (120, 121). Many of the Ras family proteins, as well as their regulators and effectors, are transformed in some way in cancer cells and immortalized cell lines used in research, with Ras mutations detected in 30% of cancer cells (120). Ras activation is important for the downstream signaling of the PI3K/Akt, Raf/MEK, and Ral pathways that regulate cell survival and proliferation through the activation and inhibition of transcription factors (122). Ras GTPases are also vital for the regulation of cell cycle progression through kinase signaling pathways as well as the control of transcription factors. The disruption of these pathways by glucosylation by LCTs, particularly TcsL, leads to cell cycle inhibition and

eventual cell death when pro-apoptotic pathways are activated and cell survival pathways are inhibited (123, 124).

#### *Localization of Rac and Ras GTPases*

Rho family GTPases have been reported throughout the cell, and Rac localization can be diverse as well. Rac is typically found associated with cellular membranes and is most often associated with the plasma membrane at the cell surface where it plays an important role in cytoskeleton regulation (125, 126). Rac1 has been localized in both cytosolic and membrane fractions by cellular fractionation and has been visualized to be found primarily on plasma membranes (125, 127, 128). Work focused on the Rac2 isoform has shown that it is located in the cytoplasm but also on endosomal membranes, and that localization to membranes increases particularly upon assembly of the NADPH oxidase complex in neutrophils (125, 129). Work has shown that despite the varied localization of Rac GTPases, the protein must be trafficked through the endosome to be activated. The authors show that activation of Rac is inhibited by preventing endocytosis and formation of early endosomes and that recruitment to the endosome is regulated by Rab5 (126). This shows another layer of complexity in the regulation of Rac GTPases using localization and trafficking to specific cellular locations.

Ras GTPase isoforms, consisting of H, K, and NRas, are localized to the plasma membrane after expression and have been shown to require plasma membrane localization to activate signaling (130). The localization of Ras is achieved through a C-terminal CAAX motif that is further processed to drive the protein to the membrane.

After expression, the CAAX motif is initially prenylated by either farnesyl protein transferase or geranylgeranyltransferase to drive localization to the endoplasmic reticulum (ER) (131, 132). Once the Ras GTPase reaches the membrane of the ER, the protein is further modified by the cleavage of the AAX from the CAAX motif and either palmitoylated in the case of HRas and NRas or methylated as is seen for KRas (133–136). KRas travels directly to the plasma membrane while HRas and Kras travel to the plasma membrane via the Golgi apparatus (131, 133). While there have been reports of Ras located in the nucleus, other data has suggested this may not be true (137, 138). Localization of Ras GTPases is limited to plasma membranes in cells and activation and signaling occurs at the inner plasma membrane.

### **Research Objectives**

Much of our understanding about the mechanism of action for the LCTs has come from work done on TcdA and TcdB of *C. difficile*. Our understanding of *C. sordellii* toxins TcsH and TcsL is based in part on the activity of TcdA and TcdB due to the high levels of homology between the toxins. This project was designed to directly test mechanisms of action in the context of TcsH and TcsL. There are already differences that are known about the toxins of *C. sordellii* from other large clostridial toxins. One main difference is the ability of TcsL to modify Ras proteins, which has been shown to lead to apoptosis of intoxicated cells (123, 124, 139). The other large clostridial toxins are unable to glucosylate Ras proteins, with the exception of variant TcdB strains. Differences between TcsH and TcsL and other large clostridial toxins, such as the



GTPases that are glucosylated, demonstrate that our understanding of one toxin is not always true for the other large clostridial toxins. Previous work looking at TcdA and TcdB has shown important differences such as differences in receptor specificity (67, 68, 89, 140) and recent work from our lab has shown that TcdB is able to induce necrosis independent of enzymatic activity, which TcdA does not (82, 141).

While work had been done on TcsL, very little had been done looking in depth at TcsH. Part of this was due to the fact that TcsH is not expressed in all pathogenic strains of *C. sordellii* as well as the lack of a gene sequence until 2013 (28), which made studying the impact of TcsH difficult. TcsL, as well as TcdA and TcdB, had been expressed recombinantly in prior studies using a *Bacillus megaterium* expression system (142) but TcsH has not been reported to be expressed recombinantly. Our lab was able to introduce *tcsH* into the *B. megaterium* expression vector to be used for study alongside recombinantly expressed TcsL.

In order to have a better understanding of TcsH and TcsL, I began a study comparing the toxins of *C. sordellii* to their homologs of *C. difficile*, TcdA and TcdB. I wanted to understand whether the differences seen in toxicity and mechanism between TcdA and TcdB would be similar to differences between TcsH and TcsL. In Chapter II, I describe the work that was done using recombinant expressions of TcsH and TcsL and assays that were performed in order to delve deeper into the differences and similarities between the two toxins, as well as to compare them to their *C. difficile* homologs, TcdA and TcdB. We know that different cell lines have different sensitivities to the large clostridial toxins, so I started by looking for a cell line that was sensitive to both TcsH and TcsL to use for comparative studies. In addition, I worked to determine the

cytotoxicity of each toxin relative to each other as well as to determine the mechanism of cell death that each toxin induces. I report data suggesting that despite high homology between TcsH and TcdA and between TcsL and TcdB, it appears that the mechanisms of cytotoxicity and cellular responses may be similar for TcsL/TcdA and TcsH/TcdB instead.

To further build on our limited knowledge of TcsL, I undertook work to study the importance and role each domain had in cytotoxicity. In the context of holotoxin, mutations were introduced to inhibit the activity of key enzymatic activities of glucosyltransferase and autoprocessing in TcsL. It was seen, and described in Chapter III, that the glucosyltransferase activity was required for induction of cytotoxicity, but that the loss of autoprocessing impaired cytotoxicity. Interestingly, the introduction of mutations to disrupt TcsL autoprocessing resulted in different glucosylation activity for Rac and Ras GTPases, which may explain differences in cytotoxicity.

The work was continued by looking at the role of a membrane localization domain (MLD) found within the GTD of TcsL and its role in intoxication. It had been reported previously that the MLD was a conserved structure found in many bacterial toxins and plays a role in toxin localization and membrane interaction (143–146). Mutations were made in the MLD of TcsL and the result on cytotoxicity and glucosylation ability were studied. I was able to show that the mutations impaired the membrane localization ability of TcsL and that the impairment led to a decrease in cytotoxicity and glucosylation in cells.

## CHAPTER II

### COMPARATIVE STUDY OF TcsH AND TcsL WITH THEIR LARGE CLOSTRIDIAL TOXIN HOMOLOGUES FROM *CLOSTRIDIUM DIFFICILE*

#### Introduction

*Clostridium sordellii* produces two large exotoxins, TcsH and TcsL, which are drivers of disease during infection. They are members of the large clostridial family and are highly homologous to TcdA and TcdB of *C. difficile* respectively (Table 1-1). While a lot of work has been done to study TcsL, almost nothing has been published on TcsH due to the fact that the TcsH wasn't sequenced until 2013 (28). Much of the knowledge for TcsH, as well as TcsL to a lesser degree, has been determined by comparison with other large clostridial toxins, particularly TcdA and TcdB (147–150). Despite the high homology, there are many differences between the LCTs and their mechanisms of action that warrant investigation of TcsH and TcsL directly.

TcsH and TcsL, like other LCTs, act upon host cells to glucosylate specific GTPases and the glucosylation is thought to lead to cytopathic and cytotoxic effects downstream. There are two main cell death pathways in cells: apoptosis and necrosis. Apoptosis is programmed cell death that is distinguished by the cell's ability to cleanly die without rupturing and risking an inflammatory response in the host (151, 152). While many stimuli lead to apoptosis, cells undergoing apoptosis exhibit several key characteristics such as the condensation of chromatin and the eventual activation of

caspases 3 and 7 (151). In comparison, necrosis is a much quicker and less organized mechanism of cell death. The cells exhibit a loss of membrane integrity, which can be measured by the release of cytosolic lactate dehydrogenase (LDH), and do not exhibit caspase activation like is seen in apoptotic cells (151). During both apoptosis and necrosis, cells experience a loss of ATP that can be measured as a marker for cell death.

Recent work from our lab has shown that TcdB, which is highly homologous to TcsL, is able to induce necrotic cell death at higher concentrations while TcdA and lower concentrations of TcdB induce apoptotic cell death (82, 141). Based on this work, I wanted to investigate the mechanism of cell death induced by TcsH and TcsL and see how that compares to the homologous toxins from *C. difficile*. The high homology between TcsL and TcdB suggested that TcsL may also induce a necrotic cell death at higher concentrations while TcsH may only induce apoptosis similar to TcdA.

## Methods

### *Toxin cloning, expression, and purification:*

TcsH was amplified from the JGS6382 strain of *C. sordellii* and inserted into a BMEG20 vector (MoBiTec) using BsrGI/KpnI restriction digest sites in the vector as reported previously (81). TcsL was amplified from the JGS6382 strain of *C. sordellii* using the same methods as TcsH and as reported in chapter III. Recombinant toxins (TcsH, TcsL, TcdA, and TcdB) were expressed in *Bacillus megaterium* using 35 mL of

overnight culture seeded into 1 L of LB. Expression of toxin was induced at an OD<sub>600</sub> of roughly 0.5 with 5 g D-xylose and grown for 4 hours at 37° C with shaking at 220 rpm. The cells were pelleted and resuspended in lysis buffer (20 mM KPi pH 7, 500 mM NaCl, DNase, and protease inhibitors (Sigma)). The bacteria were passed through an Emulsiflex for lysis and centrifugation performed at 48,000 g for 30 min. Supernatants were run over Ni-affinity, anion exchange with a gradient from 20 mM Tris pH 8 to 20 mM Tris pH 8, 600 mM NaCl, and Superdex 200 size exclusion columns, and toxins eluted in 20 mM HEPES, pH 7, 50 mM NaCl.

#### *Cell culture:*

Conditionally immortalized murine pulmonary microvascular endothelial cells (mPMVECs) were obtained from the laboratory of Ambra Pozzi (Vanderbilt University). The cells were grown at 33° C using EGM-2 (Lonza) supplemented with 10 ng/mL IFN- $\gamma$  in 5% CO<sub>2</sub>. For use, mPMVECs were transferred to 37° C for overnight growth in EGM-2 media without IFN- $\gamma$ .

HeLa cells were grown at 37° C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin.

#### *Viability assays:*

HeLas and pMPVECs were plated at 5,000 cells/well in black wall 96-well plates. The cells were treated with toxin the following day and incubated at 37° C in 5% CO<sub>2</sub> for either 24 or 48 hours. After incubation with toxin, viability was assessed by measuring the amount of ATP present by addition of CellTiterGLO (Promega), and the

luminescence read using a BioTek Synergy 4 plate reader. Relative viability was calculated by setting the mock treated sample readings as representing 100% viability.

*Caspase activation assay:*

Caspase 3 and 7 activation levels were measured using the Apo-One reagent (Promega). The reagent was added to cells in a 96-well plate, toxin was added, and the plate was incubated at 37° C. 12 hours post-intoxication, fluorescence was read using a BioTek Synergy 4 plate reader. Relative caspase activation was calculated by setting the fluorescence of untreated cells to 1 and dividing toxin-treated fluorescence values by untreated fluorescence.

*Lactate dehydrogenase release:*

Lactate dehydrogenase (LDH) release was determined using CytoTox GLO reagent (Promega). The reagent was added to cells in a 96-well plate and incubated for 15 minutes prior to intoxication. 10 nM TcsH, TcsL, TcdA, and TcdB were added to the cells and the cells were incubated at 37° C. Luminescence was read every hour for 7 hours on a BioTek Synergy 4 plate reader. Relative luminescence was determined by setting the luminescence of untreated cells to 1 and dividing toxin-treated cell luminescence by untreated luminescence.

*Intracellular calcium release:*

mPMVECs were plated at 20,000 cells/well in clear bottom, black wall 96-well plates and incubated at 37° C. The following day the cells were washed with HBSS

containing calcium and magnesium and 100 uL fresh HBSS was added to each well. 10 uL/well of calcium signaling reagent, FLUOFORTE (Enzo Life Sciences), diluted 20-fold was added. The cells were then intoxicated with recombinant TcsH, TcsL, TcdA, and TcdB over a range of concentrations. The fluorescent signal was read kinetically overnight every 30 minutes using a BioTek Synergy 4 plate reader with the sensitivity setting at 40. Relative fluorescence was determined by setting the mock treated background fluorescence for each time point as 1 and dividing toxin-treated cell fluorescence by the mock-treated fluorescence.

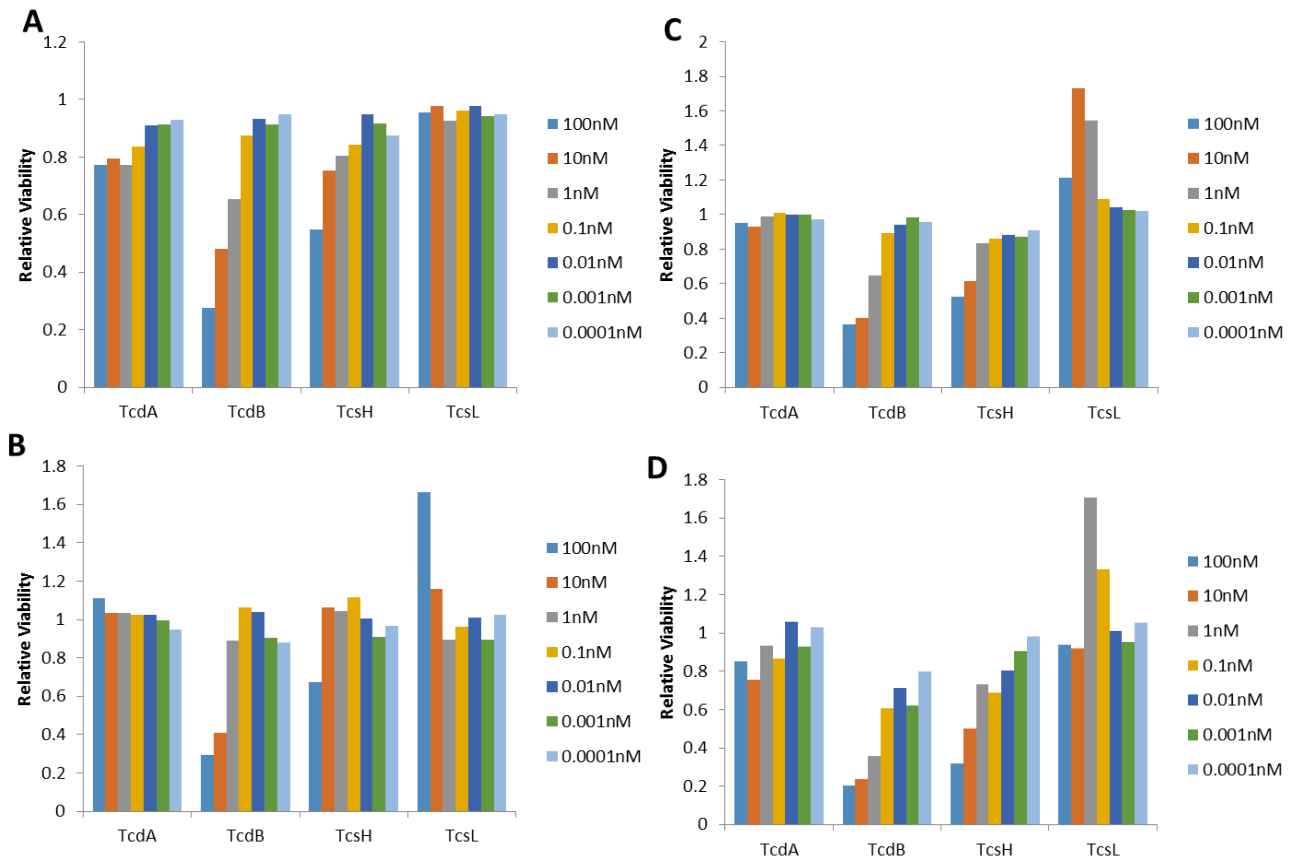
*Reactive oxygen species production:*

Reactive oxygen species (ROS) production was measured using the Total ROS Detection Kit (Enzo Life Sciences). Cells plated in a 96-well plate were incubated for 1 hour at 37° C with 5 uM oxidative stress detection reagent. The cells were then washed twice with HBSS and intoxicated in HBSS. Toxin-treated cells were incubated at 37° C for 22 hours and fluorescence was read on a BioTek Synergy 4 plate reader. Relative ROS production was determined by setting fluorescence from mock-treated cells to 1 and dividing treated cell fluorescence by the mock-treated fluorescence.

## Results

*TcsH* and *TcsL* induce cytotoxicity in endothelial cells.

Much of the previous work on *TcsH* and *TcsL* was done on cell lines that the toxins induced cell rounding in. In order to perform a study looking at the cytotoxicity and mechanisms of cell death induced by the *C. sordellii* toxins, I first needed to find a cell line that was able to effectively induce cell death. While *TcsH*, as well as *TcdB* of *C. difficile*, was induced cytotoxicity in HeLa cells, *TcsL*, as well as *TcdA*, was not able to



**Figure 2-1: Cytotoxicity in HeLa cells.** HeLa cells were treated with *TcsH*, *TcsL*, *TcdA*, and *TcdB* over a range of concentrations. Cell viability was determined by CellTiterGLO luciferase 2 (A), 8 (B), 24 (C), and 48 hours (D) after intoxication. Relative viability was calculated dividing the signal from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates.



induce noticeable cytotoxicity (Figure 2-1). Previous work in our lab by Nicole Chumbler (unpublished) has shown that TcdA is able to cause cytotoxicity in immorto-mouse derived epithelial cells (YAMC) which are conditionally immortalized at 33° C. I obtained an endothelial cell line derived from the same immorto-mouse, mPMVECs. When the mPMVECs were intoxicated, the toxins were able to induce cytotoxicity (Figure 2-2). Interestingly, both TcsH and TcdB saw induction of cytotoxicity at the highest concentrations as early as two hours after intoxication, while TcsL and TcdA did not show significant cell death until 24 hours. This was the first suggestion that the homology between TcsH/TcdA and TcsL/TcdB may not be consistent with which toxins act more similarly on cells.

#### *Apoptosis or necrosis induced by TcsH and TcsL.*

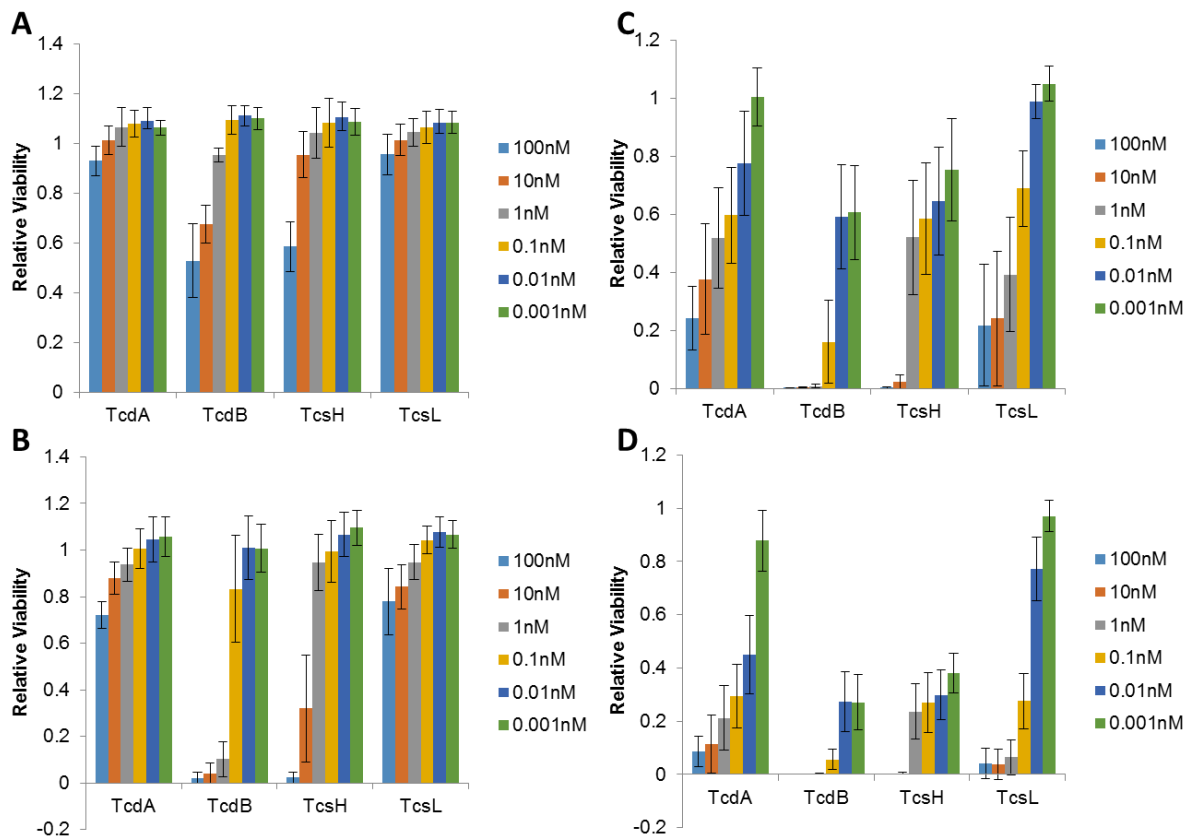
Despite the high homology seen between TcsH and TcdA and between TcsL and TcdB, the cytotoxicity induced in cells does not seem to be similar in the time frame and extent of cytotoxicity. It appears that TcsH induces cytotoxicity in a time frame and severity more similar to TcdB while TcsL induces cytotoxicity more similar to TcdA. While it has been reported previously that TcsL induces cytotoxicity through apoptosis, it has not been extensively shown how TcsH causes cytotoxicity (124, 139). Recently, our lab has shown that at higher concentrations TcdB induces a necrotic cell death in cells while TcdA induces apoptosis (82, 141). While apoptosis relies on activation of caspases 3 and 7, necrosis is marked by an increase in reactive oxygen species (ROS)

in cells and the detection of lactate dehydrogenase outside the cell (82, 152, 153).

Because of the high sequence homology between the *C. sordellii* and *C. difficile* toxins, and the similarity in cytotoxicity observed above, I decided to look for the ability of TcsH and TcsL to induce necrosis or apoptosis.

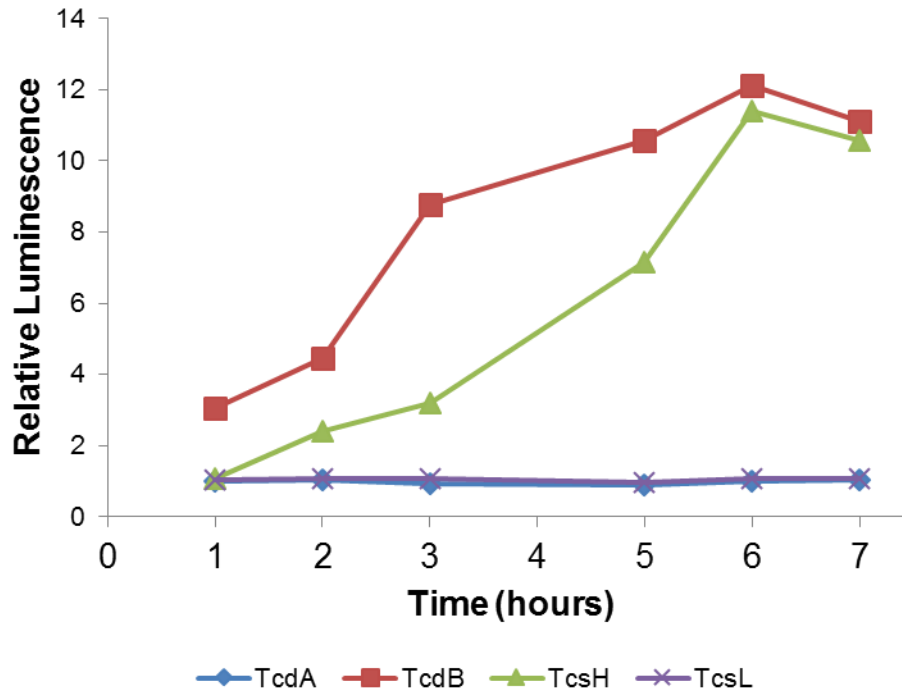
Lactate dehydrogenase (LDH) is a molecule that is typically found solubilized in the cytoplasm of cells. When the cell undergoes necrosis and loses membrane integrity, the cytoplasmic LDH is released and can be detected in the extracellular media (154).

To determine whether TcsH or TcsL induce necrotic cell death, LDH release was



**Figure 2-2: Cytotoxicity in mPMVECs.** mPMVEC cells were treated with TcsH, TcsL, TcdA, and TcdB over a range of concentrations. Cell viability was determined by CellTiterGLO luciferase 2 (A), 8 (B), 24 (C), and 48 hours (D) after intoxication. Relative viability was calculated by dividing the signal from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates. Error bars represent standard deviation of the mean across three replicates.

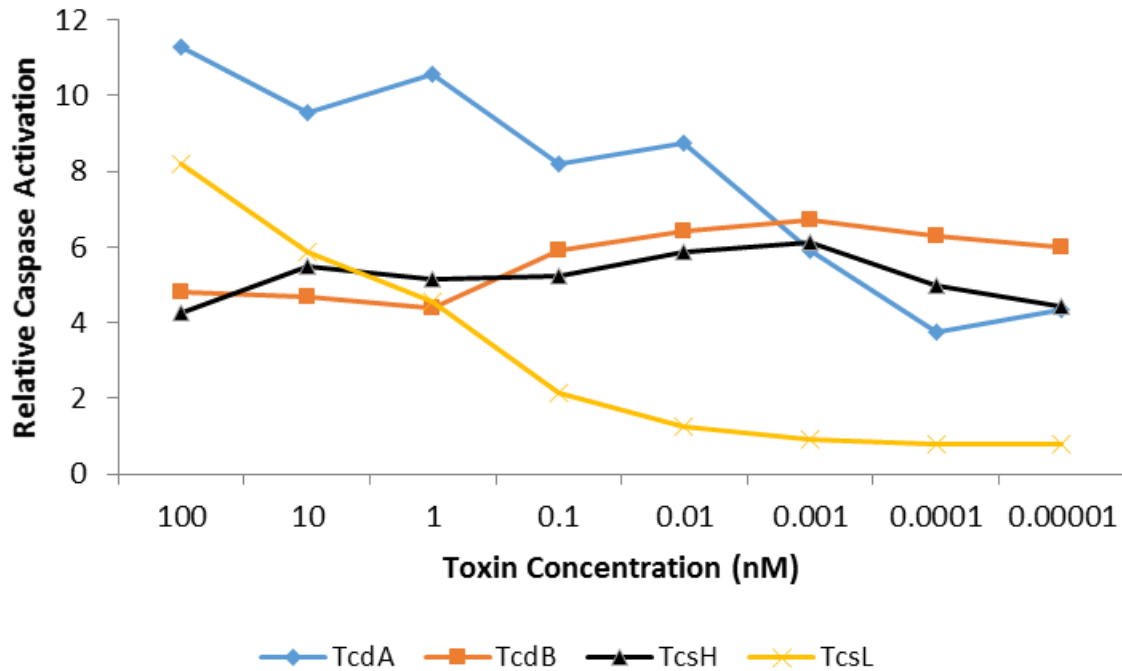
measured in mPMVEC cells. Over a time-course of 7 hours, the amount of LDH released increased for cells treated with TcsH, similar to that seen for cells treated with TcdB (Figure 2-3).



**Figure 2-3: TcsH induces LDH release in endothelial cells.** mPMVEC cells were treated with 10 nM TcsH, TcsL, TcdA, and TcdB. LDH release was measured by luminescence on a plate reader every hour for 7 hours. Relative LDH release was calculated by dividing the luminescence from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates.

An assay was also performed by Nicole Chumbler with TcsH and TcsL to look for the activation of caspase activity in epithelial YAMC cells. During apoptosis, caspases 3 and 7 are activated by cleavage of the pro-caspase forms (155) and then are able to disrupt downstream signaling pathways. To measure whether apoptosis or necrosis is induced, Nicole treated cells with TcsH and TcsL as well as TcdA and TcdB and measured the activity of caspases 3 and 7. Twelve hours after intoxication, Nicole measured the relative caspase activity based on the fluorescent signal from treated

cells. TcsL shows a concentration dependent increase in caspase activation in cells, similar to apoptosis-inducing TcdA (Figure 2-4). The levels of TcsH-induced caspase activation did not change as the concentration of toxin changed and neither did the



**Figure 2-4: TcsL induces caspase activation.** YAMC cells were treated with TcsH, TcsL, TcdA, and TcdB at various concentrations. Caspase activation was measured by fluorescence on a plate reader 12 hours post-intoxication. Relative caspase activation was calculated by dividing the fluorescence from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates.

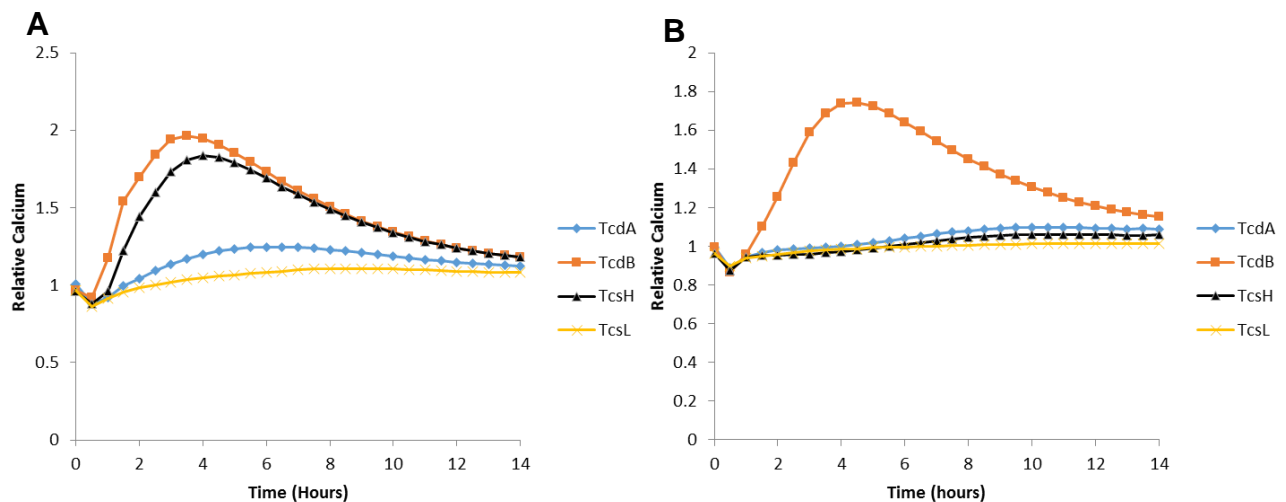
necrosis-inducing TcdB. This further suggests that TcsH induces a necrotic cell death similar to TcdB, while TcsL induces apoptosis like TcdA as has been reported in the literature.

*Signaling events induced by TcsH and TcsL intoxication.*

It has been shown that certain stresses and signals produced by cells can turn on the pathways leading to necrosis, one of which is the production of ROS (153). Work

from our lab has shown that ROS production is an important early step in the necrotic cell death induced by TcdB (141). Data also shows intracellular calcium release is increased as part of the events triggering and sustaining a necrotic response and the calcium increase occurs simultaneously with increased ROS production, although it is not yet clear which increases first (Farrow, unpublished).

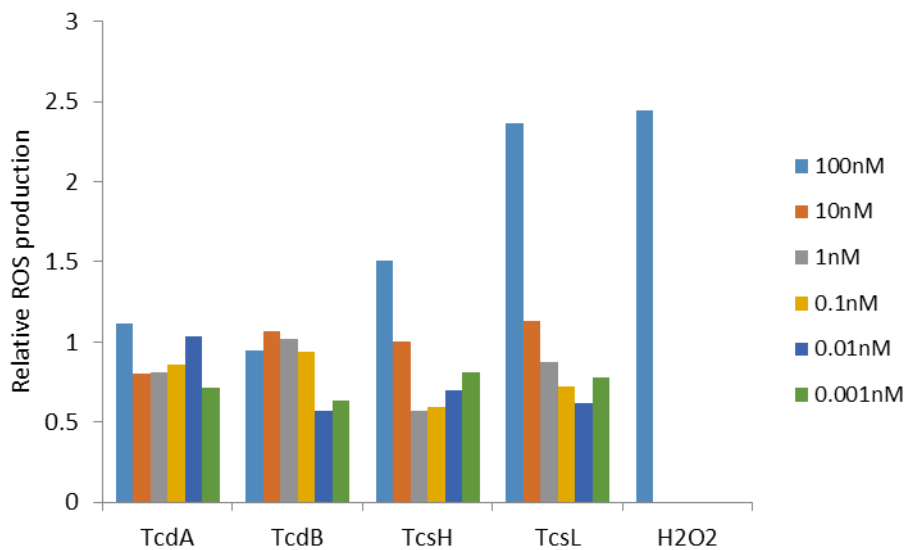
Increased levels of intracellular calcium have been associated with increased cell death and necrosis (156, 157). Melissa Farrow from our lab has also seen a release of intracellular calcium that occurs rapidly after TcdB intoxication and increases in time as the cells undergo necrosis. To understand whether TcsH may be inducing a similar intracellular calcium signaling to cause necrosis I assayed for intracellular calcium levels in endothelial cells. When endothelial cells were treated with higher concentrations of TcsH, a spike in intracellular calcium was observed, similar to TcdB-induced calcium levels, which reached a maximum by four hours (Figure 2-5). Lower concentrations of



**Figure 2-5: TcsH induces intracellular calcium release.** mPMVEC cells were treated with TcsH, TcsL, TcdA, and TcdB at 10 nM (A) and 0.1 nM (B). Intracellular calcium was measured by fluorescence on a plate reader over a course of 14 hours. Relative intracellular calcium release was calculated by dividing the fluorescence from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates.

TcsH were unable to induce a detectable increase of intracellular calcium, while TcdB was still able to induce a calcium increase at lower concentrations. This suggests that TcsH may induce necrosis through an intracellular calcium signaling pathway, but that TcsH is less potent than TcdB at inducing necrosis through this mechanism.

The production of reactive oxygen species (ROS) is also a signaling event that can lead to necrosis following cellular stress (153). TcdB-induced necrosis is dependent on ROS production from the NADPH oxidase (141). I tested for ROS production in endothelial cells to determine if TcsH induced necrosis through a ROS-dependent mechanism as well. When endothelial cells were treated with TcsH and TcsL, as well as TcdA and TcdB, a ROS response was seen in cells treated with 100 nM of both TcsH and TcsL (Figure 2-6). Interestingly, TcsL had the most robust ROS production, similar



**Figure 2-6: ROS induction by TcsH and TcsL.** mPMVEC cells were treated with TcsH, TcsL, TcdA, and TcdB at various concentrations and H<sub>2</sub>O<sub>2</sub>. Reactive oxygen species production was measured by fluorescence on a plate reader 22 hours after intoxication. Relative ROS production was calculated by dividing the fluorescence from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates.

to the H<sub>2</sub>O<sub>2</sub> positive control. TcdB, which induces ROS in epithelial cells, did not induce detectable ROS production in our assay using endothelial cells. This suggests that one or more components involved in TcdB-induced ROS are not present or different in endothelial cells. It also suggests ROS may not play the same role for *C. sordellii* toxins because TcsL is able to induce a robust ROS response but does not induce necrosis.

## Discussion

The purpose of this study was to determine the mechanism of cell death induced in cells by TcsH and TcsL and to compare to the homologues TcdA and TcdB of *C. difficile*. Based on the high structural and sequence identity between TcsH and TcdA and between TcsL and TcdB one could expect that the mechanisms of each toxin to be similar to the mechanism induced by their homologue. When a standard lab cell line, HeLa cells, were treated with TcsH and TcsL, they induced differing levels of cytotoxicity (Figure 2-1). Neither TcsL nor TcdA were able to induce cell death in HeLa cells, while cell rounding was observed. When endothelial cells were used that TcsL and TcdA could induce cytotoxicity in, TcsH was able to induce cell death at higher concentrations and in a short amount of time, which was similar to TcdB in both speed of cell death induction and that cytotoxicity was seen at the higher concentrations.

Recent work has shown that TcdB is able to induce necrosis at higher concentrations, separate from the apoptosis that it has been attributed to causing (82, 141). I wanted to see whether TcsH could also induce necrosis based on similarities seen between the levels and speed of cytotoxicity induced by TcdB. Endothelial cells

were treated with TcsH and TcdB, as well as TcsL and TcdA and the cells were assayed for LDH release and caspase activation, markers representative of necrotic and apoptotic cell death. TcsH intoxication induced LDH release at higher concentrations showing that TcsH leads to necrotic cell death in treated cells, similar to TcdB. Conversely, TcsL treated cells showed caspase activation supporting previous literature reports of TcsL inducing apoptotic cell death.

I further looked into signaling events that have been shown to be involved in cell death, and have been shown to be present during TcdB-induced necrosis (141). Intracellular calcium release was induced when cell were treated with TcdA and TcdB at higher concentrations, although TcdB was able to induce a calcium response to lower concentrations than TcsH (0.1 nM). This suggests that although calcium release may be an important step in the necrotic death seen in both TcsH and TcdB, that TcdB is more potent at inducing both intracellular calcium release as well as necrosis at lower

GTD	TcdA	TcdB	TcsH	TcsL
TcdA				
TcdB	51%			
TcsH	85%	51%		
TcsL	53%	76%	55%	

Autoprocessing	TcdA	TcdB	TcsH	TcsL
TcdA				
TcdB	56%			
TcsH	86%	58%		
TcsL	57%	79%	59%	

Translocation	TcdA	TcdB	TcsH	TcsL
TcdA				
TcdB	48%			
TcsH	79%	47%		
TcsL	47%	77%	48%	

CROPs	TcdA	TcdB	TcsH	TcsL
TcdA				
TcdB	42%			
TcsH	69%	45%		
TcsL	41%	75%	44%	

**Table 2-1: Protein sequence identity between toxin domains.** Protein sequences for TcdA and TcdB from *C. difficile* strain 10643 and TcsH and TcsL from *C. sordellii* were compared by Protein BLAST. The values shown indicate the amino acid sequence identity between each of the large clostridial toxins of each of the four individual domains: glucosyltransferase (GTD), autoprocessing, translocation, and CROPs domains.



concentrations. However, ROS production was seen in both TcsH- and TcsL-treated endothelial cells while TcdA and TcdB were not observed to produce ROS. The ROS production was more robust after TcsL-intoxication even though TcsL induced an apoptotic cell death. This suggests that ROS production in endothelial cells may be a separate event from induction of necrosis because TcdB did not induce ROS production in endothelial cells and TcsL, which induces apoptosis, produced the highest ROS response.

The necrosis induced by TcsH similarly to TcdB and the TcsL-induced apoptosis similar to TcdA shows interesting similarity between the large clostridial toxins. Based simply on sequence and structural homology, TcsL would be expected to induce necrosis similar to TcdB at higher concentrations because they share the highest homology and TcsH would be expected to induce apoptosis like TcdA. These results show that while the large clostridial toxin family share many characteristics, such as glucosylation of GTPases and overall structural arrangement, the details of intoxication cannot be assumed based on homology to other toxins. Even when we look at the homology between each domain of the toxins, the homology remains consistent and highest between TcsH and TcdA and between TcsL and TcdB (Table 2-1). The lack of large differences in specific domains suggests that the differences in toxin-induced cell death are not the result of large, obvious differences in structure and amino acid sequence and that the differences between toxins is more nuanced. This shows how important it is for research in large clostridial toxins to study each toxin individually and to thoroughly investigate the possible similarities seen between large clostridial toxins, particularly those of *C. sordellii* and *C. difficile*.

## CHAPTER III

# CLOSTRIDIUM SORDELLII LETHAL TOXIN AUTOPROCESSING AND MEMBRANE LOCALIZATION ACTIVITIES DRIVE GTPASE GLUCOSYLATION PROFILES IN ENDOTHELIAL STUDIES

### Introduction

*Clostridium sordellii* is a Gram-positive, spore-forming anaerobic bacterium that causes infections in humans and livestock (31). In humans, the bacteria enter at sites of soft tissue trauma and cause infections that lead to gas gangrene, sepsis, and, in up to 70% of cases, death (2, 10, 20, 23). *C. sordellii* produces two major toxins, the hemorrhagic toxin (TcsH) and the lethal toxin (TcsL), which are considered the major virulence factors in disease (4, 5). First, animals that are injected with purified toxins develop symptoms that mimic the symptoms of *C. sordellii* infection (4, 48). Second, antibodies that target TcsL and TcsH can protect against tissue damage (6, 7). As a toxin mediated disease, there is therefore interest in understanding the molecular mechanism of toxin action, especially that of TcsL since not all clinical isolates contain TcsH (28).

While much of what we understand about TcsH and TcsL mechanism comes from analogy to the homologous TcdA and TcdB toxins from *C. difficile*, there are several reports validating the functional similarities. Toxin activity relies upon binding to receptor(s) on the cell surface and clathrin-mediated endocytosis into the host cell (69).

Maturation of the endosome causes a conformational change in the pore-forming domain of TcsL causing it to form a pore in the endosomal membrane (71, 73). The autoprocessing domain is activated by host inositol hexakisphosphate and cleaves the GTD (77, 81, 86, 87), presumably, to permit access to substrates residing at the plasma membrane. The GTD glucosylates small GTPases, predominately Rac, Ras, Ral, and Rap (85, 90, 158). The glucosylation leads to cytoskeletal rearrangement and rounding of the cells and also causes the induction of apoptosis (139, 150, 159, 160).

Previous studies have investigated the different roles of host GTPases in the cellular response to intoxication, and show that glucosylation of H, K, and NRas lead to the apoptotic cell death seen in cells by causing cell cycle arrest (123, 124, 161). Studies have also shown that Rac1 glucosylation drives a change in cell morphology through actin cytoskeletal rearrangement causing the cytopathic effects upon cells (162). While work has been done to understand the relationship between specific GTPase glucosylation and the cellular effects induced by TcsL, an in-depth look at the role of the toxin functional activities in the context of cellular intoxication has not been reported. We have introduced mutations into TcsL to inhibit the glucosyltransferase and autoprocessing enzymatic functions and have studied the changes these mutations have on lung endothelial cells.

## Methods

### *Toxin cloning, expression, and purification:*

TcsL was amplified from the JGS6382 strain of *C. sordellii* and inserted into a BMEG20 vector (MoBiTec) using BsrGI/KpnI restriction digest sites in the vector as reported previously (81). Point mutations were introduced into the glucosyltransferase domain (F17N, R18A, D286N, and D288N) and autoprocessing domain (C698A) from the wild-type recombinant TcsL sequence using a QuikChange mutagenesis protocol. Construct names and primers used are found in the supplement (Table 1). Recombinant toxins were expressed in *Bacillus megaterium* using 35 mL of overnight culture seeded into 1 L of LB. Expression of toxin was induced at an OD<sub>600</sub> of roughly 0.5 with 5 g D-xylose and grown for 4 hours at 37° C with shaking at 220 rpm. The cells were pelleted and resuspended in lysis buffer (20 mM KPi pH 7, 500 mM NaCl, DNase, and protease inhibitors (Sigma)). The bacteria were passed through an Emulsiflex for lysis and centrifugation performed at 48,000 g for 30 min. Supernatants were run over Ni-affinity, anion exchange with a gradient from 20 mM Tris pH 8 to 20 mM Tris pH 8, 600 mM NaCl, and Superdex 200 size exclusion columns, and toxins eluted in 20 mM HEPES, pH 7, 50 mM NaCl.

Native TcsL was purified from *C. sordellii* strain JGS6382 obtained from David Aronoff (Vanderbilt University). Expression and purification was done as previously described (82).

*Cell culture:*

Conditionally immortalized murine pulmonary microvascular endothelial cells (mPMVECs) were obtained from the laboratory of Ambra Pozzi (Vanderbilt University). The cells were grown at 33° C using EGM-2 (Lonza) supplemented with 10 ng/mL IFN- $\gamma$  in 5% CO<sub>2</sub>. For use, mPMVECs were transferred to 37° C for overnight growth in EGM-2 media without IFN- $\gamma$ .

*Viability assays:*

mPMVECs were plated at 2,500 cells/well in black wall 96-well plates. The cells were treated with toxin the following day and incubated at 37° C in 5% CO<sub>2</sub> for either 24 or 48 hours. After incubation with toxin, viability was assessed by measuring the amount of ATP present by addition of CellTiterGLO (Promega), and the luminescence read using a BioTek Synergy 4 plate reader. Relative viability was calculated by setting the mock treated sample readings as representing 100% viability.

*Western blot analysis:*

Cell lysates were prepared from mPMVECs plated at 200,000 cells/mL in 10 cm dishes the day prior. Cells were intoxicated with 10 nM toxin and incubated at 37° for 0, 1, 2, and 3 hours before manual lifting from the dish. Cells were washed and resuspended in lysis buffer (250 mM sucrose, 10 mM Tris pH 7.4, 3 mM imidazole) and passed through a 27G needle 25 times. The lysates clarified by centrifugation. Samples were run on SDS-PAGE gels (Bio-Rad) and transferred to PVDF for Western analysis. Blots were probed with antibodies for unmodified Rac1 (BD, 610651), total

Rac1 (Millipore, clone 23A8), unmodified Ras (Abcam, ab52939), total HRas (Santa Cruz, sc-520), and GAPDH (Santa Cruz, sc-25778). HRP-conjugated anti-mouse and anti-rabbit antibodies (Cell Signaling, 7076 and 7074, respectively) were applied as secondary antibodies, and the blots were visualized using Pierce ECL Western Blotting Substrate (Thermo) and exposure to film. Film was scanned using the Odyssey Licor Imaging System and analyzed using Image Studio Lite.

*In vitro cleavage assay:*

200 nM TcsL and TcsL C698A were placed into MES buffer (pH 7) containing 100 mM DTT with or without 5 mM IP6. The reactions were placed at 37° C for 0, 30, 60, 90, and 120 minutes. The reactions were stopped by adding loading buffer and boiling. Samples were separated by SDS-PAGE gels and stained by SimplyBlue SafeStain Coomassie (ThermoFisher).

*In vitro glucosyltransferase assay:*

TcsL and TcsL C698A were incubated in buffer containing 50 mM HEPES, 100 mM KCl, 1 mM MnCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 0.1 mg/mL BSA (pH 7.5) with 24 uM UDP-[<sup>14</sup>C]glucose (250 mCi/mmol, PerkinElmer) and 2 uM GST-tagged Rac1 or HRas for 1 hour at 37° C. Reactions were stopped by adding loading buffer and boiling. Samples were separated by SDS-PAGE, and the dried gels were imaged using a Typhoon FLA 7000 phosphoimaging scanner.

### *Liposome binding assay:*

Liposomes were prepared using 30% DOPE, 20% cholesterol, 20% egg PC, and 30% brain PS (Avanti Polar Lipids) by combining lipid solutions in chloroform and dried by nitrogen gas and vacuum. The dried lipids were resuspended to 10 mM final concentration in 20 mM HEPES, pH 7.5, 100 mM KCl and freeze-thawed. The lipids were then passed through an extruder using a 0.2  $\mu$ M filter repeatedly. Toxin was cleaved using 2  $\mu$ M toxin, 50 mM DTT, and 5 mM IP6 in 20 mM HEPES pH 7 buffer and incubated at 37° C for 2 hours. The cleaved toxin was dialyzed against 20 mM HEPES, pH 7.5, 100 mM KCl to remove IP6 and DTT. Liposome binding reactions were prepared using 3 mM liposomes and 0.5  $\mu$ M dialyzed toxin incubated in 20 mM HEPES, pH 7.5, 100 mM KCL buffer containing 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> for 1 hour at 37° C. The reactions were separated by centrifugation at 436,000 g for 1 hour at 23° C. Pellets were resuspended in the same volume of buffer with 1% SDS added. Samples were separated by SDS-PAGE and stained by SimplyBlue SafeStain Coomassie (ThermoFisher).

### *Statistics:*

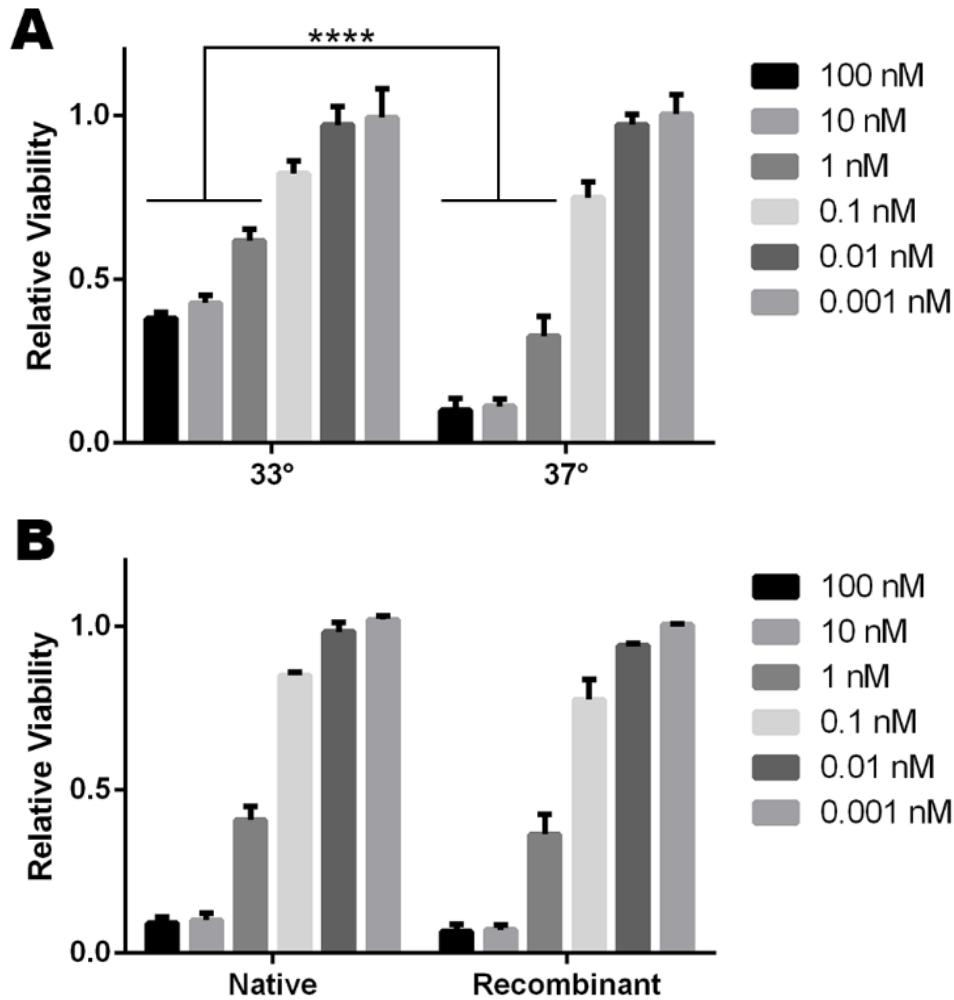
Statistics were performed on our data using two-way ANOVA and p-values were determined using Dunnett's multiple comparisons test on GraphPad Prism.

## Results

*TcsL induces cytotoxicity in mPMVEC cells.*

TcsL cytotoxicity is driven by glucosylation of Ras, and many Ras related pathways are mutated in standard cell lines to induce immortality. Much of the work done to study the impact of TcsL has used either transformed cell lines or cell lines that do not accurately represent the tissue specificity shown during *C. sordellii* infections. *C. sordellii* infection leads to edema and hypotension, and TcsL induces vascular permeability in the lungs of mice (48). This suggests that lung endothelial cells are a physiologically relevant model for studies of TcsL function. We chose conditionally immortalized murine pulmonary microvascular endothelial cells (mPMVECs), which behave similarly to primary cells when grown at 37° C but are permissive for expression of the SV40 large T antigen at 33° C (163). We used a recombinant system to allow for expression and purification of TcsL with specific point mutations (69, 81). Cells were treated with TcsL across a range of concentrations at both the permissive temperature (33° C) and the non-permissive temperature (37° C). TcsL induced significantly higher levels of cytotoxicity at 37° C (Figure 3-1A). A comparison between recombinant TcsL and TcsL purified from *C. sordellii* indicates that both forms of TcsL induce similar effects on endothelial cells with no statistical difference in cytotoxicity levels. (Figure 3-1B).

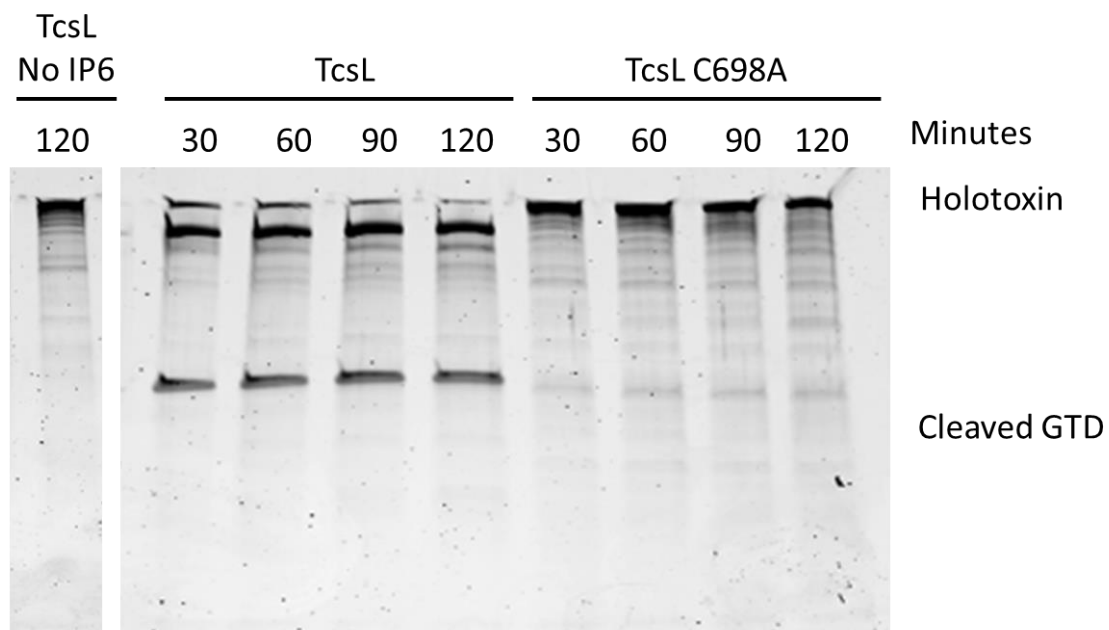




**Figure 3-1: mPMVECs as a model to study cytotoxicity.** (A) Murine pulmonary microvascular endothelial cells (mPMVECs) were incubated at 33° or 37° C and treated with TcsL over a range of concentrations. Cell viability was determined by CellTiterGLO luciferase 24 hours after intoxication. (B) The dose responses for native and recombinant TcsL were identical. Toxins were incubated with mPMVECs for 24 hours at 37° C. Relative viability was calculated dividing the signal from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates. Error bars represent standard error of the mean. \*\*\*\* shows p-values <0.0001.

*TcsL autoprocessing and glucosyltransferase activities are important for cytotoxicity.*

To determine the importance of the enzymatic activities of TcsL, mutations were introduced into the active sites of the GTD and the autoprocessing domain. The glucosyltransferase activity was ablated by the introduction of D286N and D288N mutations (DxD), and the autoprocessing activity was eliminated by introducing a C698A mutation (78, 81, 92) (Table 3-1, Figure 3-2). The endothelial cells were treated with TcsL as well as the glucosyltransferase and autoprocessing mutants (DxD and C698A, respectively). Cell viability was determined at 24 and 48 hours post-intoxication using CellTiterGLO. When cells were treated with glucosyltransferase-deficient TcsL, no cell death was seen except at the highest concentration tested (Figure 3-3). The autoprocessing mutant was attenuated but still induced cytotoxicity. These findings



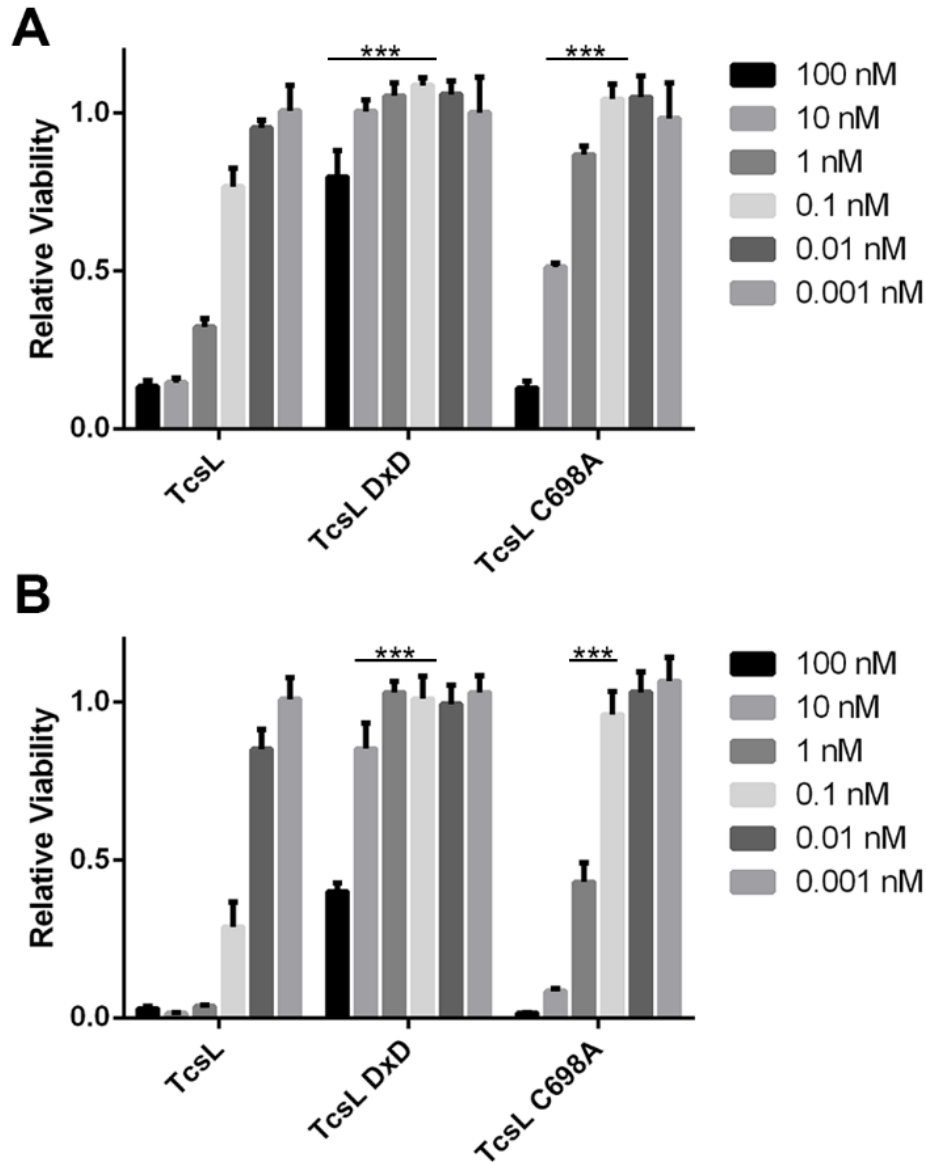
**Figure 3-2: TcsL C698A is deficient in autoprocessing ability.** *In vitro* cleavage assay was performed for TcsL and TcsL C698A by placing in a reaction with IP6 and DTT at 37° for up to 120 minutes. The samples were separated on a SDS-PAGE gel and bands corresponding to cleaved GTD are seen in TcsL lanes but only uncleaved holotoxin is present in TcsL C698A samples.

suggest that glucosyltransferase activity is required for cytotoxicity while the autoprocessing activity is important but not required for induction of cell death.

Plasmid Name	Protein Expressed	Primer Sequences Used
pBL552	TcsL in BMEG20 vector	
pBL685	TcsL C698A	5'-GTCTATAGAAATAAATTTACTAGGAGCTAATATGTTTCAGCTATAACGTTAATG-3' 5'-CATTACGTTATAGCTGAACATATTAGCTCCTAGTAAATTTATTTCTATAGAC-3'
pBL686	TcsL D286N, D288N	5'-GGTGGGGTATATTTAAATGTTAATATGTTACCAGGTATAC-3'
		5'-GTATACCTGGTAACATATTAACATTTAAATATACCCACC-3'
pBL691	TcsL R18A	5'-GGCATATGTAAAATTTGCTATTCAAGAAGATGAGTACGTAGC-3'
		5'-GCTACGTA CTCTTCTTGAATAGCAAATTTTACATATGCC-3'
pBL692	TcsL F17N	5'-GGCATATGTAAAAAATCGTATTCAAGAAGATGAGTACGTAGC-3' 5'-GCTACGTA CTCTTCTTGAATACGATTTTTTACATATGCC-3'
pBL745	TcsL F17N, R18A	5'-GGCATATGTAAAAAATGCTATTCAAGAAGATGAGTACGTAGC-3' 5'-GCTACGTA CTCTTCTTGAATAGCATTTTTTTACATATGCC-3'
pBL746	TcsL F17N, R18A, C698A	sequences above for pBL745 and pBL685

**Table 3-1: Plasmid information and primer sequences for TcsL and TcsL mutations**

We next assayed the impact of enzyme mutation on the glucosylation of Rac1 and Ras GTPases in cells. We analyzed endothelial cell lysates that had been treated with 10 nM TcsL, TcsL Dx D, and TcsL C698A. Cell lysates were analyzed by Western blots using Rac1 and Ras antibodies that are specific to unglucosylated GTPase (90, 124, 164). Once the Rac1 and Ras are modified by TcsL, the antibody can no longer recognize its epitope, and the signal is lost. The cells that were treated with TcsL showed modification after one hour, and glucosylation of both GTPases was nearly complete by 2 hours (Figure 3-4). When TcsL Dx D was used to intoxicate cells, the loss of the glucosyltransferase activity rendered the mutant unable to glucosylate

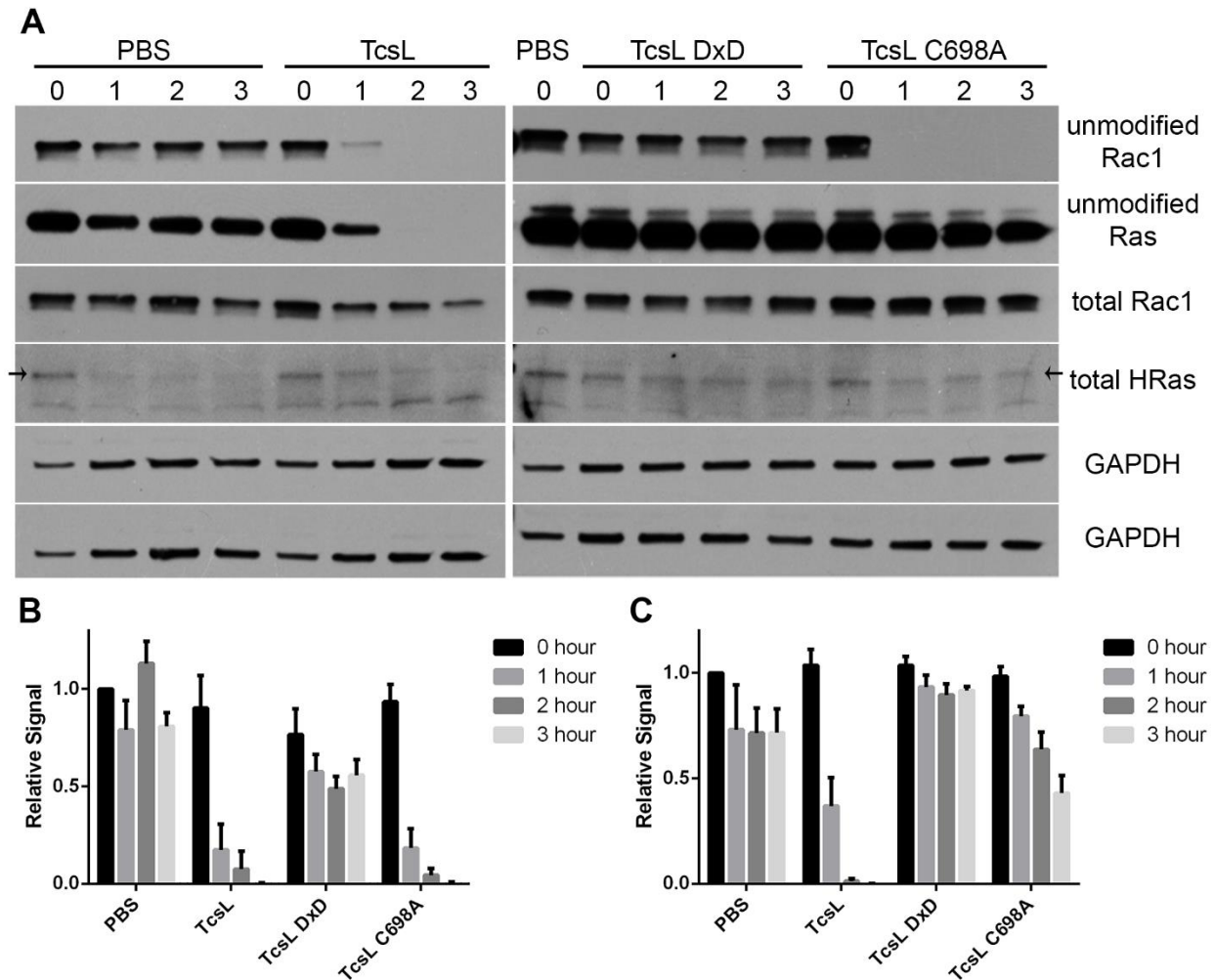


**Figure 3-3: TcsL autoprocessing and glucosyltransferase mutants are impaired in cytotoxicity.** Endothelial cells were treated with TcsL, glucosyltransferase deficient TcsL (DxD), or autoprocessing deficient TcsL (C698A) over a range of concentrations. Cell viability was determined by GLO luciferase 24 hours (A) and 48 hours (B) after intoxication. Relative viability was calculated by dividing the signal from intoxicated cells by the signal from those that were mock treated and averaging across three biological replicates. Error bars represent standard error of the mean. \*\*\* shows p-values <0.001.

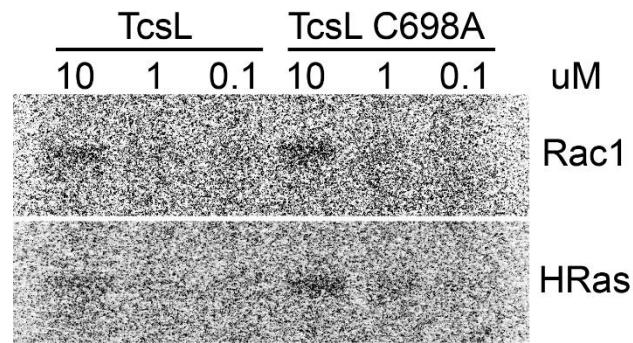
both Rac1 and Ras. Interestingly, TcsL C698A was able to quickly glucosylate Rac1, similar to wildtype TcsL, but was attenuated in its ability to glucosylate Ras GTPases.

The introduction of the autoprocessing mutation did not inhibit the ability of Rac1 or

HRas glucosylation as shown *in vitro* compared to wild-type TcsL (Figure 3-5). The difference in Rac1 and Ras glucosylation when the autoprocessing activity was ablated suggests that there is a localization difference for Rac1 and Ras.



**Figure 3-4: Kinetics of Rac1 versus Ras glucosylation differ when cells are treated with TcsL autoprocessing mutant.** Endothelial cells were treated with 10 nM TcsL, TcsL Dx/D, TcsL C698A or PBS only. The cells were incubated for 0, 1, 2, or 3 hours before lysis. (A) Lysate supernatants were analyzed by Western blot using antibodies for unglucosylated Rac and Ras, total Rac, total HRas, and GAPDH. Quantification was performed for Rac (B) and Ras (C) signal relative to PBS-treated sample at 0 hours. Error bars show standard error for the mean of four replicates.

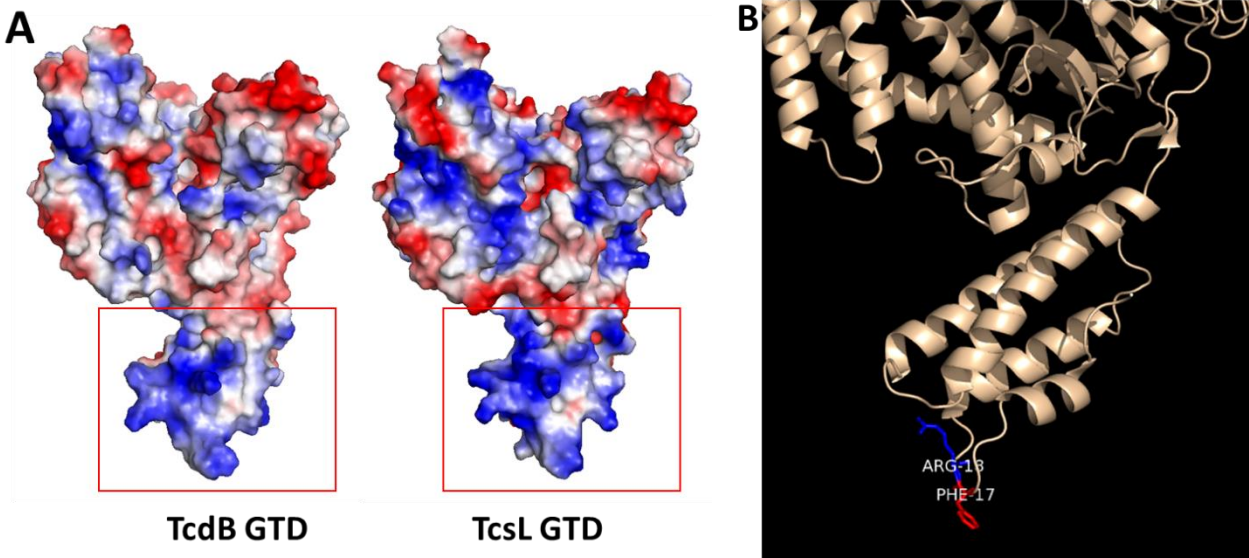


**Figure 3-5: Autoprocessing mutation does not alter glucosylation.**

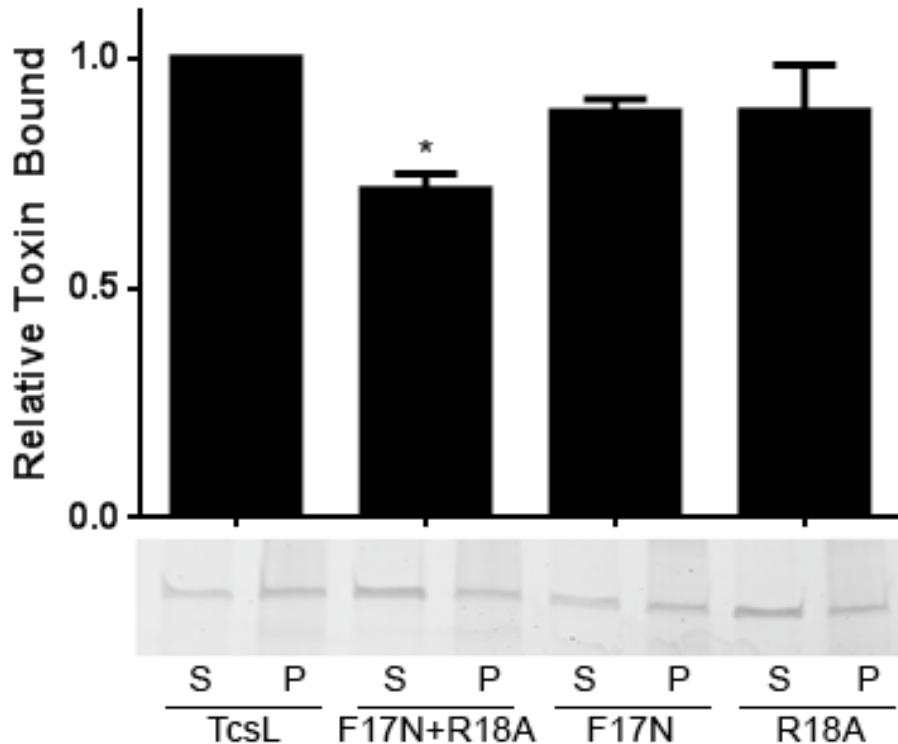
Recombinantly expressed GST-Rac1 and GST-HRas were glucosylated using TcsL and TcsL C698A in the presence of UDP-[<sup>14</sup>C]glucose for 1 hour. The protein gels were imaged and bands represent glucosylated GTPase.

*Mutations in the GTD membrane localization domain inhibit TcsL cytotoxicity.*

Previous reports have identified a membrane localization domain (MLD) on the GTD that is involved in the localization of the GTD to the plasma membrane (143, 144). To test the importance of the MLD in TcsL cytotoxicity, F17N and R18A mutations were created as well as a double mutant (F17N, R18A). These residues are found on the surface of the MLD and have been previously implicated in membrane localization (Figure 3-6) (144), which we also saw when the mutants were tested in a liposome binding assay (Figure 3-7). A triple mutant (F17N, R18A, C698A) was also created to determine the impact of both autoprocessing and MLD mutations on cytotoxicity. Endothelial cells were treated with TcsL and TcsL MLD mutants, and cytotoxicity was assessed. Relative to treatments with wild-type TcsL, cytotoxicity was significantly attenuated when cells were treated with the MLD mutants (Figure 3-8). The level of attenuation for TcsL MLD mutants is similar to the attenuation of TcsL C698A and suggests that TcsL cytotoxicity is dependent upon GTD localization to the cell membrane.



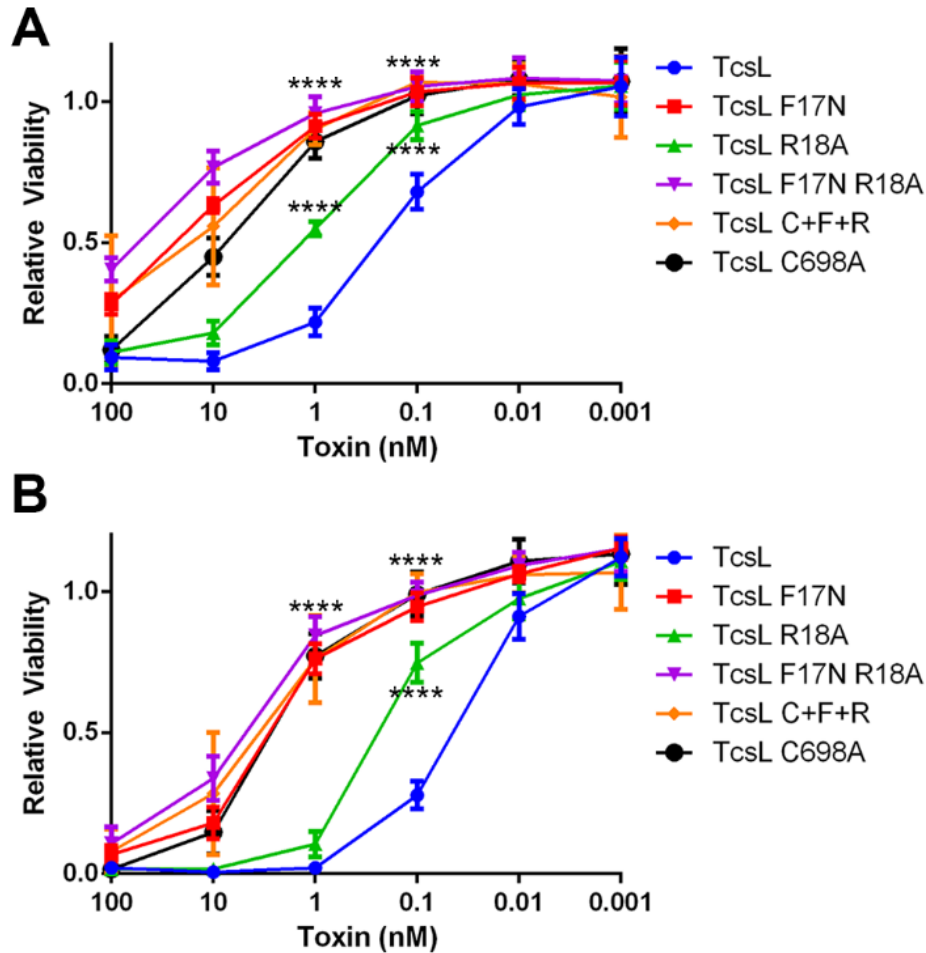
**Figure 3-6: TcsL MLD location and mutations.** A) Electrostatic maps of the GTD of TcdB and TcsL are shown. At the N-terminus, boxed in red, is the location of the conserved membrane localization domain. The homology between the TcsL MLD with TcdB can be seen. Positively charged residues are shown in blue and negatively charged residues are shown in red. Adapted from Pruitt et al. (171). B) A zoomed image of the TcsL MLD with the amino acids targeted for mutagenesis (F17 and R18) are shown at the bottom of the structure.



**Figure 3-7: MLD mutants disrupt lipid binding.** TcsL and TcsL mutants were combined with liposomes and after incubation the bound toxin was pelleted. The gel (bottom) shows both supernatant and pellet fractions for each toxin and is representative of three separate experiments. The relative values show that the bound toxin level decreases when membrane localization mutations are introduced. \* shows p-value <0.05.

To understand how MLD mutations affect the mechanism of cellular response, we next assayed the glucosylation of Rac1 and Ras by TcsL MLD mutants. Cell lysates were obtained from endothelial cells treated with TcsL and TcsL MLD mutants and analyzed by Western blot for Rac1 and Ras glucosylation. While TcsL was able to quickly glucosylate Rac1 and Ras, the MLD mutants were attenuated in capacity to glucosylate substrates in the cell (Figure 3-9). Both TcsL F17N and TcsL R18A were



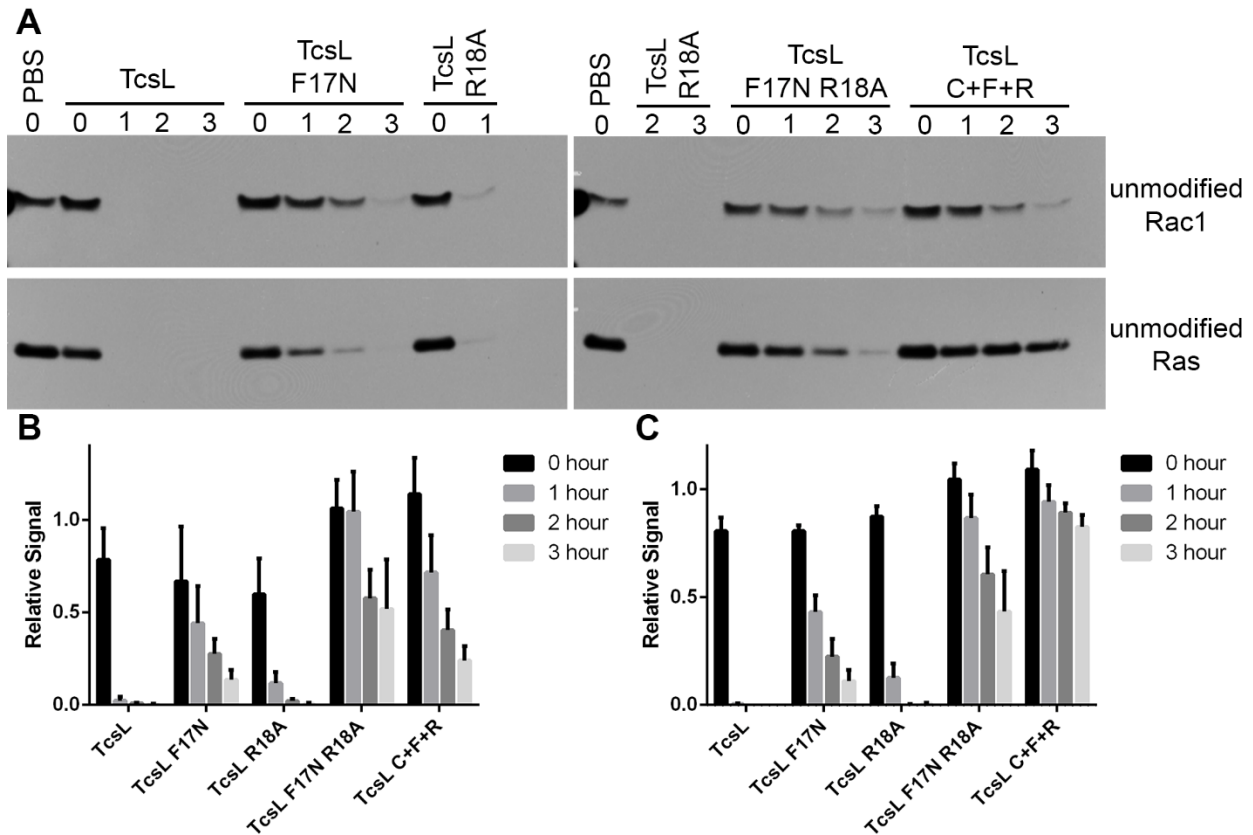


**Figure 3-8: TcsL membrane localization domain is important for cytotoxicity.**

Endothelial cells were treated with various concentrations of TcsL, TcsL membrane localization mutants (F17N, R18A, and F17N R18A), TcsL C698A, or the triple mutant TcsL F17N R18A C698A (C+F+R). Cell viability was determined by GLO luciferase 24 hours (A) and 48 hours (B) after intoxication. Relative viability was calculated by comparing the signal to cells that were mock treated and represents the average of three replicates. Error bars represent standard error of the mean. \*\*\*\* shows p-values <0.0001.

delayed in their glucosylation of Rac1 and Ras, and the double mutant, TcsL F17N R18A, was the most attenuated in its glucosylation of Rac1 and Ras. The triple mutant (F17N, R18A, C698A) was also inhibited in both Rac1 and Ras glucosylation. These observations indicate that the MLD interaction with the membrane is important for the

glucosylation of both Rac1 and Ras and that the MLD mutation prevents the autoprocessing-deficient TcsL from efficiently glucosylating Rac1.



**Figure 3-9: Membrane localization domain is required for efficient Rac modification.** Endothelial cells were treated with 10 nM TcsL, TcsL F17N, TcsL R18A, TcsL F17N+R18A, or TcsL C698A+F17N+R18A for 0, 1, 2, or 3 hours. Cells were lysed using detergent-free lysis and supernatants were stained for unmodified Rac and unmodified Ras GTPase (A). The signals relative to untreated samples were quantified for unmodified Rac (B) and unmodified Ras (C) and averaged across three replicates. Error bars represent standard error of the mean.

## Discussion

The purpose of our study was to understand the impact of the different TcsL enzymatic activities in the context of lung endothelial cells. Although many cell lines have been used in the study of TcsL-induced effects, we wanted to find a cell line that

would be a good, physiologically accurate model for the effects seen during infection. Symptoms seen commonly in *C. sordellii* infection are edema, hypotension, and multi-organ failure, indicating that the toxins act strongly upon host microvasculature. Our use of conditionally immortalized murine pulmonary microvascular endothelial cells allowed us to induce a primary cell-like state that removed the potential interference of common mutations associated with immortalization. Figure 3-1 shows that TcsL is a potent cytotoxin in these cells and, thus, allowed us to assess the impact of enzymatic activities in the cell death mechanism.

When endothelial cells were treated with TcsL and the DxD and C698A mutants, we saw cytotoxicity was inhibited for the glucosyltransferase mutant and impaired in the autoprocessing mutant. The inhibition of cytotoxicity when treating cells with TcsL DxD supports previous research showing that glucosylation of the host cell GTPases leads to arrest of the cell cycle and induction of apoptosis (123). The decrease in cytotoxicity seen with TcsL C698A suggests that the autoprocessing activity is important in TcsL cytotoxicity (Figure 3-3).

Previous studies investigating the role of autoprocessing in the *C. difficile* toxins suggest that preventing GTD release through inactivation of the autoprocessing activity has only modest effects on cytopathic responses (165), and, in the case of TcdB, no impact on toxin-induced necrosis (82). The observations beg the question, why has the autoprocessing activity been retained in this family of toxins? A key difference between TcsL and the *C. difficile* toxins is that TcsL is more active in the modification of Ras GTPases (90, 164), and Ras inactivation has been linked to TcsL-induced cell death (123). We therefore assayed whether TcsL and the TcsL C698A mutant were capable

of modifying both Rac and Ras. The experiments in Figure 3-3 reveal that while autoprocessing is not required for the modification of Rac, it is important for the efficient modification of Ras.

The differential ability to glucosylate the GTPases when the GTD is not cleaved from the holotoxin suggests a difference in the localization of Rac and Ras GTPases. It has been reported that Rac cycles to the endosomes where it is activated before trafficking back to the membrane (126). TcsL C698A does not release the GTD, and it remains bound to the endosome. The GTD may then encounter Rac that has been trafficked to the endosome for activation. Ras GTPases, however, are trafficked to and found in abundance at the plasma membrane after translation (133). We therefore propose that the GTD that remains tethered to the endosome does not immediately encounter and glucosylate Ras proteins. We propose that the slow Ras glucosylation is due to endosomal membranes, with the tethered GTD, recycling back to the cell surface. The delay in Ras glucosylation is enough to cause the decrease in cytotoxicity.

Previous studies have identified a MLD on the TcsL GTD that is conserved across all large clostridial toxins and important in membrane localization. The MLD may be important for GTD to insert into the plasma membrane and help tether it to the membrane (145, 146). We looked at the impact of MLD point mutations on both cytotoxicity (Figure 3-8) and the glucosylation of host GTPases (Figure 3-9). Our work shows that the introduction of MLD mutations inhibits the ability to induce cytotoxicity and delays the glucosylation of both Rac and Ras GTPases, supporting the importance of the MLD in tethering GTD to the cell membrane where it can interact with and glucosylate the GTPases. Interestingly, when we combined the MLD mutations with the

autoprocessing mutation, we saw a decrease in cytotoxic ability as well as glucosylation of both Rac and Ras. While TcsL C698A was able to efficiently glucosylate Rac, the loss of efficient Rac glucosylation with the introduction of MLD mutations suggests that it is not enough for the GTD to be tethered to the endosome but also relies on the ability of the GTD to interact with the endosomal membrane through the MLD.

Our studies have shown the impact of mutating the enzymatic domains of TcsL. While the glucosyltransferase activity is needed for Rac modification and cytotoxicity, autoprocessing-deficient TcsL is impaired in cytotoxicity but efficient in its modification of Rac. We also show that the interaction with the cell membrane, not just proximity, is needed for efficient glucosylation of GTPases. The increased understanding of each toxin domain during host intoxication provides a foundation for more targeted approaches to study toxin-induced cellular events.

## CHAPTER IV

### CONCLUSIONS AND FUTURE DIRECTIONS

#### Conclusions

TcsH and TcsL are the primary virulence factors of *C. sordellii*, whose infections lead to serious illness and have a high mortality rate. Due to the fact that *C. sordellii* infection is a toxin-mediated disease, a better understanding of TcsH and TcsL will help to understand the disease and provide us further insights into potential ways to combat or prevent serious infections. Much of our understanding of the functions and mechanisms of action for *C. sordellii* toxins, particularly TcsH, has been derived from studies of homologous LCTs. In my thesis work, I have shown that TcsH and TcsL induce cell death by different mechanisms and that TcsL glucosylation depends on autoprocessing and membrane localization.

In Chapter II, I have presented work comparing the cell death mechanisms induced by TcsH and TcsL, and compared these to the cytotoxicity induced by TcdA and TcdB of *C. difficile*. I have shown that TcsL induces apoptosis based on the activation of caspases and lack of necrotic death markers, which supports what has been shown previously in the literature. TcsH induces a necrotic cell death at higher toxin concentrations that is marked by LDH release and onset of cytotoxicity seen two hours after intoxication. After looking for common signaling events that occur during

many cell death pathways, I show that TcsH induces intracellular calcium release at necrotic concentrations and that both TcsH and TcsL induce ROS production.

This work shows the differences between *C. sordellii* toxins and their homologues from *C. difficile*. TcsH/TcdA and TcsL/TcdB share high sequence and structural homology which may suggest that the toxins act in similar manners. However, the cell death mechanisms induced by the homologous toxins are different. TcsH induces a necrotic cell death at higher concentrations which is similar to what was seen and reported for TcdB, while both TcsL and TcdA induce apoptosis. Interestingly, TcsH induced intracellular calcium release and ROS production at high concentrations. In contrast, TcdB treated endothelial cells did not show evidence of ROS production. These data suggest that while TcsH and TcdB share some similarities in their necrosis induction, the mechanisms for each toxin are unique and differences exist despite the homology between the LCTs.

In Chapter III, I have shown work describing the importance of the enzymatic activities and membrane localization of TcsL. Introduction of point mutations to inhibit activity lead to the conclusion that TcsL cytotoxicity is dependent upon glucosyltransferase activity. I have also shown that autoprocessing activity is important but not necessary for TcsL-induced cytotoxicity. Ras glucosylation depends on autoprocessing of TcsL, while Rac glucosylation was found to be independent of autoprocessing. Further work showed that the MLD was important for the induction of cytotoxicity and MLD mutants displayed decreased in glucosylation activity in cells.

This work has helped us to increase our understanding of TcsL, one of the main virulence factors in *C. sordellii* infections. We built on our previous understanding of

LCT domains and enzymatic activities to determine which were important and what role they played during toxin-induced glucosylation and cytopathicity. Each enzymatic activity tested is important for cytotoxicity while autoprocessing is not required. Autoprocessing activity is also not necessary for Rac glucosylation but is important for Ras glucosylation, suggesting that differential localization of the TcsL GTD determines the glucosylation profile. The MLD was also shown to be important for glucosylation and cytotoxicity of TcsL by the introduction of mutations which decreased cytotoxicity and delayed glucosylation. By combining the autoprocessing and MLD mutants, I showed the MLD is important for interacting with the host membrane to facilitate glucosylation and that the MLD is not simply for GTD localization.

### **Future Directions**

#### *Determine mechanism of TcsH-induced necrosis*

In Chapter II, I showed that TcsH is able to induce a necrotic cell death in endothelial cells at a high concentration. I also showed that TcsH induces an intracellular calcium release and low levels of ROS production as well. While this suggests that calcium and ROS signaling may play a role in TcsH-induced necrosis, I would like to look further into the mechanism driving necrosis in endothelial cells. I want to identify host factors that are important for TcsH-induced necrosis and determine what role they play in the signaling events that lead to cell death. To this point, no work has been done to determine the mechanism used by TcsH to induce cytotoxicity and any knowledge of the process would help to identify host factors, protein complexes, or



signaling pathways that can be targeted for therapeutics to reduce the toxin's harmful effects during infection.

To determine the mechanism I would first look to identify host factors involved in the induction of necrosis. This can be done using several approaches: a siRNA screen and a CRISPR knockout screen. The siRNA screen would knockdown protein expression levels in the host cells to identify proteins conferring sensitivity to toxin-induced necrosis, similar to what was done with TcdB in our lab. A CRISPR-knockout screen would be similar but would allow for the complete knockout of protein expression using the CRISPR-Cas system developed previously (166).

Once potential host proteins are identified, I can begin testing their role in TcsH-induced necrosis. The first priority would be to confirm that they are important in necrosis by looking for changes in LDH release of TcsH-treated cells when the target protein is knocked down as well as to see whether the levels of activated caspases are altered. Once several proteins are identified, I can begin to create a pathway of signaling and interactions by removing components and testing for disrupted interactions and signaling events downstream.

The importance of the observed ROS and intracellular calcium in the induction of necrosis will also be evaluated. The ROS production can be inhibited by treating endothelial cells with compounds, such as tempol, DPI, ebselen, or NAC, that are known inhibitors (141). The decrease in ROS production can then be measured and the cells will be tested for a correlated decrease in necrosis that would support the importance of ROS production as a precursor in necrotic cell death. The same can be done using inhibitors of intracellular calcium release to test for the importance of

intracellular calcium signaling for TcsH-induced necrosis. To determine whether intracellular calcium release or ROS production provide signaling crosstalk, the levels of each can be tested under the same conditions to determine if a change in the levels of one alters the other.

#### *Determine the importance of TcsH enzymatic activities*

Very little work has been done to this point to study TcsH and no in-depth work has been done looking at the importance and role of the enzymatic activities of the TcsH domains on intoxication. Similar to the work presented in Chapter III for TcsL, mutations can be introduced to inhibit the enzymatic activity of the glucosyltransferase and autoprocessing domains and the impact upon cytotoxicity and cytopathicity can be determined. Having an increased understanding of the importance of TcsH enzymatic activities can help to better understand the toxin and design the best inhibitors to prevent TcsH from damaging host tissues during *C. sordellii* infection. While the field currently holds that the activity of each domain is important for intoxication, the ability of TcsH to induce necrosis at higher concentrations similar to TcdB emphasizes a need to test the role of each enzymatic activity. Work from our lab has shown TcdB is able to induce cell death independent of autoprocessing or glucosyltransferase activity, suggesting these enzymatic activities may not be necessary in TcsH as well (82, 141).

Mutations will be introduced into recombinantly expressed TcsH that inhibit the autoprocessing activity (C700A) and glucosyltransferase activity (D285A D287A). Endothelial cells will be treated with the mutant toxins and compared with wild type TcsH looking for changes in glycosylation levels of Rac1 and the amount of cytotoxicity

induced. A shift in the ability of TcsH to induce necrosis, as well as a shift in the concentration of toxin required, could also be tested using the mutants by measuring changes in the levels of LDH release in cells. If TcsH does induce necrosis by a mechanism similar to TcdB, we would not expect to see a change in necrosis when the enzymatic activities are inhibited.

This work would help us to better understand the importance of the enzymatic activity and domains found in TcsH. By understanding which activities are important for the cytotoxicity and cytopathicity of TcsH, we can determine which toxin activities would be most effective to target and inhibit for therapeutic purposes and reduction of tissue damage observed during *C. sordellii* infection. The work would also help to increase our understanding of the similarities between the LCTs. If TcsH does not require its enzymatic activities to induce necrosis, similar to what has been observed for TcdB, it would support the idea that the necrotic mechanism and signaling pathways may be a conserved characteristic of LCTs and necrosis may be induced by other toxins but has not yet identified.

I initially started working on this future direction as part of my project but experienced difficulties recombinantly expressing TcsH. After experiencing differences in cytotoxicity between preparations of purified TcsH, it was noticed that TcsH from different preparations ran at slightly different sizes on a protein gel. I sequenced TcsH from several isolates of the toxin-expressing *B. megaterium* and found that deletions were present in most of the colonies. The mutations were localized to the CROPs region of TcsH and the location and length of the deletions was random. The deletions also occurred rapidly after the expression plasmid was transformed into *B. megaterium*,

making it difficult to consistently express full-length TcsH. Moving forward with a project relying on recombinant expression of TcsH and TcsH mutants would rely on developing a successful expression system. One possibility is to introduce recombination deletions into *B. megaterium* system that would prevent deletions from recombination events. Another possibility is to switch to expression using *B. subtilis*, which has recombination deficient strains available for expression. The drawback to this approach is that *B. subtilis* forms spores and the ability to do so would need to be removed prior to safe expression of TcsH in the laboratory. A third approach would be to engineer a codon-optimized TcsH gene that would reduce the number of nucleotide repeats in the CROPs region and use codons that are more commonly expressed by *B. megaterium*. The sequence optimization would hopefully decrease the ability of the bacteria to perform recombination and allow them to more easily express the toxin.

#### *Identify TcsL and TcsH surface binding receptors*

Research has shown that TcsH and TcsL enter cells through receptor-mediated endocytosis, but no work has identified the receptors for each toxin. Future work focused on identifying the cell surface receptors responsible for binding TcsH and TcsL would provide valuable information about the early entry and intoxication process for each toxin. Our lab has a siRNA library that can be used to knockdown genes in endothelial cells and changes in cytotoxicity can help identify potential toxin binding targets. Further screening to test changes in toxin binding can make use of fluorescently labeled toxin, either by direct labeling or the introduction of a fluorescent tag, to test for a decrease in binding ability by FLOW cytometry. Toxin receptors can also be validated

using binding competition assays including treatment of cells with antibodies against the cell receptor or pre-treatment of toxin with soluble domains or peptides from the potential receptors.

To further the understanding of the TcsH and TcsL receptors, work can also be done to identify the binding sites between toxin and receptor. Expression constructs can be generated for truncated TcsH and TcsL as well as individual toxin domains. These can be used in pull-down experiments combined with purified full-length receptors or truncated receptor constructs to narrow down the peptides required for a binding interaction between toxin and receptor. After narrowing down the binding site on each protein, structural studies can be done to identify the amino acids responsible for the binding interaction. Our lab regularly performs crystallography studies and determining the crystal structure of toxin bound to its receptor would allow for identification of precise amino acids involved in the interaction.

The amino acids identified by crystallography as important for toxin binding would be tested by introducing point mutations both into the toxin and the receptor. The mutated toxin and receptor expression constructs can be tested by pull-down assays to determine if the binding efficiency is decreased. A FLOW cytometry-based binding assay can also be performed by treating cells with mutated toxin or treating cells that express mutated receptors and looking for a decrease in binding fluorescence of TcsH or TcsL. Another possible assay that our lab has recently utilized to confirm the importance of specific amino acids in the toxin-receptor binding interaction is to incorporate unnatural amino acids that can be photo-cross-linked to the binding partner (167). If the site of the unnatural amino acid is important for binding, after exposing a

mixture of receptor and toxin, a bound fragment at a higher molecular weight will be present on a protein gel.

The identification of TcsH and TcsL receptors and the specific amino acids involved will allow for advances in treatment of *C. sordellii* infection. Knowing the exact binding epitopes for the toxins allows for the production of antibodies that can disrupt and block binding of the toxins. By blocking the interaction with receptors, the toxins' damaging effects in host tissues can be decreased and hopefully provide healthcare workers more time to treat and control *C. sordellii* infections and ultimately lead to a decrease in the mortality rate.

#### *Identify compounds that inhibit TcsH and TcsL cytotoxicity*

Another possible direction to pursue for TcsH and TcsL is the identification of chemical compounds that can inhibit cytotoxicity. As a toxin-mediated disease, the damage caused during *C. sordellii* infection can be greatly reduced and the survival rate increased if we can provide effective treatment to patients that inhibits TcsH and TcsL.

To identify chemical compounds that can inhibit toxin-mediated cytotoxicity, we can make use of the Vanderbilt high-throughput core to screen chemical compounds for their ability to decrease TcsH and TcsL cytotoxicity. Compounds that decrease the cell death induced by the toxins can then be followed up to determine the toxin mechanism that is disrupted and inhibited. The compounds can be tested for their ability to inhibit cell entry and trafficking, autoproducting, glucosylation, or another important step of intoxication. The compounds can also be studied to see if the chemical can be optimized for an increased inhibition and testing can also be done to determine the

safety of using the compound in a clinical setting. The compound can also be tested in an animal model of *C. sordellii* infection to determine the level of protection the compound can provide by inhibiting TcsH or TcsL.

Although we have learned a great deal already about TcsH and TcsL cytotoxicity in cells, there is still much to be studied. The knowledge gained from these studies will also help us to better understand LCTs and the range of activities and impacts they are responsible for in cells and tissue. An increased understanding of these toxins will be important for directing research and the development of more effective treatments for *C. sordellii* infections.

## APPENDIX

### LIST OF PUBLICATIONS

Craven R and Lacy DB. “*Clostridium sordellii* lethal-toxin autoprocessing and membrane localization activities drive GTPase glucosylation profiles in endothelial cells.” *mSphere*, **2015**, 1(1):e00012-15.

Cox RG, Erickson JJ, Hastings AK, Becker JC, Johnson M, Craven RE, Tollefson SJ, Boyd KL, and Williams JV. “Human Metapneumovirus Virus-Like Particles Induce Protective B and T Cell Responses in a Mouse Model.” *Journal of Virology*, **2014**, 88(11): 6368-6379.

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