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REGULATION OF TOXIN SYNTHESIS BY CLOSTRIDIUM DIFFICILE

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A

DISSERTATION

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By

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DEDICATION

I dedicate this work to my grandmother, who encouraged and helped instill in me a passion for hard work, perseverance, progress, knowledge, and the curiosity to explore the unknown. Grandma, your life has been a great source of inspiration for me!

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ABSTRACT

REGULATION OF TOXIN SYNTHESIS BY *CLOSTRIDIUM DIFFICILE*

Clostridium difficile is the leading definable cause of nosocomial diarrhea worldwide due to its virulence, multi-drug resistance, spore-forming ability, and environmental persistence. The incidence of *C. difficile* infection (CDI) has been increasing exponentially in the last decade. Virulent strains of *C. difficile* produce either toxin A and/or toxin B, which are essential for the pathogenesis of this bacterium. Current methods for diagnosing CDI are mostly qualitative tests that detect the bacterium, the toxins, or the toxin genes. These methods do not differentiate virulent *C. difficile* strains that produce active toxins from non-virulent strains that do not produce toxins or produce inactive toxins. Based on the knowledge that *C. difficile* toxins A and B cleave a substrate that is stereochemically similar to the native substrate of the toxins, uridine diphosphoglucose, a quantitative, cost-efficient assay, the Cdifftox activity assay, was developed to measure *C. difficile* toxin activity. The concept behind the activity assay was modified to develop a novel, rapid, sensitive, and specific assay for *C. difficile* toxins in the form of a selective and differential agar plate culture medium, the Cdifftox Plate assay (CDPA). This assay combines in a single step the specific identification of *C. difficile* strains and the detection of active toxin(s). The CDPA was determined to be extremely accurate (99.8% effective) at detecting toxin-producing strains based on the analysis of 528 *C. difficile* isolates selected from 50 tissue culture cytotoxicity assay-positive clinical stool samples. This new assay advances and improves the culture methodology in that only *C. difficile* strains will grow on this

medium and virulent strains producing active toxins can be differentiated from non-virulent strains. This new method reduces the time and effort required to isolate and confirm toxin-producing *C. difficile* strains and provides a clinical isolate for antibiotic susceptibility testing and strain typing. The Cdifftox activity assay was used to screen for inhibitors of toxin activity. Physiological levels of the common human conjugated bile salt, taurocholate, was found to inhibit toxin A and B *in vitro* activities. When co-incubated *ex vivo* with purified toxin B, taurocholate protected Caco-2 colonic epithelial cells from the damaging effects of the toxin. Furthermore, using a caspase-3 detection assay, taurocholate reduced the extent of toxin B-induced Caco-2 cell apoptosis. These results suggest that bile salts can be effective in protecting the gut epithelium from *C. difficile* toxin damage, thus, the delivery of physiologic amounts of taurocholate to the colon, where it is normally in low concentration, could be useful in CDI treatment. These findings may help to explain why bile rich small intestine is spared damage in CDI, while the bile salt poor colon is vulnerable in CDI.

Toxin synthesis in *C. difficile* occurs during the stationary phase, but little is known about the regulation of these toxins. It was hypothesized that *C. difficile* toxin synthesis is regulated by a quorum sensing mechanism. Two lines of evidence supported this hypothesis. First, a small (<1 KDa), diffusible, heat-stable toxin-inducing activity accumulates in the medium of high-density *C. difficile* cells. This conditioned medium when incubated with low-density log-phase cells causes them to produce toxin early (2-4 hrs instead of 12-16 hrs) and at elevated levels when compared with cells grown in fresh medium. These data suggested that *C. difficile*

cells extracellularly release an inducing molecule during growth that is able to activate toxin synthesis prematurely and demonstrates for the first time that toxin synthesis in *C. difficile* is regulated by quorum signaling. Second, this toxin-inducing activity was partially purified from high-density stationary-phase culture supernatant fluid by HPLC and confirmed to induce early toxin synthesis, even in *C. difficile* virulent strains that over-produce the toxins. Mass spectrometry analysis of the purified toxin-inducing fraction from HPLC revealed a cyclic compound with a mass of 655.8 Da. It is anticipated that identification of this toxin-inducing compound will advance our understanding of the mechanism involved in the quorum-dependent regulation of *C. difficile* toxin synthesis. This finding should lead to the development of even more sensitive tests to diagnose CDI and may lead to the discovery of promising novel therapeutic targets that could be harnessed for the treatment *C. difficile* infections.

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CHAPTER 1: THE NATURAL HISTORY OF *CLOSTRIDIUM DIFFICILE* AND PATHOGENESIS

1.1 The History of *C. difficile*

Clostridium difficile was first isolated in 1935 by Hall and O'Toole (87), who were investigating the development of normal bacterial flora in neonates. They collected feces of new-born babies from sterile diapers, suspended the feces in sterile water, and examined prepared slides microscopically using the Gram's method (84) and methylene blue staining. Primary cultures were prepared and tested for the presence of aerobic bacteria on blood agar and eosin methylene blue lactose agar plates. The presence of anaerobic bacilli was also tested using dextrose broth constricted tubes and deep iron brain medium. Different bacterial species were isolated and identified from the feces of these neonates including various species of *Streptococcus*, *Micrococcus*, *Lactobacillus*, and anaerobic bacilli. The most interesting among them was an obligate anaerobic bacillus (Fig. 1.1) that was observed to be an actively motile, heavy-bodied rod with elongated sub-terminal or nearly terminal spores of about the same diameter as the rods (87). This obligate anaerobic bacillus was associated with *Kopfchenbacterien* in feces from three of the infants. Both bacteria appeared and disappeared at about the same time under similar conditions. According to Hall and O'Toole (87), the isolation of each of the two was complicated when both were present, but this anaerobic bacillus was more difficult to isolate than the *Kopfchenbacterien.* This bacterium was also much more difficult to study, due to its slower growth and lack of distinctive morphological properties.

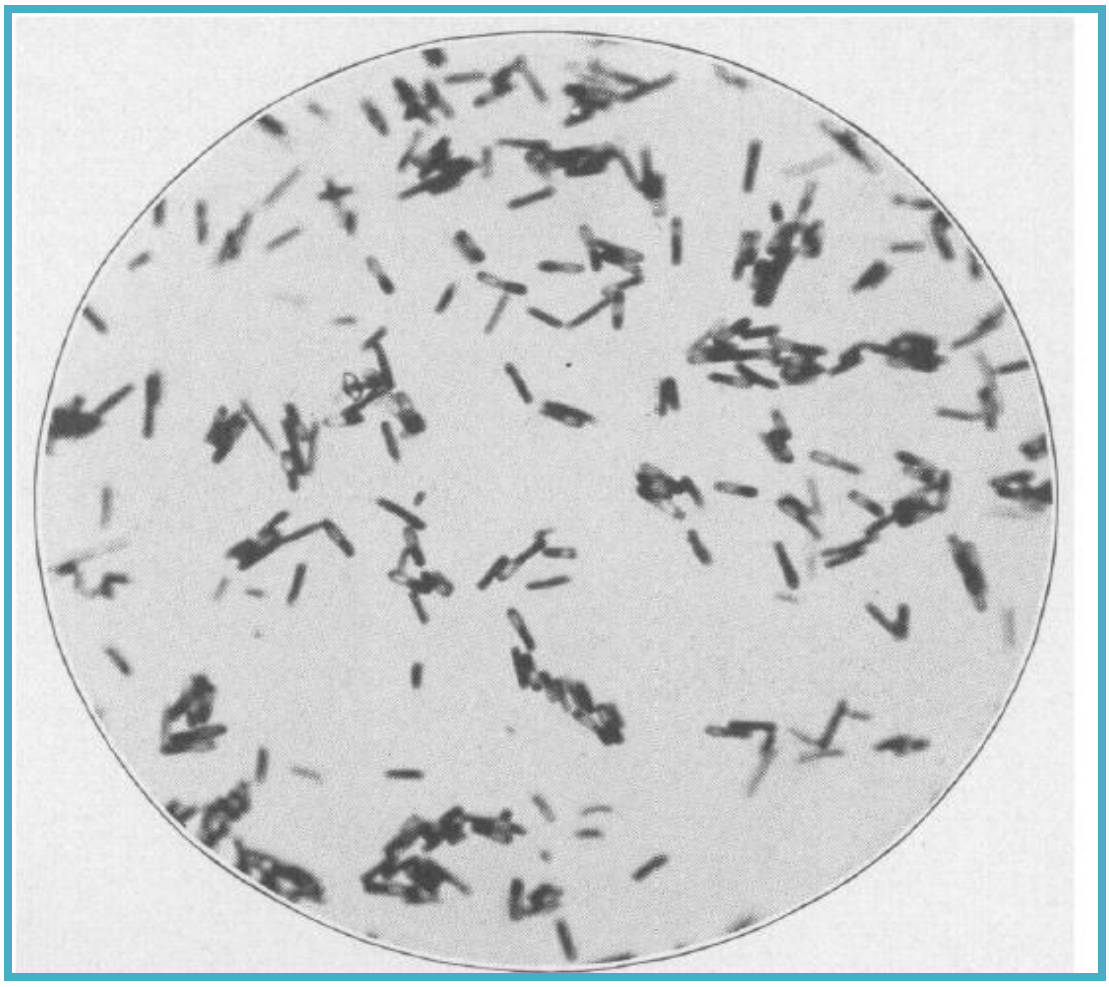

Figure 1.1: *Bacillus difficilis* (now *Clostridium difficile*) grown for 48 hrs on a blood agar slant under alkaline pyrogallol. The Gram stain (84) showed vegetative rods and spores (87). Kingdom: Bacteria; Phylum: Firmicutes; Class: Clostridia; Order: Clostridiales; Family: Clostridiaceae; Genus: *Clostridium*; Species: *Clostridium difficile* (87).

Based on Hall and O'Toole's account (87), the colonies of this anaerobic bacillus generally emerged in deep agar after 48 hrs of incubation and initially appeared minute, flat, and opaque. After 3 days, the colonies appeared lobulated in shape with a diameter of about 1 mm. Single colonies on blood agar slants under alkaline pyrogallol were irregular in form, flat, and non-hemolytic.

Dextrose broth cultures of 13 isolated strains of this new anaerobic bacillus were incubated for 48 hrs under anaerobic conditions at 37^{o}C and subcutaneously inoculated into guinea pigs. Following inoculation, the guinea pigs refused to eat and exhibited moderate to marked edema on the belly, breasts, and at the site of inoculation. Postmortem observation showed marked subcutaneous edema with or without congestion, but with no emphysema. Smears from the subcutaneous site of inoculation in one animal that died within 4 hrs showed numerous Gram-positive rods, but no spores or leukocytes were observed (87). In all the animals, no evidence of septicemia was apparent, but phagocytic leukocytes were found with few free bacteria. A guinea pig injected with a culture filtrate died within 24 hrs, whereas boiling of the culture filtrate for one minute completely destroyed the toxicity. These observations indicated that the new anaerobic *bacillus* produced and released soluble exotoxins into the medium. Hall and O'Toole (87) thus, named this new anaerobic bacterium *Bacillus difficilis* (now called *Clostridium difficile*), due to the difficulties in its isolation and culture.

1.2 The Life Cycle of *C. difficile*

C. difficile exist either as actively dividing vegetative cells or as spores (Fig.1.2). The vegetative cells are obligate anaerobes and sensitive to oxygen. In order to survive under aerobic conditions, *C. difficile* forms oxygen resistant spores. Thus, *C. difficile* vegetative cells excreted in feces must transform into a spore form to survive aerobically (110). It is therefore, widely acknowledged that the *C. difficile* spores initiate the infection process. The spores serve as the dissemination form of this pathogen and germination in the gastrointestinal tract is essential for pathogenesis. This is because only the vegetative cells cause disease.

Little is known about sporulation and germination in Clostridial species. However, the physiological and morphological changes that occur during sporulation in the well-studied *Bacillus* species are similar to that of Clostridial species (214). Sporulation is initiated under conditions of nutrient limitation or other unfavorable conditions, when the cells can no longer maintain vegetative growth. In *Bacillus* species, signal transduction systems that control sporulation initiation have been broadly studied and consist of extended variants of two-component signal transduction systems (sporulation phosphorelay) (25). Sporulation-associated sensor histidine kinases sense various environmental and cellular cues when the cells can no longer sustain vegetative growth. As a result, a specific histidine located in the catalytic domain of sensor histidine kinases becomes autophosphorylated (198). The phosphoryl group is then transferred to an aspartate on the Spo0F response regulator and this leads to activation of the Spo0A response regulator transcription factor via the Spo0B phosphotransferase (198). The

phosphorylation of the active site Spo0A aspartate promotes binding to a specific target sequence (the “0A box”) in or near the promoters of genes under Spo0A control, leading to either activation or repression of these genes (198).

The phosphoryl groups on sensor kinases activate two-component systems and phosphorelays. Sensor kinases generally consist of an N-terminal signal input domain and a catalytic C-terminal kinase domain containing the dimerization and histidine phosphotransfer sub-domain and an ATP binding sub-domain (25, 198). The Spo0F in the sporulation phosphorelay can be phosphorylated by multiple sensor kinases (199). In *B. subtilis,* there are five sensor kinases, KinA to KinE, with conserved active sites that influence sporulation (108). One sensor kinase is normally responsive to a single specific signal ligand; hence, incorporating several kinases increases the variety of the signals that can be sensed and allows various different signals to influence Spo0A activation.

In Bacillus and Clostridium species, Spo0A is highly conserved (214). The regions upstream of genes most likely controlled by Spo0A in Clostridia contain “0A boxes”, suggesting that the mechanism of Spo0A-mediated gene control is similar to that of Bacillus. In Clostridium and Bacillus species, the most important residues of Spo0A that mediate interaction with the nucleotides of the “0A box” are highly conserved (235). However, protein sequence homology analysis indicates that the genomes of sequenced Clostridium species do not appear to encode homologues of Spo0F and Spo0B (199).

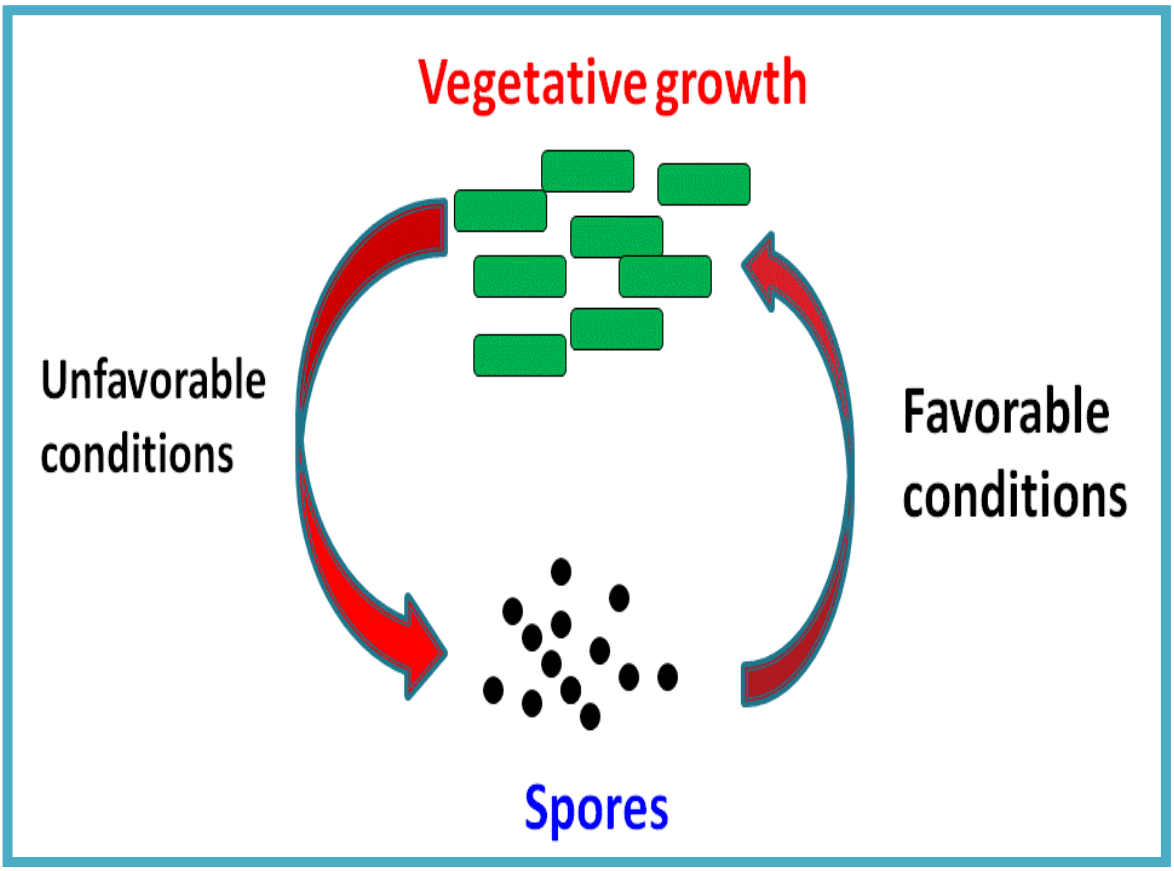

Figure 1.2: The life cycle of *C. difficile*. This bacterium grows anaerobically as vegetative rods. When faced with an unfavorable condition or when efforts to maintain vegetative growth fail, it forms spores. The spores survive the unfavorable condition and germinate when favorable conditions return.

The general consensus has been that the sporulation initiation pathway in Clostridium species does not involve a multi-component phosphorelay, but utilizes a two-component system to sense signals via sporulation-associated sensor kinases that phosphorylate Spo0A directly (200, 229).

Activated Spo0A regulates several post-exponential phase phenomena (214). To sustain their pathogenic lifestyle, bacterial spore-formers appear to have evolved to allow Spo0A to control virulence as well as survival responses (200). For instance, sporulation and toxin production in *Clostridium perfringens* involves Spo0A, which binds to the putative “OA box” upstream of the Cpe cytotoxin (100).

To date, no conclusive evidence exists that links sporulation and toxin production in *C. difficile*. Furthermore, many reports describing direct correlations between toxin production, sporulation, and stationary-phase events (57, 114, 117) have been subsequently disputed by other reports demonstrating negative correlations or no link at all (3, 118). These ambiguities have been made difficult by differences in the strains and the growth media used in these studies, as well as the lack of effective *C. difficile* genetic tools to generate mutants in key genes to enable definite analysis of the pathways involved.

Upon *C. difficile* sporulation initiation, an asymmetrically placed division septum is formed that divides the cell into two unequal compartments (the mother cell and a forespore). Each of these two compartments contains one copy of the chromosome. The larger, mother cell compartment engulfs the forespore leading to maturation (95). The process of maturation involves addition of a peptidoglycan cortex and

several layers of proteins that coat around the forespore. The mother cell finally lyses and the spore is released into the environment (92).

The spore is metabolically dormant when released from the mother cell, but is resistant to various harsh environmental conditions, such as high temperature, oxygen, pH, alcohols, etc. Under suitable conditions, the spores germinate and grow as vegetative cells. Germination in *B. subtilis* can be artificially induced by different compounds such as L-alanine or a mixture of asparagine, glucose, fructose, and potassium ions (195). The *B. subtilis* receptors that have been identified to be involved in sensing these environmental signals are GerA, GerB, and GerK (105). A large amount of calcium dipicolinate is released subsequent to sensing of the germinant, leading to hydration of the core, degradation of the cortex, and resumption of metabolism (191). Homologs of GerA, GerB, and GerK have been identified in many Bacillus and Clostridial species except *C. difficile*. This suggests that *C. difficile* spores respond to a different kind of environmental signal for germination (163, 190).

In *C. difficile*, germination and outgrowth of the spores have not been well studied due to the absence of good genetic tools. Cholate derivatives of bile salts, such as taurocholate and the amino acid glycine, act as co-germinants of *C. difficile* spores (195) and improve the germination of *C. difficile* spores from environmental surfaces and stool samples (17, 223). Lysozyme and thioglycolate also improve the colony formation of *C. difficile* spores (115, 227). Unfortunately, the mechanism by which these molecules stimulate germination of *C. difficile* spores is unknown. The formation of spores by *C. difficile* is a significant impediment in overcoming hospital-

acquired *C. difficile*-associated diseases and recurrence. The spores contribute to the survival of this bacterium after treatment of surfaces with antiseptics and antibiotic therapy, which disrupts the colonic microflora and precipitates *C. difficile* infection, colonization, and overgrowth in the intestinal tract (220).

1.3 Epidemiology of *C. difficile* Infection

The incidence of *C. difficile* infection (CDI) in acute care hospitals in the United States during the early 1990s was maintained at stable rate of 30 to 40 cases per 100,000 population (143). By 2001, the incidence of CDI had risen to almost 50 per 100,000 population. The incidence rate in 2005 (84 per 100,000) was almost triple that of 1996 (31 per 100,000) with concomitant increases in the severity and fatality of this infection (132, 166). Currently, the number of cases of CDI in hospitals in the United States exceeds 250,000 per year (over 80 cases per 100,000) (226, 237), with the total cost of treatment estimated between 1 billion and 3.2 billion U.S. dollars annually (124, 160). The number of cases occurring in the community and non-hospital healthcare facilities appears to make CDI the most common form of bacterial diarrhea in the U.S. Morbidity and mortality resulting from CDI in recent years have increased significantly as a result of changes in the virulence of the causative strains, the expanding number of the elderly and immunocompromised in the population, and antibiotic usage patterns (143, 160, 177, 237). As a result, *C. difficile* has been identified as the causative organism associated with 10-25% of the cases of antibiotic-associated diarrhea, 50-75% of the cases of antibiotic-associated colitis, and 90-100% of the cases of pseudomembranous colitis (13, 61).

C. difficile cells overpopulate the human gastrointestinal tract after the normal gut microflora has been reduced by antibiotic therapy. Thus, antibiotic therapy can be considered the most significant risk factor for developing CDI (14). It appears that the antibiotic therapy allows *C. difficile* to overcome the normal gut microbiota colonization resistance mechanisms against CDI, which include occupying the space required for *C. difficile* proliferation, direct impairment of *C. difficile* growth or germination, siphoning nutrients or germinants from *C. difficile*, and shaping the host innate and adaptive immune responses. CDI predominantly affects the elderly and immunocompromised patients in hospitals and nursing homes (143, 166). CDI is the most frequent cause of morbidity and mortality among elderly hospitalized patients (194). Nevertheless, other populations are also at risk of the infection, such as young and healthy individuals who have not undergone antimicrobial therapy or were not exposed to a health care environment. Severe CDI that results in either death or colectomy has also been described in young women (156).

Asymptomatic carriage of *C. difficile* in children is estimated to be about 50% or higher (129). Interestingly, infants have been reported to have high levels of toxigenic *C. difficile* and toxins in their stools, but present no clinical symptoms (4, 18, 38). Although, it is not known why neonates are unusually refractory to CDI, several theories have been proposed. First, the membrane receptors required for toxin binding in the colon may be absent in neonates. Secondly, mucins directly inactivate the *C. difficile* toxins (74). Third, the thick layer of colonic mucus in neonates may mask the toxin receptors. Fourth, the intestinal epithelial cells in neonates have reduced sensitivity to intoxication than adult cells, and this may

contribute to the asymptomatic carriage (33). In fact, infant hamsters are also insensitive to CDI (135, 183).

The emergence of hypervirulent high toxin producing strains of *C. difficile* has contributed to the increasing incidence of CDI. McDonald, et al. (143, 222) examined various *C. difficile* isolates collected from eight health care facilities in six states (Illinois, Pennsylvania, Maine, Georgia, New Jersey, and Oregon) during CDI outbreaks between 2000 and 2003. A single strain was reported to account for half of the isolates from five of the facilities (143). Moreover, 82% of the stool samples from another outbreak in Quebec, Canada were positive for the same strain (143). In the 1980s, this epidemic strain was initially identified by restriction endonuclease analysis and named BI, but it is currently referred to as North American Pulsed Field Type 1 (NAP1) and PCR ribotype 027 (i.e., BI/NAP1/027, or NAP1/027) (143). The unique characteristics of this virulent NAP1/027 strain are increased toxins A and B production, fluoroquinolone resistance, and production of the binary toxin. Also, high-level of gatifloxacin and moxifloxacin resistance has been reported in recent isolates, but not in the original NAP1 strains. Resistant strains may have a competitive advantage in the hospital environment where fluoroquinolone use is widespread (165).

1.4 The *C. difficile* Pathogenicity Island

The essential *C. difficile* virulence factors are two large toxins that are chromosomally encoded by the genes *tcdA* (toxin A) and *tcdB* (toxin B). The toxin genes together with the genes that encode the proteins TcdR, TcdE, and TcdC lie

within a 19.6 kb pathogenicity locus (Fig. 1.3) in the *C. difficile* genome (21, 89). TcdA (308 kDa) is known to function as an enterotoxin causing gastrointestinal damage, whereas TcdB (269 kDa) is a highly potent cytotoxin (138).

The *tcdR* gene, which lies upstream of *tcdB* in the pathogenicity locus, encodes an RNA polymerase sigma factor that regulates transcription from the toxin promoters and from its own promoter (140, 141). TcdR is homologous to transcriptional activators of several Clostridium species and families of RNA polymerase sigma factors found in many organisms (140, 149). Proteins that have been found in other pathogenic Clostridia similar to TcdR include the sigma factors that control the tetanus neurotoxins (*TetR*) in *Clostridium tetani*, botulinum toxin (*BotR*) in *Clostridium botulinum,* and UV-inducible bacteriocin (*UviA*) in *Clostridium perfringens* (56, 176). The first evidence for the role of TcdR in *C. difficile* toxin regulation was reported by Moncrief (149) and his co-workers using *E. coli* as a surrogate host. These results were supported by similar experiments using *C. perfringens* as a surrogate host (140) and later in *C. difficile* (141). Biochemical and genetic evidence suggest that the role of TcdR is indispensable for initiation of transcription from the *tcdA* and *tcdB* promoters (140). Furthermore, the expression of TcdR and the toxin genes are both influenced in parallel by the growth phase, growth temperature, and the composition of the growth medium (119). The same expression pattern is observed for all the genes in the pathogenicity locus except *tcdC*, which is highly expressed during the rapid exponential growth phase and less expressed during the stationary phase (103).

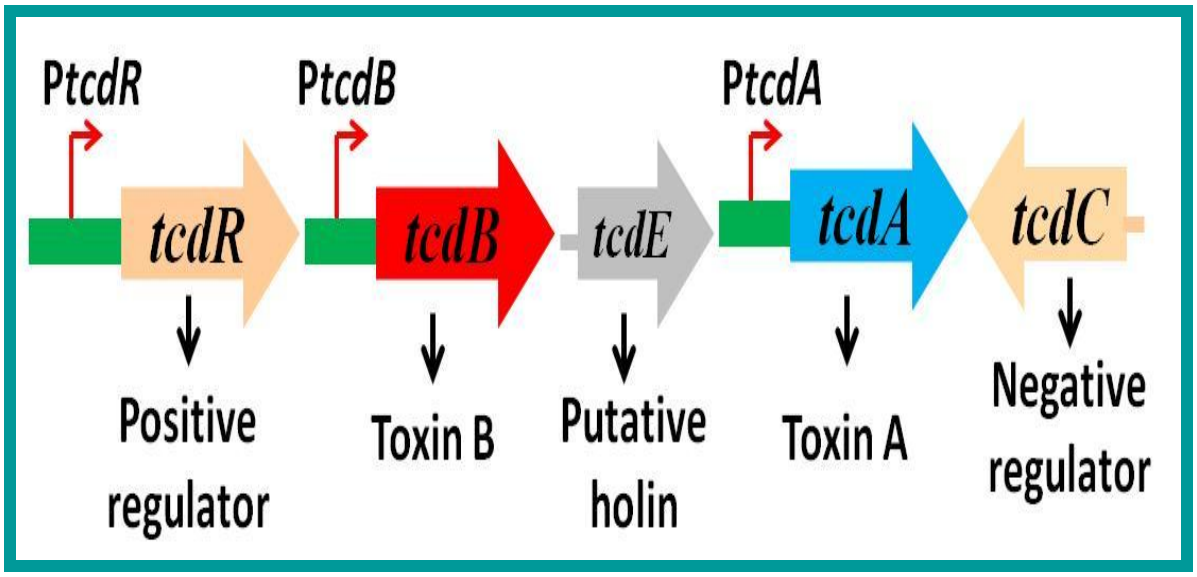

Figure 1.3: The pathogenicity locus of *C. difficile*. The *tcdA* and *tcdB* genes encode toxins A and B, respectively. The *tcdR* gene encodes a sigma factor that controls the transcription of the toxin genes, whereas the *tcdC* encodes a protein has been proposed to play a negative role in toxin gene regulation by antagonizing TcdR. *TcdE* codes for a protein similar to holin, that is suggested to have a role in the release of the toxins. The exact role of TcdE and the mechanism of TcdC-mediated repression of toxin synthesis are unclear.

Genetic and biochemical evidence suggest that TcdC negatively regulates toxin production by disrupting the capability of TcdR-containing RNA polymerase to recognize the *tcdA* and *tcdB* promoters (140, 141). Thus, it has been proposed that *tcdC* encodes a negative regulator of toxin production (103) and this concept has been supported by qualitative functional genetics and *in vitro* protein interaction studies (30, 142). Evidence to support this hypothesis includes the inverse transcription pattern of *tcdC* in relation to the toxin genes, and the emergence of epidemic strains (NAP1/027 strains) with deletions or frame-shift mutations in the *tcdC* gene that produce high toxin levels (30, 39, 103, 139, 142, 143, 222). Moreover, all NAP1/027 isolates from the 1980s and 1990s, like those from recent outbreaks, carry *tcdC* mutations (132, 143). These reports highlight the importance of *tcdC* in the pathogenesis of *C. difficile*. However, Cartman and co-workers (32) found no association between toxin production and the *tcdC* genotype when they deleted the *tcdC* gene by allelic exchange. Furthermore, restoration of the Δ117 frame-shift mutation and the 18-nucleotide deletion that occur naturally in the *tcdC* gene of some virulent *C. difficile* strains such as R20291 did not alter toxin production (32). This suggests that *C. difficile* toxin production is controlled by a complex mechanism probably involving many different factors.

Between the toxin genes is a small open reading frame, *tcdE*, which encodes a putative holin, a protein whose activity is thought to allow the release of the toxins from the cell (205). The *tcdE* open reading frame encodes a small, hydrophobic protein with 166 amino acids comprising a short hydrophilic stretch at the N-terminus and a series of charged residues at the C-terminus (205). TcdE is

predicted to contain three transmembrane domains with structural features and a primary sequence similar to class I holins. Holins are small membrane proteins encoded by double-stranded DNA phages required for lysis of host cells following completion of intracellular phage development (221, 233). Holins oligomerize in the plasma membrane of the host cell forming a disruptive lesion, which enables the transport of prophage-encoded endolysin (a muralytic enzyme) across the membrane (47-49). The prophage-encoded endolysin hydrolyzes the murein of the host cell leading to cell lysis and release of the phage particles. Even though, most holins are associated with terminal lysis of phage-infected bacteria, some holin-like proteins are known to be involved in the release of proteins from uninfected bacteria (47-49).

TcdE was initially suggested to play a role in the secretion of *C. difficile* toxins due to its homology to holins. Govind and Dupuy (81) have demonstrated empirically that TcdE is required for efficient secretion of the *C. difficile* toxins and facilitates release of toxins without inducing cell lysis or general membrane permeability. On the other hand, Olling et al. (162) insertionally inactivated the *tcdE* gene and observed no delay or inhibition of toxin release. Olling, et al. (162) further stated that inactivation of TcdE did not either alter the kinetics of toxin release or the absolute level of secreted toxins A and B, suggesting that TcdE does not account for the pathogenicity of *C. difficile*. Moreover, no significance difference was observed between the wild-type and *tcdE*-deficient *C. difficile* when the secretome was analyzed by mass spectrometry, thus, excluding the proposed secretory role of TcdE (162). In *C. difficile*, *tcdE* encodes a 19-kDa protein but when expressed in *E.*

coli, TcdE appears as a 19 and 16-kDa protein. The truncated 16-kDa protein was associated with bacterial cell death, suggesting that TcdE does not exhibit pore-forming function in *C. difficile*, since only the non-lytic full length 19-kDa protein is present (162).

1.5 The Large *C. difficile* Toxins

The toxins A and B are the essential virulence factors in *C. difficile* pathogenesis and belong to a family of the large Clostridial glucosylating toxins (73, 123, 138, 185, 219). Strains that do not produce either of these toxins are not associated with disease (61, 219). Both toxins have similar enzymatic cleavage activities (50, 112, 113) and are cytotoxic to cultured cells; however, toxin B is 100-1,000-fold more potent than toxin A (111, 217, 219). *C. difficile* toxins A and B share high amino acid sequence identity. These toxins are structurally similar to each other (Fig. 1.4) with an N-terminal enzymatic domain composed of a glucosyltransferase domain and an autocatalytic cysteine proteinase domain, a central translocation domain encompassing a hydrophobic region, and a C-terminal receptor binding domain made up of Clostridial repetitive oligopeptides (CROPs) (106, 217).

The N-terminus of the toxins harbors the glucosyltransferase activity, which is the biologically active domain, and a domain with the conserved catalytic triad (Asp587-His653-Cys698) of a cysteine protease, which mediates toxin autocleavage during internalization in the host cell (203).

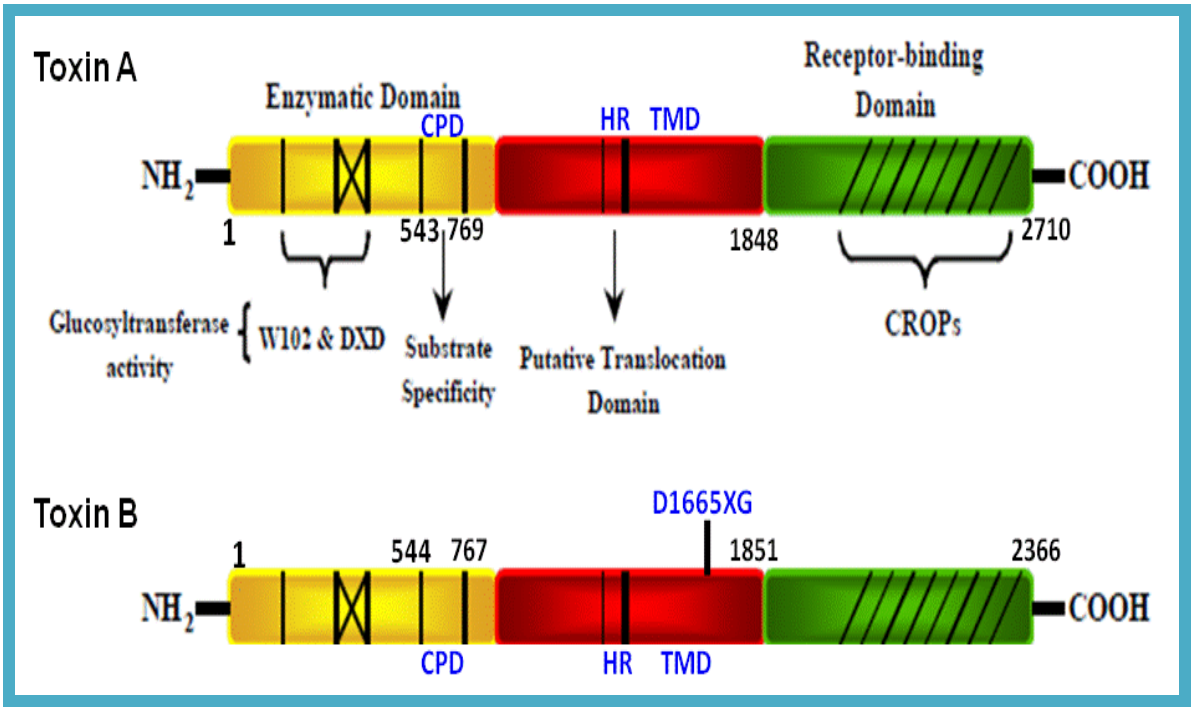

Figure 1.4: Structural comparison of *C. difficile* toxins A and B. These toxins have three domains: an N-terminal enzymatic domain consisting of a glucosyltransferase domain and an autocatalytic cysteine protease domain (CPD); a central translocation domain (TMD) encompassing a hydrophobic region (HR); and a *C*-terminal receptor binding domain containing the Clostridial repetitive oligopeptides (CROPs). The DXD (Asp-X-Asp) motif and a conserved tryptophan (W102) present in the glucosyltransferase domain are involved in Mn^{2+} and UDP-glucose binding. The DXG (Asp-X-Gly) motif in the TMD region of TcdB has an aspartate protease activity, which may be involved in toxin cleavage (203).

The crystal structure of the TcdB glucosyltransferase domain has been determined and the essential amino acid residues involved in the glucosyltransferase reaction or substrate binding have been identified (179). The Asp-X-Asp (DXD) motif and a conserved tryptophan (W102) play a role in Mn^{2+} and UDP-glucose binding (179). There is limited information concerning the transmembrane domain and its function. The transmembrane domain comprises more than 50% of the total amino acid content of the toxins. It also includes a hydrophobic region whose role may be for membrane insertion.

The CROPs of the receptor binding domain has 21-, 30-, and 50- repetitive amino acid residues. The CROPS of TcdA contains between 30 and 38 contiguous repeats, whereas that of TcdB has between 19 and 24 repeats (85, 96). The CROPs may be involved in the initial target cell interaction and receptor binding by the toxins. The crystal structure of the receptor binding domain of TcdA showed a solenoid-like structure that has been proposed to increase the surface area of proteins and enable protein-protein or protein-carbohydrate interactions (85, 96). TcdA has been reported to bind to the trisaccharide, Galα1-3Galβ1-4GlcNAc, carbohydrate antigens, components in human milk, and glycosphingolipids (122, 184, 208, 213). The crystal structure of TcdA was solved in complex with the synthetic carbohydrate, Gal-α1-3Gal-β1-4GlcNAc (85). On the contrary, a functional α-galactosyltransferase does not exist in humans, suggesting that Gal-α1-3Gal-β1-4GlcNAc cannot be an intestinal receptor in human (107). The disaccharide Gal-β1-4GlcNAc, which is present in humans, has therefore been suggested to be part of the host receptor (107). Little is known about the TcdB receptor, but it has been

suggested that the TcdB receptor appears to be at the basolateral sites, whereas the TcdA receptor is on the apical sites on the host intestinal cells (201). However, researchers have been unsuccessful in identifying the actual host receptor for the toxins. The interaction between the receptor binding domain of the toxins and the host cell receptors initiates receptor-mediated endocytosis (65, 116, 213).

Some *C. difficile* isolates (less than 10%) including the epidemic NAP1/027 strain also produce a third toxin that is unrelated to the pathogenicity locus called the *C. difficile* binary toxin (CDT) (76). This toxin is a two-component ADP ribosyltransferase encoded by the genes *cdtA* (enzymatic component) and *cdtB* (binding component). The role of the binary toxin in *C. difficile* pathogenesis is not clear. Toxins A- and B-negative binary toxin-positive strains cause fluid accumulation in rabbit ileal loops, but no diarrhea or death in hamster models (72). Moreover, *C. difficile* strains that produce the binary toxin in the absence of toxins A and B do not appear to cause disease (120). The production of the binary toxin by NAP1/027 epidemic strains has renewed speculation that this toxin may act synergistically with toxins A and B in causing severe colitis (12, 132, 143).

1.6 The Mechanism of *C. difficile* Toxin Entry into the Host Cell

During infection, toxins A and B are released into the intestinal lumen where they bind to surface receptors on colonic epithelial cells via their receptor-binding domain (Fig. 1.5). They are then internalized by the host cell by receptor-mediated endocytosis (52, 96). The acidic environment within the endosomes activates the cysteine protease activity of the toxins, which cleaves and releases the

glucosyltransferase domain located at the N-terminus into the cytosol of the mammalian host (58, 98, 168, 178, 186). A host cofactor, inositol hexakisphosphate, serves as a trigger of the cysteine protease-mediated autocatalytic cleavage of the toxins (178).

Small GTPases (8-28 kDa) are characterized by C-termini that are polyisoprenylated, and an intrinsic ability to bind to guanine nucleotides. In addition, they serve as molecular relays that transmit signals when bound to GTP and discontinue signal transmission when bound to GDP (203). Small GTPases are subdivided into the subfamilies of Rho, Rab, Ras, Ran and Arf, and Ran. The Rho subfamily members (Rho GTPases or Rho proteins) such as RhoA, Rac1, and Cdc42 are the major known intracellular targets of TcdA and TcdB (111).

Once in the cytosol, the glucosyltransferase effector domain of the toxins mono-O-glucosylates low molecular weight GTPases of the Rho family (RhoA, Rac1, and CDC42) using cellular uridine diphosphoglucose (UDP-glucose) as the glucose donor (111, 112). In the GDP-bound form, Rho GTPases are inactive and associate with guanine nucleotide dissociation inhibitors, which keep the GTPases in the cytosol. Rho GTPases interact with different effectors to control several signaling processes upon activation by guanine nucleotide exchange factors (203). They regulate many host cell functions, such as epithelial barrier functions, adhesion, phagocytosis, cytosecretion, immune cell migration, and immune cell signaling (106, 111).

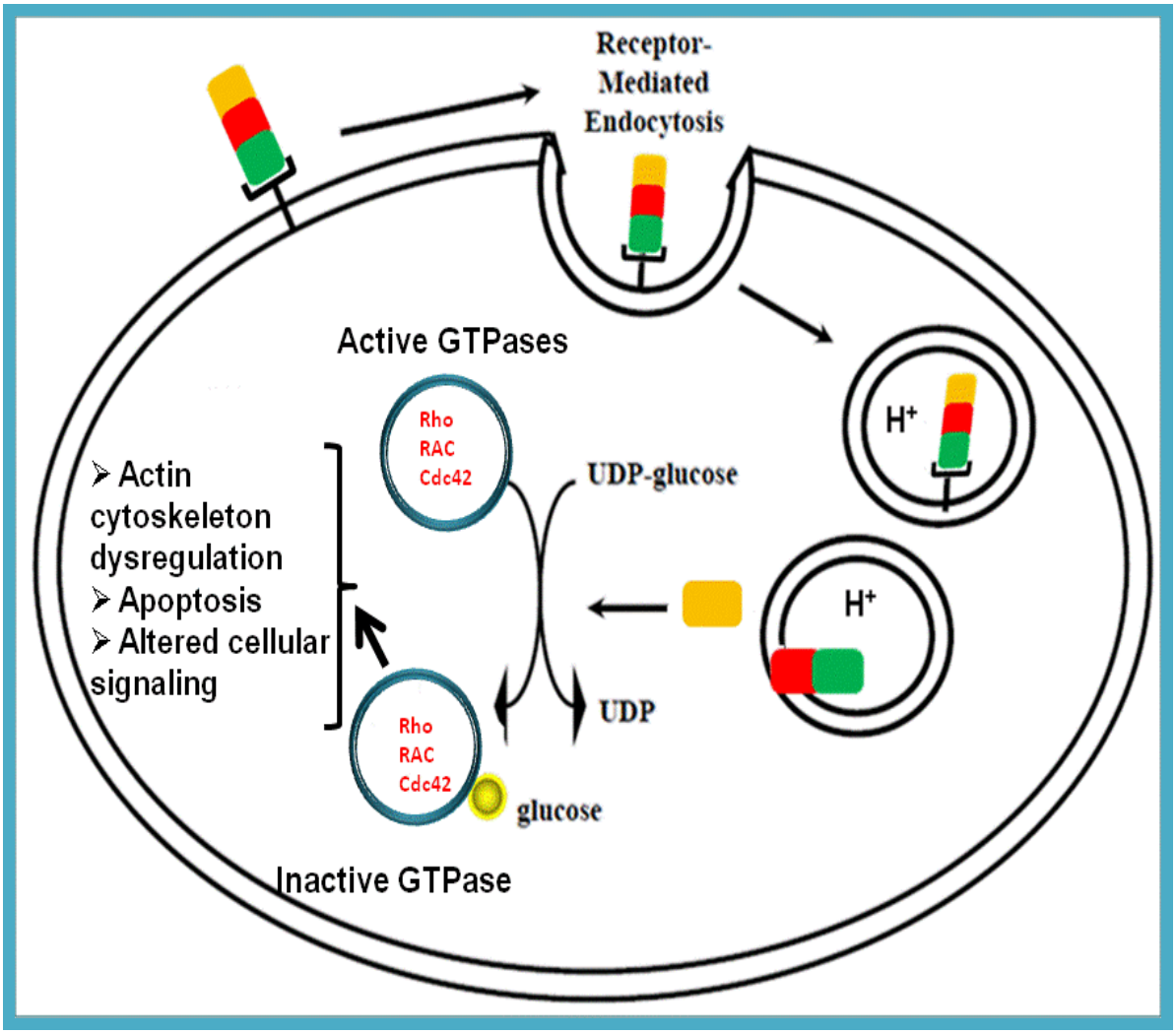

Figure 1.5: Mechanism of *C. difficile* toxins entry into host cells. The toxins bind to unknown host cell receptors and are subsequently internalized by receptor-mediated endocytosis (178). The low pH within the endosomes stimulates the autocatalytic cysteine protease activity of the N-terminally located enzymatic domain, resulting in the release of the glucosyltransferase domain. The released glucosyltransferase inactivates small molecular weight GTPases by monoglucosylation (219).

Monoglucosylation of the Rho GTPases by the toxins interrupts their normal functions leading to various deleterious effects including massive fluid secretion, apoptosis, cell rounding, actin cytoskeleton dysregulation, loss of tight junction integrity, acute inflammation and necrosis of the colonic mucosa, and altered cellular signaling (69, 98, 101, 111, 112). Cellular intoxication by the toxins also induces the release of various immunomodulatory mediators from epithelial cells, phagocytes, and mast cells resulting in inflammation and accumulation of neutrophils (171, 210). The clinical manifestations of CDI are highly variable: ranging from asymptomatic carriage, mild self-limiting diarrhea, to severe pseudomembranous colitis.

1.7 Treatment of *C. difficile* Infections

Alteration of the normal flora by antibiotic therapy leads to the development of CDI in susceptible individuals. Thus, an essential step in the treatment of CDI is the immediate cessation of the inciting antibiotic. Treatments with concomitant antibiotics are associated with significant prolongation of diarrhea and an increased risk of recurrence (133, 152). Patients with the typical manifestations of CDI such as diarrhea, abdominal pain, nausea, and vomiting with a positive diagnostic assay are given oral metronidazole or vancomycin (155, 194). Metronidazole is preferred over vancomycin as the first-line therapy for mild to moderately ill persons due to its lower cost relative to vancomycin, comparable clinical effectiveness, and to prevent the spread of vancomycin-resistant enterococci (5, 28). The recommended dosage for metronidazole is 500 mg three times daily or 250 mg four times daily for 10 to 14

days. Oral vancomycin is recommended at a dosage of 125 mg four times daily and is the preferred treatment for more severe cases. Intravenous vancomycin has no effect on *C. difficile* colitis since the antibiotic is not excreted significantly into the colon.

Recurrent CDI frequently results from reinfection with the same or a different strain of *C. difficile* (225). Recurrence mostly occurs within 1-3 weeks after discontinuation of antibiotic usage. One-half of recurrent episodes appear to be reinfections rather than relapses of infection with the original strain (11, 157), although mixed initial infections may be present initially. Approximately 25% of the cases of recurrence occur in patients treated with metronidazole or vancomycin (63, 145, 224). The main risk factors for recurrent CDI are severe underlying medical disorders (weakened immune system), age (> 65 years), and the need for ongoing therapy with concomitant antibiotics during CDI treatment (125, 152, 207). Patients with at least one episode of recurrent *C. difficile* have a 45-65% chance of additional episodes (144). The mechanism of CDI recurrence following initial infection is not fully understood. A number of possible factors may contribute to CDI recurrence: one, persistent spores from the initial infection that escape mechanical clearance by peristalsis (207) and two, a weak host immune response to toxins A and B. High serum antibody levels against toxins A and B have been observed in asymptomatic carriers of *C. difficile* (126). Patients with recurrent CDI tend to have lower antibody levels than patients with a brief, single episode of diarrhea (2, 125). Antibiotic resistance does not appear to be a factor in recurrence. Fidaxomicin, a drug

recently approved by FDA for the treatment of *C. difficile*, has been reported to significantly lower the recurrence rate of CDI (133).

Other non-antibiotic treatment options that have been advocated include infusion of stools from healthy donors (1), adjunctive use of monoclonal antibodies specific to the toxins (134), probiotics (145), and the use of non-toxigenic *C. difficile* strains to out-compete the toxigenic strains.

1.8 Regulation of *C. difficile* Toxins A and B

Various physiological and environmental factors such as nutrient limitation, the presence of branch-chained amino acids and biotin, and sub-inhibitory antibiotic concentrations have been reported to influence toxin production. However, the exact mechanism and the key players involved in the regulation of *C. difficile* toxins A and B synthesis are not clear.

CodY, a universal regulator of gene expression in low G+C Gram-positive bacteria, has been reported to repress *C. difficile* toxin genes expression (51). Dineen, et al. reported that all of the five genes of the *C. difficile* pathogenicity locus were derepressed when the *codY* gene was inactivated (51). CodY was found to bind with high affinity to a DNA fragment containing the promoter region of the *tcdR* gene. In the presence of GTP and branched-chain amino acids, binding of CodY to the *tcdR* promoter region was enhanced and this suggests a link between nutrient limitation and the expression of *C. difficile* toxin genes (51). Environmental stresses, such as alteration of the oxidation-reduction potential, exposure to sub-inhibitory concentrations of antibiotics, and extreme temperatures have also been reported to

affect toxin production (119, 154). To date, no conclusive link has been defined regarding these environmental factors and their effect on *C. difficile* toxin synthesis. Furthermore, no specific regulatory or signaling mechanism connecting these factors has been defined.

Transcription of the toxin genes in *C. difficile* has been reported to be affected by a universal signaling molecule involved in quorum signaling (130), a mechanism by which bacteria respond and adapt to their micro-environmental changes through coordinated gene expression. In quorum signaling, a small diffusible signaling molecule produced by one species of bacteria can act as an intraspecies and/or interspecies communication signal. Gram-negative bacteria produce various types of N-acyl-homoserine lactones (autoinducer-1[AI-1]) that are synthesized by enzymes encoded by the *luxI* family of genes. Another signaling molecule termed autoinducer-2 (AI-2), which is synthesized by enzymes encoded by the *luxS* family of genes has been identified to be involved in quorum signaling in both Gram-negative and Gram-positive bacteria and mediates interspecies communication (26, 34, 228, 230). AI-2/LuxS-mediated regulation is important in controlling different activities in a variety of bacterial species (230). *C. difficile* produces AI-2 that is able to induce bioluminescence in a *Vibrio harveyi* reporter strain (130). AI-2-containing cell-free supernatants from mid-log phase of *C. difficile* and *E. coli* DH5α producing recombinant *C. difficile* LuxS increased the transcript levels of TcdA, TcdB, and TcdE (130). However, Carter, et al. reported that AI-2 has no significant effect on either the timing of toxin production or the amount of toxins produced by *C. difficile* (31). Therefore, it is still not clear whether AI-2/LuxS is involved in *C. difficile* toxin

production. Moreover, no other known quorum signaling system has been shown to influence the production of these toxins.

1.9 Preliminary Observations on *C. difficile* Toxin Synthesis

Analysis of growth and toxin synthesis in *C. difficile* strain 630 revealed a prolonged stationary phase during which the toxins are produced (Fig. 1.6). No toxins were detected during the lag or exponential growth phases. Toxin synthesis appeared to be delayed until stationary phase, suggesting that these toxins are not constitutively expressed. These observations indicated that toxin synthesis in *C. difficile* is regulated and uncoupled from growth. There are various physiological processes and environmental changes that occur during the stationary phase, which may influence toxin synthesis. Notable among these factors are the decreasing nutrient levels, increase in cell density, and accumulation of metabolic products or wastes. The toxins were produced during a growth phase when the cell density was at its peak. Thus, toxin synthesis appeared to be coordinated and delayed until high cell density. These results implied that a toxin-inducing factor may be accumulating during growth, which reaches a threshold at stationary phase leading to activation of toxin synthesis.

These observations led to the hypothesis that the synthesis of *C. difficile* toxins A and B is cell density-dependent and involves a signaling pathway that responds to extracellular cues for regulation.

It is important to note that the transcription of the toxin genes (*tcdA* and *tcdB*) begins during the early stationary phase (103), usually between 10-14 hrs of

growth. However, the toxins were detected by enzyme linked immunosorbent assay (ELISA) during the late stationary phase, between 32-36 hrs (Fig. 1.6). This discrepancy between detection of the *tcdA* and *tcdB* transcripts and toxins A and B protein suggested that the ELISA assay used to detect the presence of the toxins was probably not sensitive enough to detect the toxins during the early stationary phase. As a result, a new functional, quantitative, and sensitive assay was developed as part of this study, which was able to detect the toxins as expected during the early stationary phase.

1.10 Significance of this Study

Treatment of *C. difficile* infection (CDI) is hampered by recurrence, multi-drug resistance, and drugs with reduced functional activity in the colon. A large number of *C. difficile* isolates are resistant to the majority of antibiotics currently used in hospitals and outpatient settings (6, 16, 164, 193). As a result of the dwindling number of antibiotics available to effectively clear this infection and prevent recurrence, there is renewed interest in exploring a non-antibiotic treatment approach to CDI. Non-antibiotic treatment options that have been proposed include, infusion of stools from healthy donors (1), adjunctive use of monoclonal antibodies specific to the toxins (134), probiotics (145), and the use of non-toxigenic *C. difficile* strains to out-compete the toxigenic strains.

The spectrum of diseases caused by *C. difficile* is variable and the severity of CDI depends on the level of toxins produced (3). This suggests that toxin production is a critical and significant determinant of *C. difficile* pathogenicity.

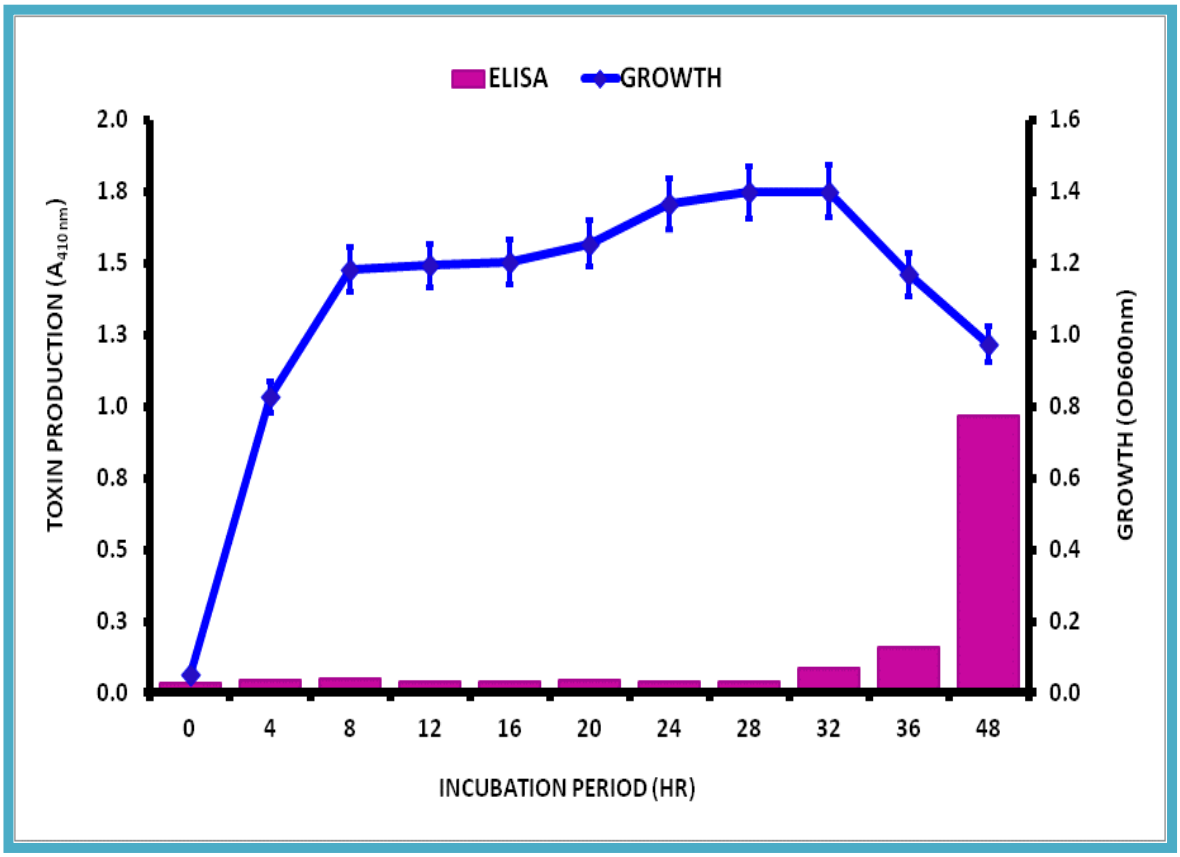

Figure 1.6: Growth of *C. difficile* in relation to toxin production. Toxin synthesis was detected during the late stationary phase of growth. An overnight culture was diluted 1:100 with fresh brain heart infusion medium and incubated anaerobically at 37^{o}C. Aliquots of the culture were taken every 4 hrs for O.D. 600 nm measurement and toxin detection by ELISA using the Wampole *C. difficile* TOX A/B II assay (TechLab, Blacksburg, VA).

Therefore, as the toxins play an essential role in *C. difficile* pathogenesis, inhibition of either toxin synthesis or toxin activity are promising approaches to combat CDI. Disruption of the regulatory mechanisms that control toxin synthesis and other virulence factors, which do not affect growth, is less likely to pose a selective pressure on the bacterium, and thus minimizes the risk of resistance development compared to antibiotics.

The overall goal of this study was to investigate whether the synthesis of toxins A and B in *C. difficile* is regulated by quorum signaling and to identify and characterize the key players involved in this regulation. The data from this study may provide insight into the processes that govern the synthesis of these toxins. This may lead to the discovery of novel genes, molecules, or pathways that could be targeted therapeutically for the treatment of *C. difficile* infection.

This project began with the development of a more sensitive *C. difficile* toxin activity assay needed to investigate the hypothesis stated above. Chapter 2 describes the purification of the toxins, validation of the toxin activity assay (the Cdifftox activity assay), and the biochemical characterization of the toxin-substrate kinetics. This new assay was modified into an agar-based version (the Cdifftox agar plate assay) for use in the clinical isolation and diagnosis of *C. difficile* from stool samples (Chapter 3). The Cdifftox activity assay was also utilized to identify an inhibitor (taurocholate) of toxin activity. Chapter 4 describes how taurocholate protected intestinal epithelial cells from the damaging effects of the toxins. The development of this more sensitive and functional assay enabled various

experiments to be conducted, which ultimately helped to confirm the hypothesis and led to the conclusion that the *C. difficile* toxins A and B are regulated by quorum signaling (Chapter 5).

CHAPTER 2: HARNESSING THE GLUCOSYLTRANSFERASE ACTIVITIES OF *CLOSTRIDIUM DIFFICILE* FOR FUNCTIONAL STUDIES OF TOXINS A AND B

Darkoh, C., H. B. Kaplan, and H. L. DuPont.

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INTRODUCTION

Currently, only one non-radioactive assay (the tissue culture cytotoxicity assay) is available for the detection of the activities of the toxins. However, quantitative analysis of toxin activity using this method is tedious and requires the maintenance of a tissue-culture system, which makes it costly in terms of time and effort. In order to investigate the regulation of the *C. difficile* toxins, determining the exact period during which the toxins are produced was necessary and important. This required a more sensitive and quantitative toxin assay, since the ELISA assay initially used was not sensitive enough to detect the toxins during the early stationary phase. It was also important to know whether active toxins were being released by the *C. difficile* strains under the experimental conditions.

This chapter describes the development and validation of a quantitative assay (Cdifftox activity assay) (41), developed as part of this project that enables detection of the *C. difficile* toxins A and B in a culture supernatant. The method was based on the inherent properties of these toxins to cleave p-Nitrophenyl-β-D-glucopyranoside (PNPG), a chromogenic substrate with stereochemical characteristics similar to the natural substrate of the toxins, UDP-glucose. This assay was found to be cost-efficient, sensitive, quantitative, and enables measurement of the cleavage activities of toxins A and B.

2.1.1 Molecular Basis of the Cdifftox Assay

The *C. difficile* toxins on entering the host cell cytoplasm via receptor mediated endocytosis recognize UDP-glucose and cleave the O-glycosidic bond between the

UDP and glucose. The glucose liberated is attached to small molecular weight GTPases resulting in their inactivation. This assay harnesses the ability of these toxins to cleave O-glycosidic bonds by replacing the UDP in UDP-glucose with a colorimetric molecule (p-Nitrophenol) that changes color when the O-glycosidic bond is cleaved (Fig. 2.1). Under optimum conditions of temperature, pH, and unlimited amount of the PNPG substrate, the p-Nitrophenol released is directly proportional to the amount of the toxins present. Thus, the amount of the toxins A and B released into the supernatant can be indirectly estimated based on the cleavage of the colorimetric substrate.

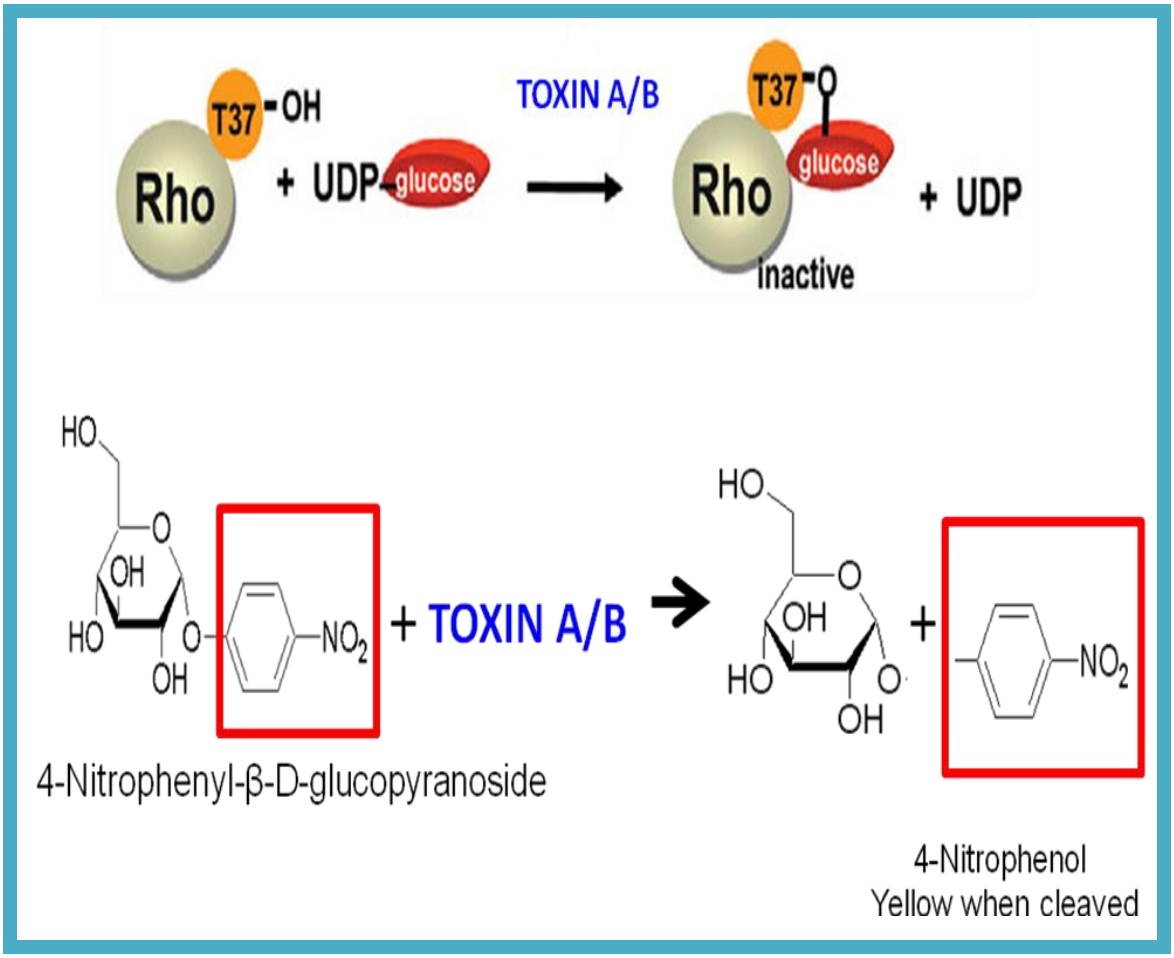

Figure 2.1: Molecular basis of the Cdifftox activity assay. After the toxins are internalized into the host cell via receptor-mediated endocytosis, the glucosyltransferase domain recognizes and cleaves UDP-glucose. The glucose released is attached to Rho-GTPases leading to their inactivation. In this assay, UDP-glucose was replaced by the substrate, p-Nitrophenyl-β-D-glucopyranoside. Cleavage of this substrate results in the production of a colorimetric molecule, p-nitrophenol, which can be monitored spectrophotometrically.

MATERIALS AND METHODS

2.2.1 Bacterial Strains and Growth Conditions

Toxigenic *C. difficile* strains ATCC numbers 43255 (*tcdA*+/*B*+), BAA-1805 (*tcdA*+/*B*+; NAP1), 700057 (*tcdA*-/*B*+), and BAA-1382 (*tcdA*+/*B*+) were purchased from the American Type Culture Collection (Manassas, VA). Clinical isolates were obtained from stool samples of hospitalized patients with antibiotic-associated diarrhea suspected to be *C. difficile* positive (see below). The bacteria were grown in brain heart infusion (BHI)-based medium (Becton Dickinson and Company, Cockeysville, MD) or on BHI-agar containing cefoxitin (8 µg/ml) and D-cycloserine (250 µg/ml) and were incubated anaerobically in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 at 37°C in a Controlled Atmosphere Anaerobic Chamber (PLAS LABS, Lansing, MI). The substrates were purchased from Biosynth International (Itasca, IL) (41).

2.2.2 Sample Storage Condition

The clinical isolates were either stored short-term in chopped meat broth (BD Diagnostics, NJ) at room temperature or in 15% glycerol stocks at -80°C. The purified toxins and eluents were stored at 4°C for a maximum of one month or until use with no loss of activity (41).

2.2.3 The Cdifftox Activity Assay

The assay consists of the Cdifftox substrate reagent composed of 5 mM PNPG, 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 100 µM $MnCl_2$. The assay was performed in Costar sterile polystyrene 96-well plates (Corning Inc., NY) by adding to each well 200 µl of sample or culture supernatant fluid containing the toxins and 100 µl of the reagent. The plate was incubated at 37^{o}C for 1-4 hrs and each reaction was stopped by the addition to the well of 40 µl of 3 M Na_2CO_3. Cleavage of the substrate was monitored by measuring the absorbance at 410 nm using a SPECTRA max Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). To identify the best substrate for the assay, a number of substrates were evaluated including: p-nitrophenyl-α-D-glucopyranoside, p-Nitrophenyl-β-D-glucopyranoside, 4-aminophenyl-α-D-glucopyranoside, 4-aminophenyl-β-D-glucopyranoside, 5-benzyloxy-3-indoxyl-β-D-glucopyranoside, 5-bromo-6-chloro-3-indoxyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-α-D-glucopyranoside, 6-chloro-3-indoxyl-α-D-glucopyranoside, 6-chloro-3-indoxyl-N-acetyl-beta-D-glucosaminide, and 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (41). P-Nitrophenyl-β-D-

glucopyranoside was selected as the substrate of choice because its cleavage by the toxins was the most efficient and sensitive, and had the lowest background. A molar extinction coefficient for *p*-nitrophenol of ε = 17700 $M^{-1}cm^{-1}$ was used in the calculations (192). One unit of toxin activity was defined as the amount of the toxins required to cleave one micromole of the PNPG substrate per hour under the experimental conditions (41).

2.2.4 Enzyme-linked immunosorbent assay (ELISA)

For comparison, the presence of toxins A and B in samples was also evaluated using the Wampole *C. difficile* TOX A/B II assay (TechLab, Blacksburg, VA). This assay was performed using the protocol provided by the manufacturer (41).

2.2.5 Comparison of *C. difficile* Toxin Detection by Cdifftox Activity Assay and ELISA.

An overnight culture of *C. difficile* strain 630 was diluted 1:100 with fresh reduced BHI medium and incubated anaerobically at 37^{o}C for 48 hrs. Aliquots of the culture were taken every 4 hrs for O.D. 600 nm measurement and toxin testing using the Cdifftox activity assay and ELISA. For the toxin assay, the culture was centrifuged at 10,000 rpm for 10 min and 200 µl of the supernatant fluid was used for each assay (41).

2.2.6 Purification of *C. difficile* Toxins A and B

To purify the toxins, *C. difficile* strain (ATCC 43255) was cultured anaerobically for 5 days at 37^{o}C in Spectra/Por dialysis bags (50 ml) with a molecular weight cut-off of 100 kDa (Spectrum Laboratories, Rancho Dominguez, CA). Purification of the toxins was performed according to established methods (146, 202, 218) with some modifications. Briefly, the culture was centrifuged at 10,000 x g for 10 minutes at 4^{o}C and the resulting supernatant was filtered through a 0.45 µm membrane filter (Millipore, Billerica, MA). To further eliminate low molecular weight proteins, the filtered supernatant was concentrated using a Pierce Concentrator (Thermo Scientific, Rockford, IL) with a molecular weight cut-off of 150 kDa. The concentrated supernatant was precipitated by the addition of ammonium sulfate (450 g/L) and incubated overnight at 4°C with gentle stirring, and subsequently centrifuged at 6,000 x g at 4°C for 20 min. The precipitate was washed and dissolved in 50 mM Tris-HCl buffer (pH 7.4). The sample was loaded onto a fast flow DEAE-Sepharose CL-6B (GE Healthcare Life Sciences, Piscataway, NJ) anion column pre-equilibrated with buffer D (50 mM Tris-HCl [pH 7.4] containing 50 mM NaCl) at a flow rate of 2 ml/min. The

column was washed with buffer D (approximately 350 ml) until all unbound proteins were removed. Toxin A was eluted first with a linear gradient of NaCl (50-250 mM) in buffer D. The elution continued for toxin B with a NaCl gradient of 250-1000 mM in buffer D, after a washing step with 250 ml of buffer D. The fractions (10 ml) were assayed for the presence of toxins by incubating 200 µl with 10 mM PNPG for 3 hrs at 37^{o}C. The toxin-positive fractions were pooled and concentrated for further purification (41).

The pooled fractions from the DEAE-Sepharose column were further purified by gel filtration chromatography. A 1 cm X 100 cm glass Econo column (Bio-Rad Laboratories, Gaithersburg, MD) was packed with Sephacryl S-300 high resolution beads (GE Healthcare Life Sciences) and calibrated using the following standards purchased from Bio-Rad Laboratories: vitamin B12 (1.35 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), g-globulin (158 kDa), and thyroglobulin (670 kDa). The concentrated toxins were applied to the column and eluted with buffer D at a flow rate of 0.5 ml/min. Fractions (5 ml) were assayed for the presence of the toxins using 200 µl as described above. The purity of the purified toxins was evaluated by electrophoresis through a 5% acrylamide: bisacrylamide PAGE gel (187). The protein concentration of samples was determined using Bradford assay (19) with bovine serum albumin as the standard (41).

2.2.7 Western Blot Analysis

C. difficile toxins A and B (100 µg each) were separated on 5% PAGE gels. The proteins were transferred from the PAGE gel onto Immun-Blot PVDF membranes (BioRad, Hercules, CA) using a Trans-Blot cell (BioRad). The membranes were incubated with individual mouse monoclonal antibodies specific for *C. difficile* toxins A or B, as the primary antibodies (Abcam, Cambridge, MA). The WesternDot 625 Western Blot kit (Invitrogen, Carlsbad, CA) was used to probe the membranes for the presence of each toxin. Briefly, the membrane was incubated with Biotin-XX goat anti-mouse IgG secondary antibody and following washing, incubated with the Qdot 625 streptavidin conjugate according to the manufacturer's instructions. Imaging and analysis of the treated membrane was performed using a UVP BioDoc-It Imaging system (Upland, CA) (41).

2.2.8 Determination of Km and Vmax

A series of experiments were performed to determine the Michaelis-Menten constant (Km) and maximum velocity (Vmax) for the PNPG cleavage activities of each toxin. To determine the amount of the toxins necessary for the experiments, different amounts of each toxin ranging from 30 µg to 120 µg were evaluated with 10 mM of PNPG as substrate. Different substrate

amounts were also tested using 55 µg of toxin A and 100 µg of toxin B. A graph of toxin activity as a function of time was plotted and the amount of each toxin that gave the best linear relationship, but occurred slowly enough for the reaction to be monitored was chosen for the assay. Based on this analysis, 55 µg of toxin A and 100 µg of toxin B were used for the kinetics experiments. Each experiment was repeated four times and the average used for the analysis (41).

2.2.9 Effect of pH and Temperature on Toxin A and B Activity

The effect of pH and temperature on the activity of the toxins was investigated to determine the optimum temperature and pH for activity. For the pH experiment, the following buffers were used: glycine-HCl buffer (pH 2-3); citrate buffer (pH 4-6); Tris-HCl buffer (pH 7-10); disodium phosphate-sodium hydroxide buffer (pH 11-12); and KCl-NaOH (pH 13). Each pH experiment was initiated by incubating 100 µg of toxin A or toxin B with 10 mM of PNPG, in the appropriate buffer followed by incubation at 37^{o}C for 4 hrs. The reaction was monitored by measuring the absorbance at 410 nm.

The effect of temperature of incubation on the PNPG cleavage activity was tested in 1.5 ml microcentrifuge tubes using the same conditions as described previously, except that the temperature of incubation was 4, 10, 15, 20, 25, 30, 35, 40, 45, and 50^{o}C (41).

2.2.10 Inhibition Assay

To identify compounds that inhibit the activity of *C. difficile* toxins A and B, several compounds including sodium taurocholate, dimethyl sulfoxide, phenylmethylsulfonyl fluoride, and dimethyl formamide were tested. Different concentrations of these agents (0, 50, 100, 200 and 300 mM) were added to 55 µg of either toxin A or toxin B in buffer D in a total reaction volume of 300 µl and incubated at 37^{o}C for 10 minutes. After the toxin-inhibitor incubation period, 10 mM of the PNPG substrate was added and incubated at 37^{o}C for 1 hr. Absorbance at 410 nm was measured and the percent inhibition was calculated as follows (41):

$$\text{Percent Inhibition } (\%) = \left[\frac{\text{Specific activity with inhibition}}{\text{Specific activity without inhibition}} \right] \text{X } 100\%$$

2.2.11 Application of the Cdifftox Activity Assay to Culture Supernatants from Clinical *C. difficile* Isolates

Stool samples obtained from patients suspected to be infected by *C. difficile* were obtained from St. Luke's Hospital (Houston, Texas) in an IRB-approved study. Single colonies, obtained independently from each patient's stool sample streaked onto BHI-agar media containing cefoxitin (8 µg/ml) and D-cycloserine (250 µg/ml), were inoculated into 10 ml of BHI medium and incubated anaerobically at 37^{o}C for 72 hrs resulting in an O.D. 600 nm of about 1.3-1.4. After centrifugation at 10,000 x g for 10 min at 4^{o}C, 250 µl of the supernatant was incubated with 50 µl of Cdifftox substrate reagent containing 30 mM PNPG at 37^{o}C for 3 hrs. The assay was quantitated spectrophotometrically at an absorbance of 410 nm. The isolates were not specifically typed to the strain level, but confirmed to be *C. difficile* based on PCR amplification of the genes that encode the toxins (*tcdA* and *tcdB*), as well as toxin production. Culture supernatants from 18 clinical isolates in addition to 4 ATCC strains [BAA-1805 (*tcdA*+/*B*+; NAP1), 700057 (*tcdA*-/*B*+), 43255 (*tcdA*+/*B*+) and BAA-1382 (*tcdA*+/*B*+)] were analyzed (41).

2.2.12 Polymerase Chain Reaction Amplification of *C. difficile* Toxin Genes

The presence of the toxin genes (*tcdA* and *tcdB*) and the 16S rRNA gene in the clinical isolates was confirmed by PCR amplification. Genomic DNA was isolated from 1 ml of culture at an optical density of 0.75 at 600 nm using the DNAEasy kit (Qiagen, Valencia, CA). Amplification was performed using Phire Hot Start DNA Polymerase (Finnzymes, Woburn, MA). The following primers were used: toxin A (F-5'TGATGCTAATAATGAATCTAAAATGG-TAAC3'and R 5'ACCACCAGCTGCAGCCATA3'); toxin B (F-5'GTGTAGCAA-TGAAAGTCCAAGTTTACGC3' and R-5'CACTTAGCTCTTTGATTGCTGCA-CCT3') and 16S rRNA (F-5'ACACGGTCCAAACTCCTACG3' and R-5'AGGCGAGTTTCAGCCTACAA3'). The DNA was amplified with an initial denaturation of 98^{o}C for 30 s and 36 cycles of 98^{o}C for 10 s, 62^{o}C for 10 s and 72^{o}C for 10 s with a final extension of 72^{o}C for 1 min. The PCR products were analyzed using 1.5% agarose gel electrophoresis (41).

2.2.13 Physico-Chemical Analysis of *C. difficile* Toxins A and B

The ProtParam program on the ExPASy Proteomics Server (68) was used to evaluate the physical and chemical properties of toxins A and B. This analysis was performed computationally using the amino acid sequences with accession numbers YP_001087137.1 and YP_001087135.1 (190) for toxins A and B, respectively (41).

2.2.14 Data Analysis

All the data were analyzed and plotted using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). The nonlinear regression method was used to calculate the Km and Vmax values. Paired t-test was used to compare the performance of the new Cdifftox Activity assay in detecting the toxins in comparison with ELISA. In all cases, statistical significance was defined as $p<0.05$ (41).

RESULTS

2.3.1 Purification of *C. difficile* Toxins A and B

Clostridium difficile toxins A and B were purified seven-fold to characterize and evaluate their substrate cleavage specificities. The native toxins were purified from culture supernatant obtained from the toxin A- and B- positive strain (ATCC 43255) cultured in a dialysis bag of 100 kDa molecular weight cut-off (MWCO). The proteins in the culture supernatant were precipitated with ammonium sulfate, resuspended, and applied to a fast flow DEAE-Sepharose anion exchange chromatography column. After elution with a 50 mM to 1 M NaCl step gradient, two peaks were observed by UV detection and confirmed by Bradford assay (Fig. 2.2A). The initial narrow peak was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to contain a protein corresponding to the molecular weight of toxin A (308 kDa) and the second broad peak was determined to contain a protein corresponding to toxin B (269 kDa). The Cdifftox activity assay was used to identify fractions that contained PNPG cleavage activity. The assay revealed two toxin-positive fractions that corresponded to the two protein peaks observed (Fig. 2.2A). The antibody-based ELISA assay, which is immuno-reactively specific for both toxins A and B, identified the presence of toxins A and B in the PNPG active fractions (Fig. 2.2B). Specifically, all of the fractions that tested positive using the Cdifftox activity assay also tested positive using the ELISA assay, and all of the fractions that were negative for the Cdifftox activity assay were also negative using the ELISA assay. These results indicated that the Cdifftox activity assay detects the activities of *C. difficile* toxins A and B (41).

To complete the purification of the *C. difficile* toxin A and B, the toxin-positive fractions eluted from the DEAE-Sepharose column were pooled, concentrated using a filter with a 150 kDa MWCO, and applied to a Sephacryl S-300 gel filtration column. After elution with buffer D, three predominant peaks were observed by UV detection and confirmed by the Bradford protein assay (Fig. 2.3A). Examination of the fractions using the Cdifftox activity assay showed that the PNPG cleavage activity was present in two peaks of different molecular weights. The fractions with PNPG cleavage activities were confirmed by the ELISA assay to contain the toxins (Fig. 2. 3B). Based on the elution profiles of the gel filtration standards used, the fractions in the first peak corresponded to toxin A (308 kDa) and those in the second PNPG-active peak corresponded to toxin B (269 kDa) (41).

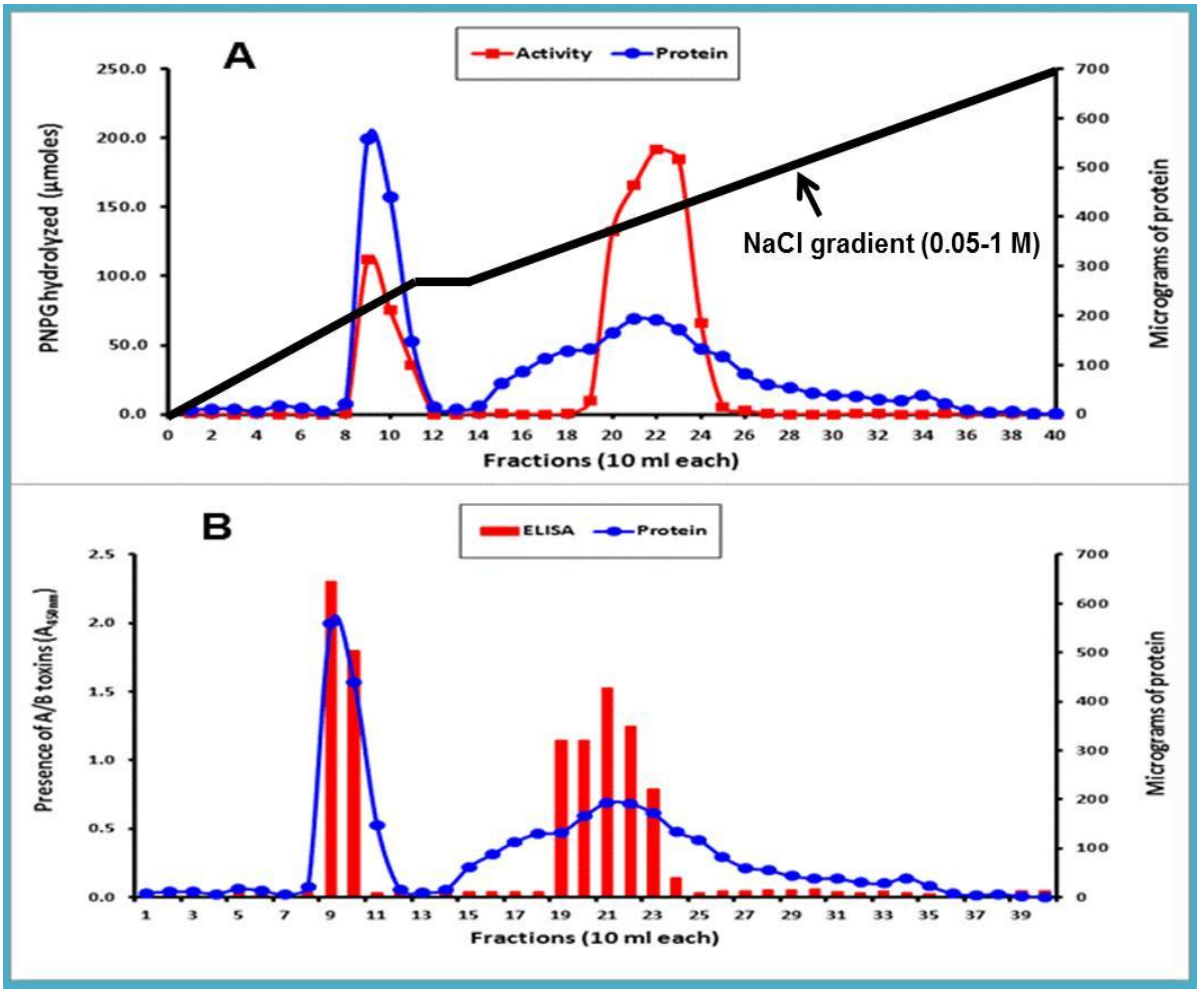

Figure 2.2: Elution profile of the proteins in *C. difficile* culture supernatant separated by DEAE-Sepharose anion exchange chromatography. Fractions (10 ml) were examined using the Cdifftox Activity assay (**A**) and the antibody-based ELISA assay (**B**). The Cdifftox Activity assay was performed by incubating 200 µl of each fraction in 50 mM Tris-HCl containing 50 mM NaCl (pH 7.4) with PNPG substrate reagent at 37^{o}C for 4 hrs. The assay was monitored by measuring absorbance at 410 nm. The protein concentration was determined using Bradford protein assay (BioRad). The ELISA assay was performed using the Wampole *C. difficile* TOX A/B II assay (TechLab, Blacksburg, VA) (41).

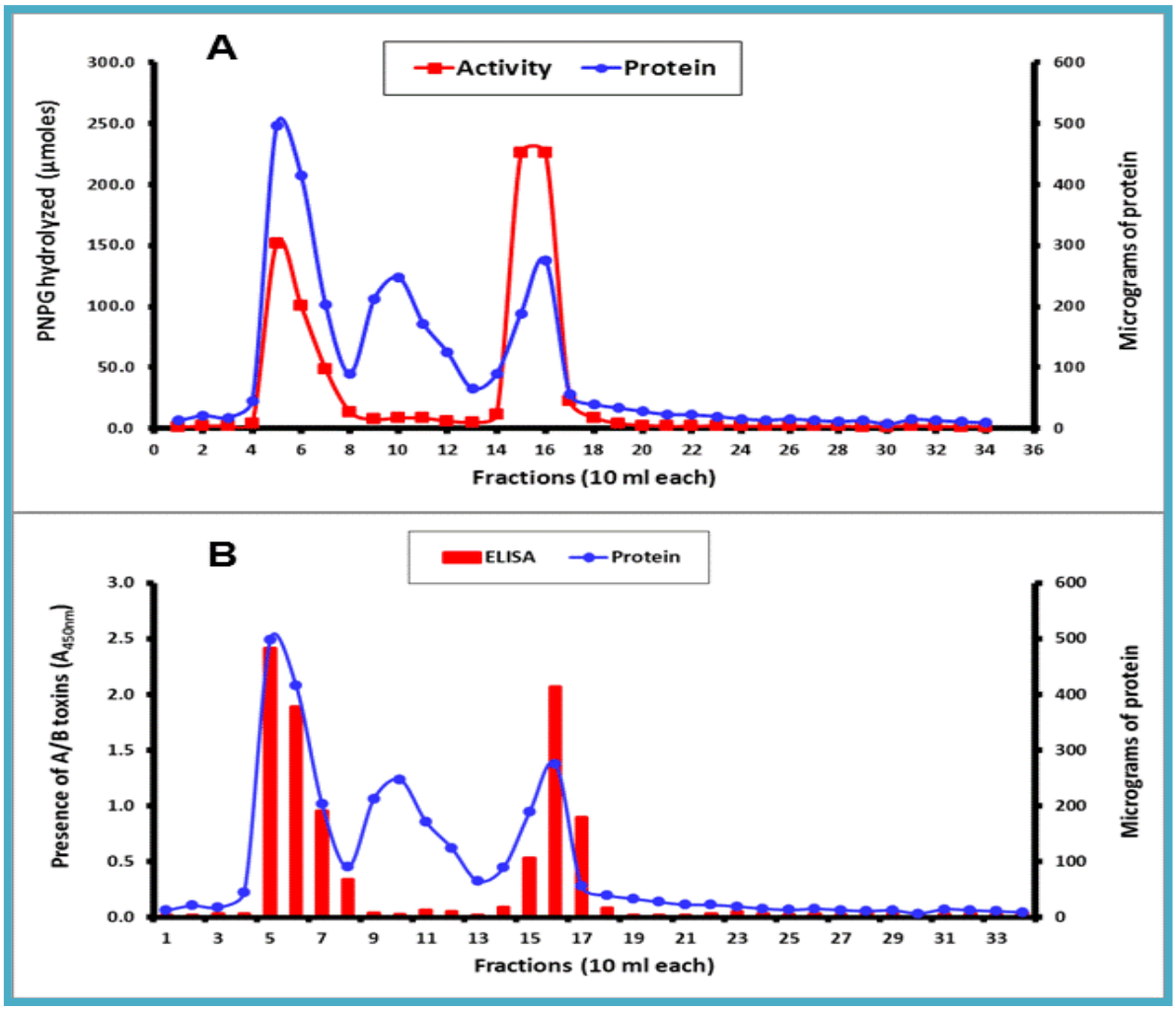

Figure 2.3: Elution profile of the pooled *C. difficile* toxin-positive fractions purified by Sephacryl S-300 gel filtration chromatography. Fractions (5 ml) were examined using the Cdifftox activity assay (A) and the antibody-based ELISA assay (B). The Cdifftox Activity assay was performed by incubating 200 µl of each fraction in 50 mM Tris-HCl containing 50 mM NaCl (pH 7.4) with PNPG substrate reagent at 37^{o}C for 4 hrs. The assay was monitored by measuring absorbance at 410 nm. The protein concentration was determined using Bradford protein assay (BioRad). The ELISA assay was performed using the Wampole *C. difficile* TOX A/B II assay (TechLab, Blacksburg, VA) (41).

The fractions showing sufficient toxin activity were pooled and concentrated for further analysis by PAGE. The results from the PAGE gel revealed a single visible band in each of the two pooled fractions representing toxins A and B (Fig. 2.4A). This established that each toxin was purified to homogeneity. The total PNPG substrate cleavage activities of the toxins from each of the purification steps are shown in Table 1. The total enzyme units of cleavage activities of the toxins were enriched by 158-fold. The final substrate cleavage activities of the purified toxins were 0.821 U/mg and 4.7 U/mg for toxins A and B, respectively (41).

2.3.2 Characterization of Toxin A and B Activity

To confirm that both toxins A and B cleave the PNPG substrate, Western immunoblot analysis was performed. Single bands were observed in each of the samples that had PNPG activity and contained either purified toxin A or B due to their specific reactivity with monoclonal antibodies that recognize toxin A or B, respectively (Fig. 2.4B). Moreover, toxin A-specific monoclonal antibody did not recognize toxin B, and the antibody specific for toxin B did not recognize toxin A. These results demonstrate that both toxins A and B cleave the PNPG substrate. This is consistent with the reported *in vivo* activity of these toxins, in that they both cleave the same cellular substrate, UDP-glucose (41, 111-113) (41).

The effects of pH and temperature on the functional activities of the toxins were evaluated. The pH experiments were performed using 5 different buffers to establish a wide range of buffering capacities. Both toxins A and B demonstrated optimal PNPG cleavage activities within a pH range of 7-9 (Fig. 2.5A). In contrast to toxin A, which showed significant activity within the pH range of 6 to 12, toxin B displayed a more narrow range of PNPG cleavage activity within the pH range of 7 to 10. This is consistent with the pathophysiological environment of the colon, where *C. difficile* causes disease. The pH of the colon varies from 6.4 ± 0.6 to 7.5 ± 0.4 (121). Both toxins showed activity optima at a temperature range of 35-40^{o}C, with toxin A showing a broader range of activity than toxin B (Fig. 2.5B) (41).

Table 1: Summary of *C. difficile* toxins A and B purification from crude culture supernatant (41).

PURIFICATION STEP	PROTEIN AMOUNT (ug)		TOTAL ACTIVITY (Unit)[a]		SPECIFIC ACTIVITY (U/ug)		PURIFICATION (FOLD)[b]
	TOXIN A	TOXIN B	TOXIN A	TOXIN B	TOXIN A	TOXIN B	
Crude culture supernatant	965000		779720		0.808		1
150 KDa Concentration	801000		973215		1.215		1.50
DEAE-Sepharose CL-6B	2230	1910	1014	3085	0.455	1.615	2.56
Sephacryl S-300	1410	805	1158	3775	0.821	4.689	6.82

[a]One unit of toxin activity was defined as the amount of the toxins required to cleave one micromole of the PNPG substrate per hour under the experimental conditions.

[b]Fold purification was calculated using the combined specific activities of toxins A and B.

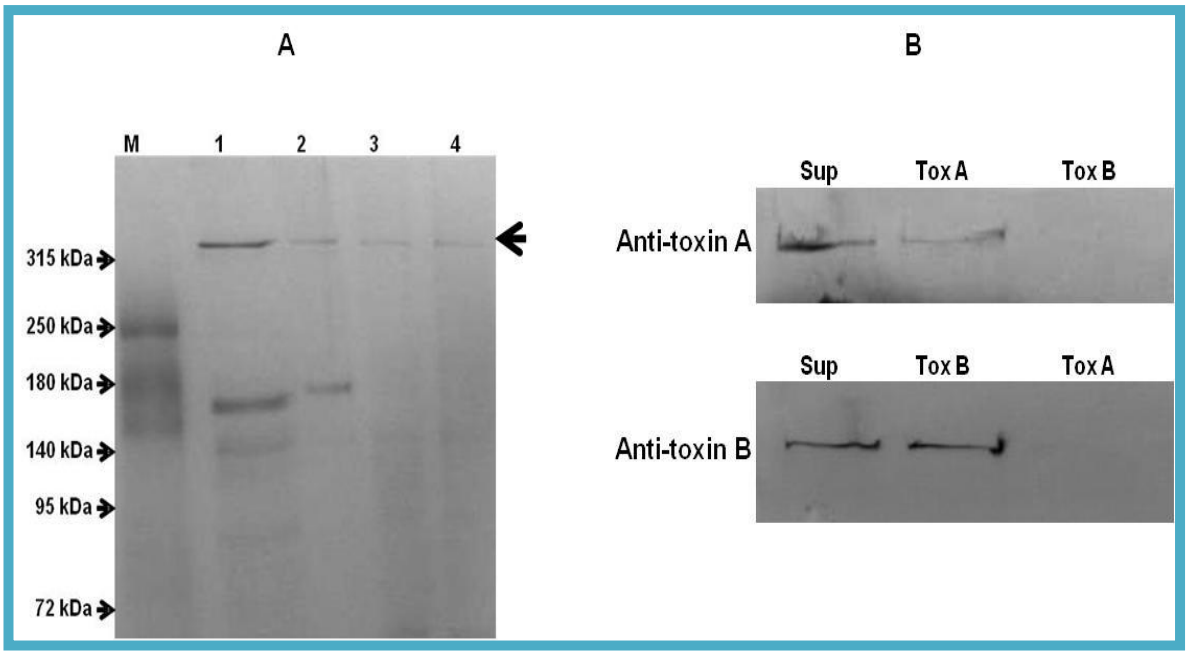

Figure 2.4-A: Polyacrylamide gel electrophoresis (PAGE) analysis of *C. difficile* toxins A and B purification by anion exchange and gel filtration chromatography. Proteins (50 µg each) were separated through a 5% PAGE gel. M= ProSieve QuadColor molecular weight marker (Lonza Rockland Inc., ME); 1= concentrated supernatant; 2= pooled and concentrated fractions from anion exchange; 3 = pooled and concentrated fractions from gel filtration (toxin A); 4= pooled and concentrated fractions from gel filtration (toxin B). The arrow indicates the location of toxins in the gel. **B**: Western immunoblot analysis of *C. difficile* toxins A and B after gel filtration chromatography. Proteins (85 µg each) were separated by electrophoresis through a 5% PAGE gel and transferred onto PVDF membranes. Each membrane was probed using mouse monoclonal primary antibodies specific for toxins A or B. The WesternDot 625 Western Blot kit (Invitrogen, Carlsbad, CA) was used for the detection of the bound antibodies. Sup = crude culture supernatant; Tox A = Toxin A; Tox B = Toxin B (41).

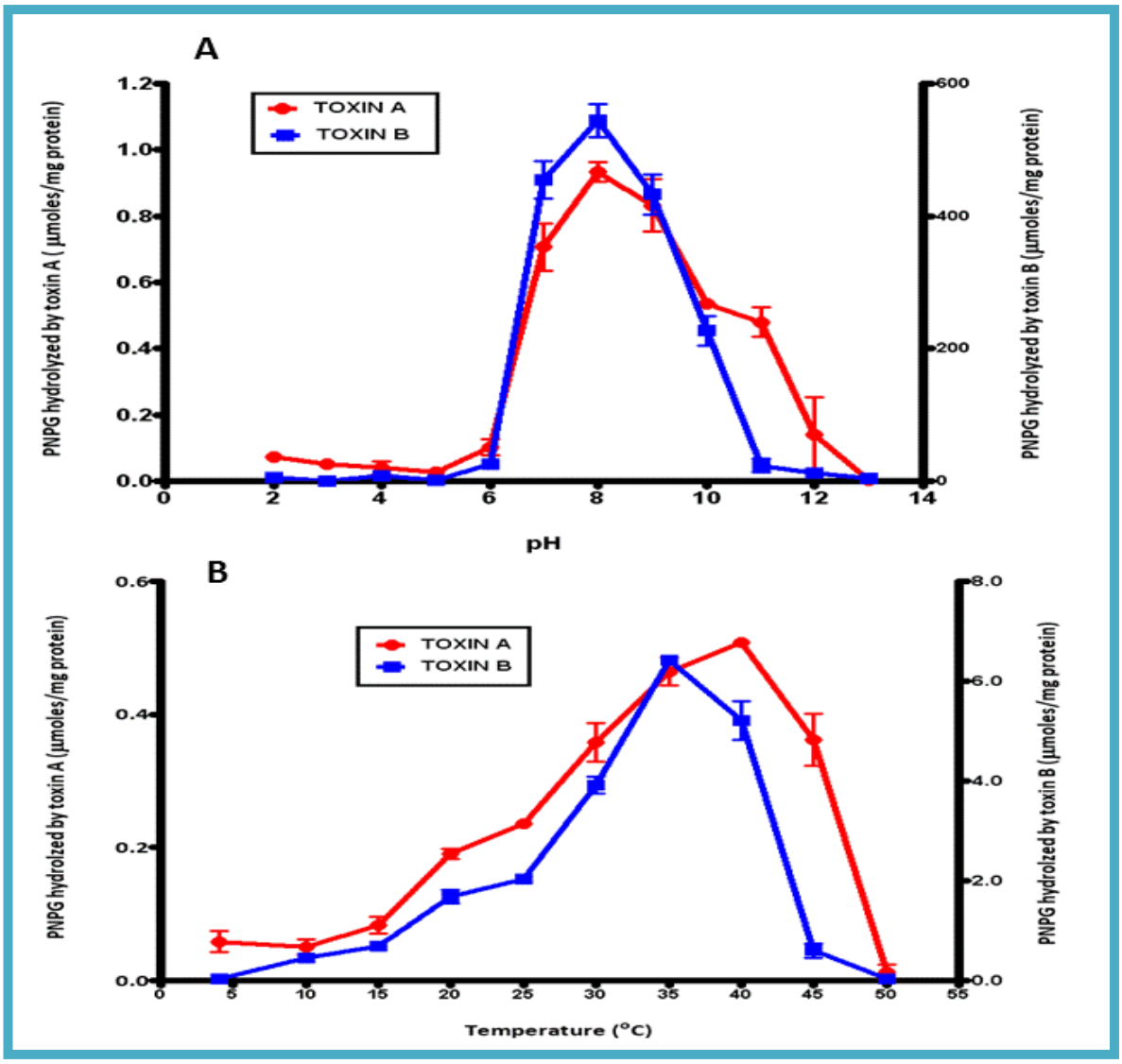

Figure 2.5: Effect of pH **(A)** and temperature **(B)** on the PNPG cleavage activities of toxins A and B. For the pH experiment, the Cdifftox activity assay was performed by incubating 100 µg of toxin A or B with 10 mM PNPG at 37°C for 4 hrs in buffers at the various pH values shown. For the temperature experiment, the Cdifftox activity assay was performed by incubating 100 µg of toxin A or B in 50 mM Tris-HCl containing 50 mM NaCl (pH 7.4) with 10 mM PNPG at the temperatures indicated for 4 hrs. The assay was monitored by absorbance at 410 nm. Error bars represent standard deviation between two replicate experiments (41).

The amino acid sequences of the toxins were analyzed using the ProtParam program (68) to assess their physico-chemical characteristics. Based on the ProtParam analysis, toxin A has 588 total charged residues out of 2710 residues, of which 54% and 46% are negatively and positively charged, respectively. Toxin B has more charged residues (597 out of a total of 2366 residues); 66% and 34% are negatively and positively charged, respectively. These data support the lower isoelectric point (IEP) of 4.42 estimated for toxin B compared to that of toxin A (5.51). The implication of this lower IEP for toxin B is a wide pH range for the maintenance of its overall negative charge at physiological pH. Toxin A is computed to be more stable with an instability index (86) of 29.6 compared to that of 36.5 for toxin B. However, both toxins are estimated to have relatively long *in vitro* half-lives based on the N-terminal end rule (8, 77, 212) of 30 hrs. These computational data suggest that toxin A should function in and tolerate a wider range of physiological and environmental conditions than toxin B (41).

To better define the activities of toxins A and B, a kinetic analysis was performed. The affinities and enzymatic cleavage abilities of each toxin for the PNPG substrate was assessed. Initially, to determine the amount of each toxin that cleaves the substrate at a measurable rate under the experimental conditions, different amounts of the toxins were evaluated at constant substrate concentration. As expected, this resulted in a dose-dependent cleavage of the substrate with increasing toxin amounts (Fig. 2.6). Different concentrations of the substrate were also tested at constant toxin amount. Increasing substrate concentrations led to an increase in cleavage products as the incubation time increased (Fig 2.7) (41).

The activity of both toxins could be fit to the Michaelis-Menten curve, indicating a single active site reaction (Fig. 2.8). The Michaelis-Menten constant (Km) values of the toxins for the PNPG substrate were determined by non-linear regression to be 1.04 mM for toxin A and 0.24 mM for toxin B. The maximum velocity (Vmax) for toxin A was 1.5 µmoles/mg/min, whereas that for toxin B was 6.4 µmoles/mg/min. These data indicate that the affinity of toxin B for the PNPG substrate is more than 4-fold higher than toxin A. Moreover, the rate of cleavage of the PNPG substrate was 4.3-fold faster for toxin B than toxin A. These results agree with assays of the relative damage by toxins A and B to tissue culture cells, in which toxin B was found to be more potent than toxin A (112, 217, 219) (41).

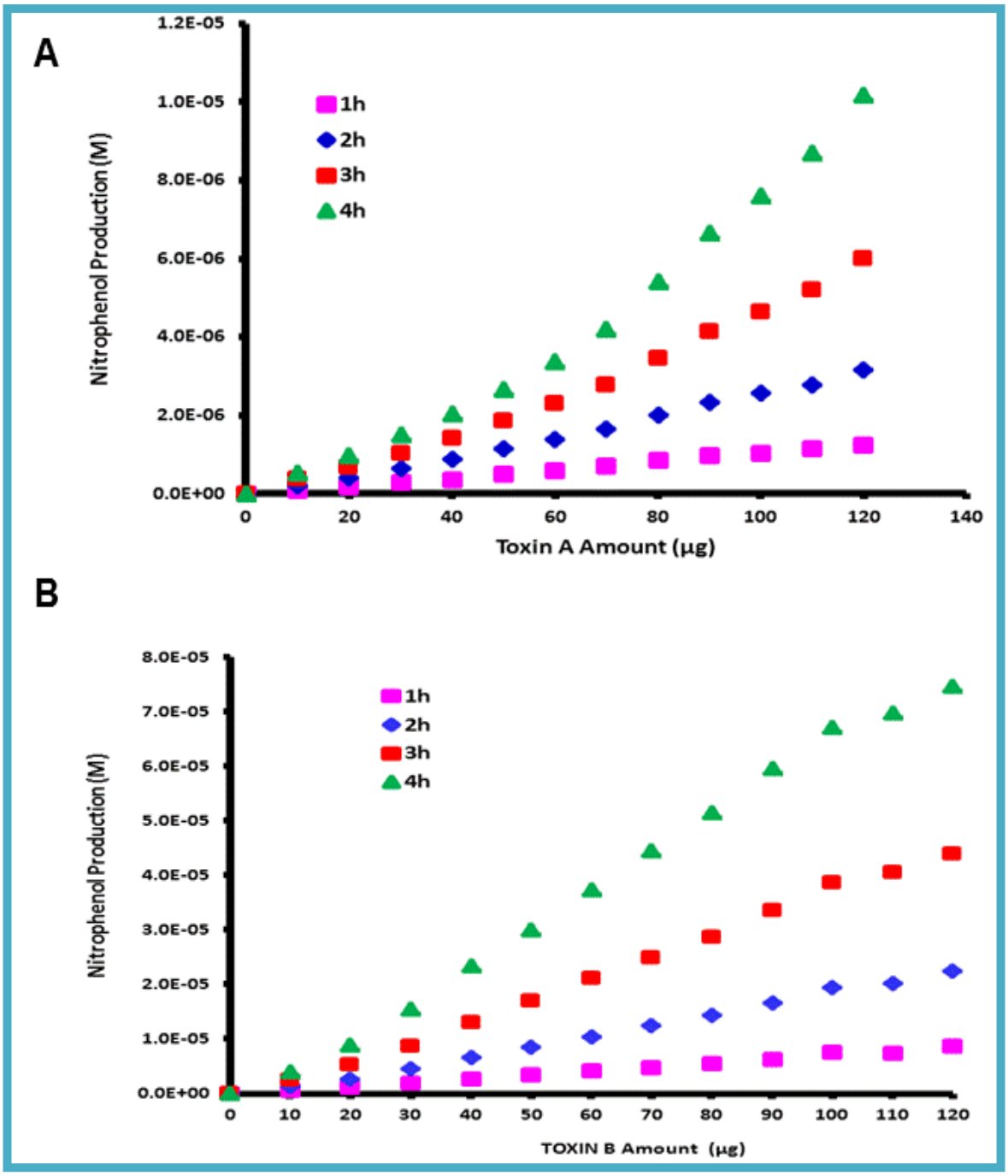

Figure 2.6: The PNPG substrate cleavage by different amounts of toxin A (**A**) and toxin B (**B**). Different amounts of the purified toxins were incubated with 10 mM PNPG substrate at 37^{o}C for 4 hrs. The cleavage of the substrate was monitored at wavelength 410 nm.

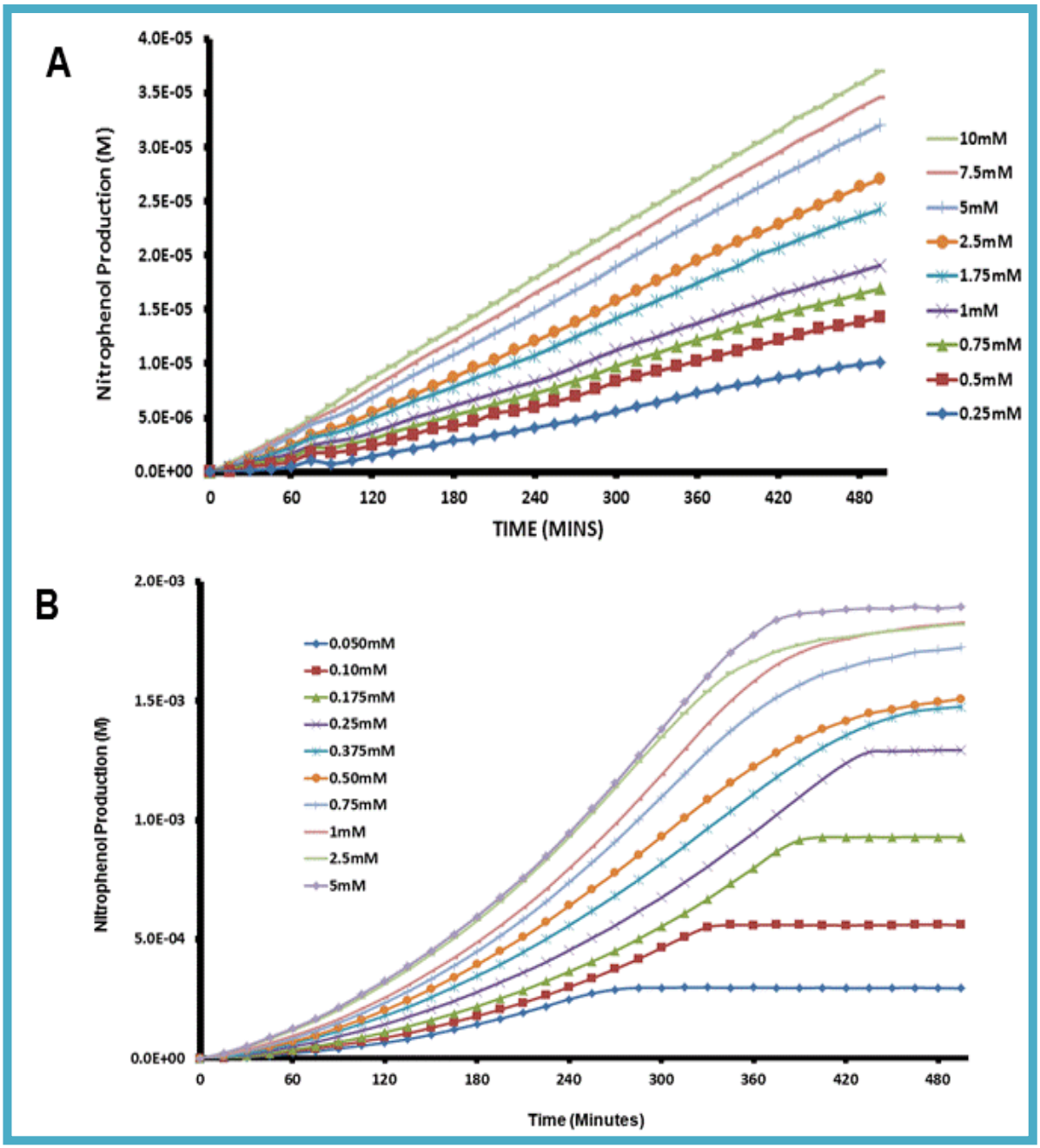

Figure 2.7: The PNPG substrate cleavage by 55 µg of toxin A (A) and 100 µg of toxin B (B). Different concentrations of the PNPG substrate were incubated with constant amount of the toxins at 37^{o}C for 8 hrs. The cleavage of the substrate was monitored at wavelength 410 nm.

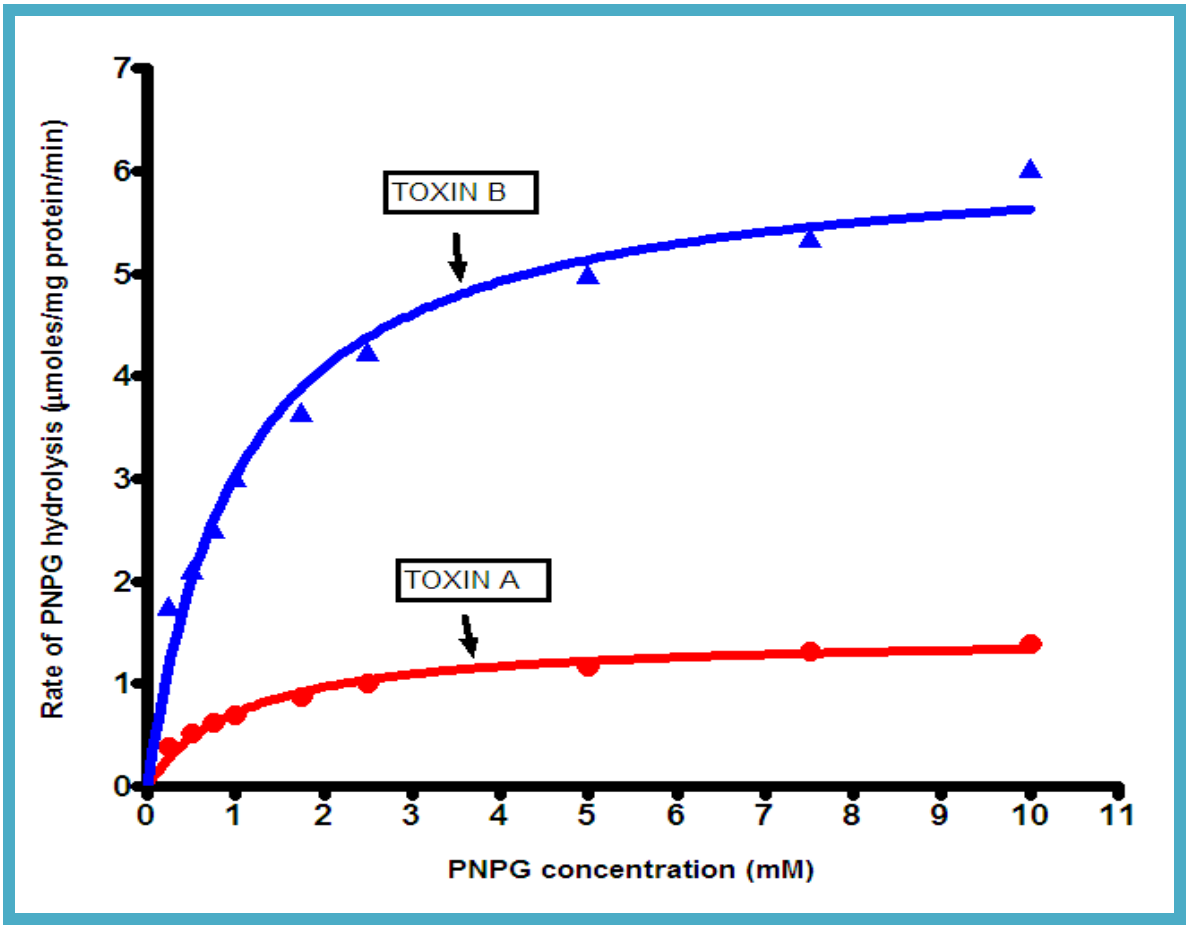

Figure 2.8: The Michaelis-Menten plot for the PNPG cleavage by *C. difficile* toxins A and B based on non-linear regression method. For toxin A: Km = 1.04 ± 0.06 mM and Vmax = 1.50 ± 0.03 µmoles/mg/min. For toxin B: Km = 0.24 ± 0.02 mM and Vmax = 6.40 ± 0.12 µmoles/mg/min. Error bars represent standard deviation from four replicate experiments (41).

2.3.3 *C. difficile* Activity Inhibition

To further characterize the toxin-substrate interactions, I sought to identify molecules or compounds that could inhibit the activities of toxins A and B. After testing several potential molecules (see Materials and Methods), sodium taurocholate was observed to inhibit the activities of both toxins. The addition of 300 mM of sodium taurocholate reduced the activities of toxins A and B within one hour of incubation by 71% and 86%, respectively (Fig. 2.9). Interestingly, taurocholate and phosphatidylserine (both negatively charged lipids) have been reported to inhibit β-glucosidases in a non-competitive manner (35, 83, 99, 167). These results support the idea that the cleavage of the PNPG substrate is due to the glucosyltransferase/hydrolase activities of the toxins (41).

2.3.4 Evaluation of the Period *C. difficile* Toxins are Synthesized

The *C. difficile* toxins A and B are produced and released during the stationary phase of growth. To test the sensitivity of the new Cdifftox activity assay in comparison with ELISA, the growth of *C. difficile* and toxin synthesis were examined. *C. difficile* cells were monitored for growth and the culture supernatant fluid was tested for the toxins over a 48-hr period. Growth was determined by optical density measurement at wavelength 600 nm whereas toxin production was determined using the new Cdifftox activity assay (41) and ELISA. The new Cdifftox activity assay detected the toxins between 12-16 hrs of growth whereas the ELISA test detected the toxins between 32-36 hrs (Fig. 2.10). Detection of the toxins by the Cdifftox activity assay during the early stationary phase is consistent with the period during which the transcription of the toxin genes occurs.

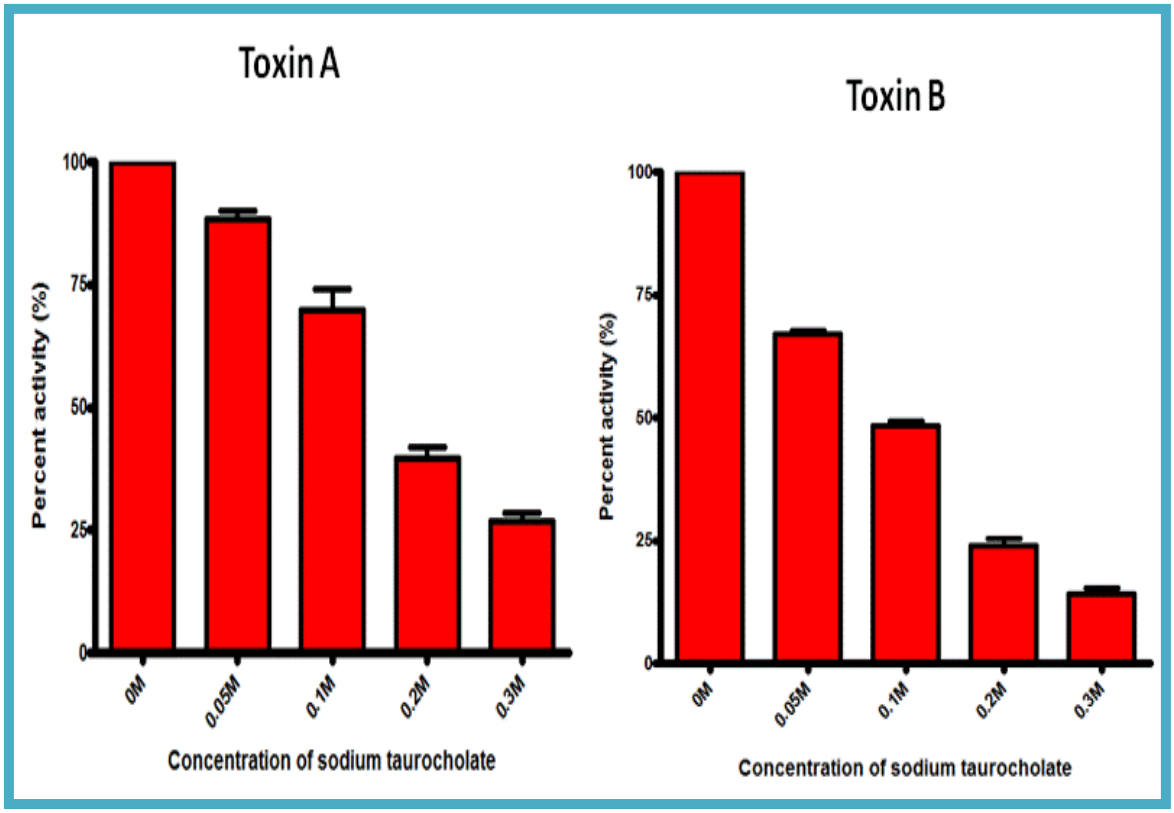

Figure 2.9: Dose-response inhibition by sodium taurocholate of toxin A and B PNPG cleavage activities. These experiments were performed by incubating 55 µg of each toxin for 1 hr with the amount of sodium taurocholate indicated at 37^{o}C in 30 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, and 10 mM of the PNPG. Error bars indicate standard deviation from three different experiments (41).

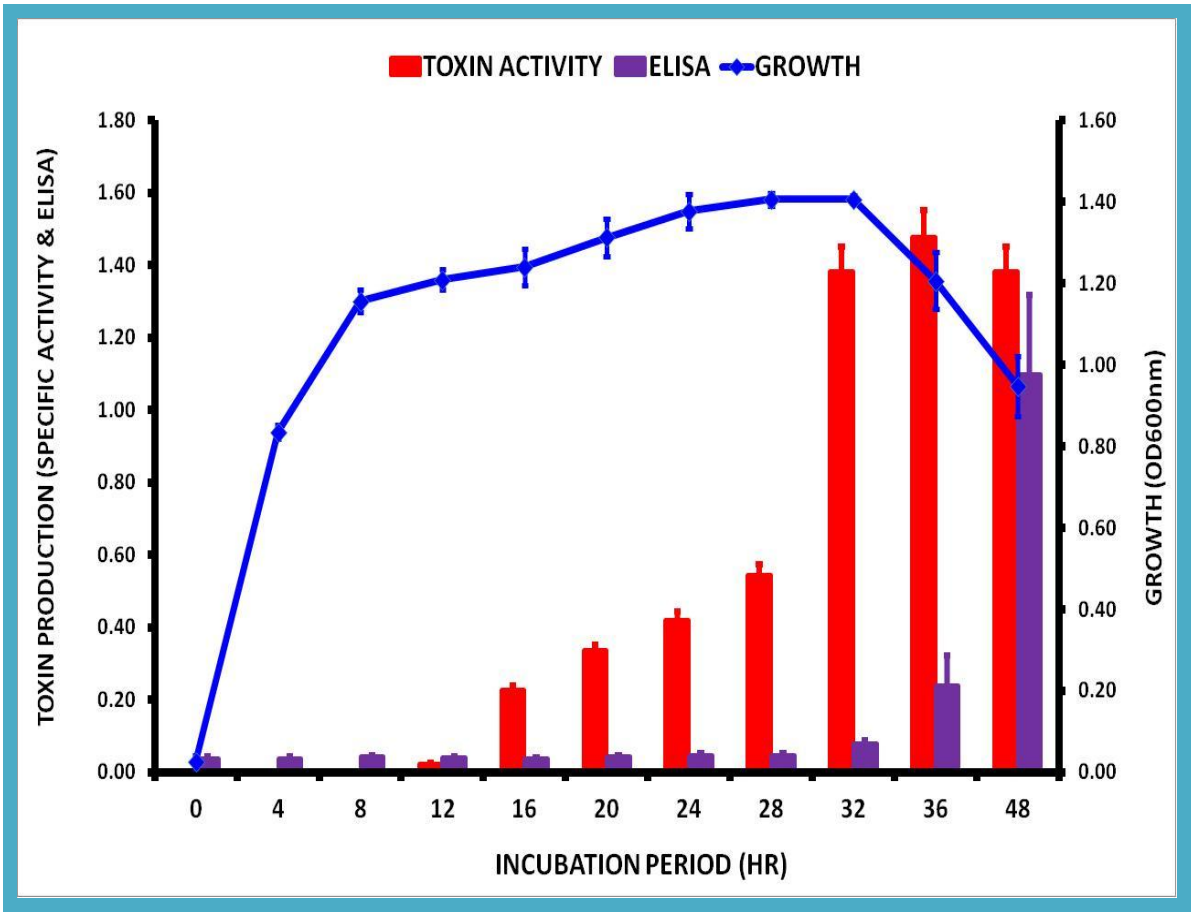

Figure 2.10: Comparison of toxin detection by the Cdifftox activity assay and ELISA. An overnight culture of *C. difficile* strain 630 was diluted 1:100 in fresh reduced BHI medium and incubated anaerobically at 37^{o}C. Aliquots were taken every 4 hrs for O.D. 600 nm measurement and toxin testing using the new Cdifftox activity assay (red) and ELISA (purple).

2.3.5 Analysis of Toxin Activity in *C. difficile* Supernatant Fluid

To evaluate the capability of this new Cdifftox activity assay to detect *C. difficile* toxins A and B in culture supernatant, *C. difficile* strains were isolated from clinical stool samples. Cultures were prepared from 18 independent clinical isolates from different patients and their supernatants were tested for toxins A and B using the Cdifftox activity and ELISA assays. All the culture supernatants from the clinical isolates determined to be positive for the toxins by the Cdifftox activity assay were also positive by the ELISA assay (Fig. 2.11) (41). The toxin-negative culture supernatants were negative in both assays. Genomic DNA was isolated from each strain and PCR amplification analysis was performed with specific primers to identify the genomes encoding the *C. difficile tcdA* (toxin A) and *tcdB* (toxin B). The toxin gene-positive isolates matched those that were toxin-positive by the Cdifftox activity and ELISA assays (data not shown). Paired t-test analysis showed both ELISA and Cdifftox Activity assay correlated significantly in detecting the presence of the toxins ($p = 0.001$). However, there was not always a correlation between the amount of ELISA signal and the Cdifftox activity. This was expected as the ELISA is not quantitative, whereas the Cdifftox assay is quantitative. Interestingly, some of the isolates that were confirmed by PCR to encode *tcdA* and *tcdB* and tested positive with the Cdifftox activity assay, initially tested negative with the ELISA assay. However, these isolates became ELISA positive after longer incubation of the culture, suggesting that the Cdifftox activity assay is more sensitive than the ELISA assay. All together, these data illustrate that the Cdifftox activity assay is a sensitive and reliable method to detect and assess the functional activities of *C. difficile* toxins A and B in culture supernatant fluid (41).

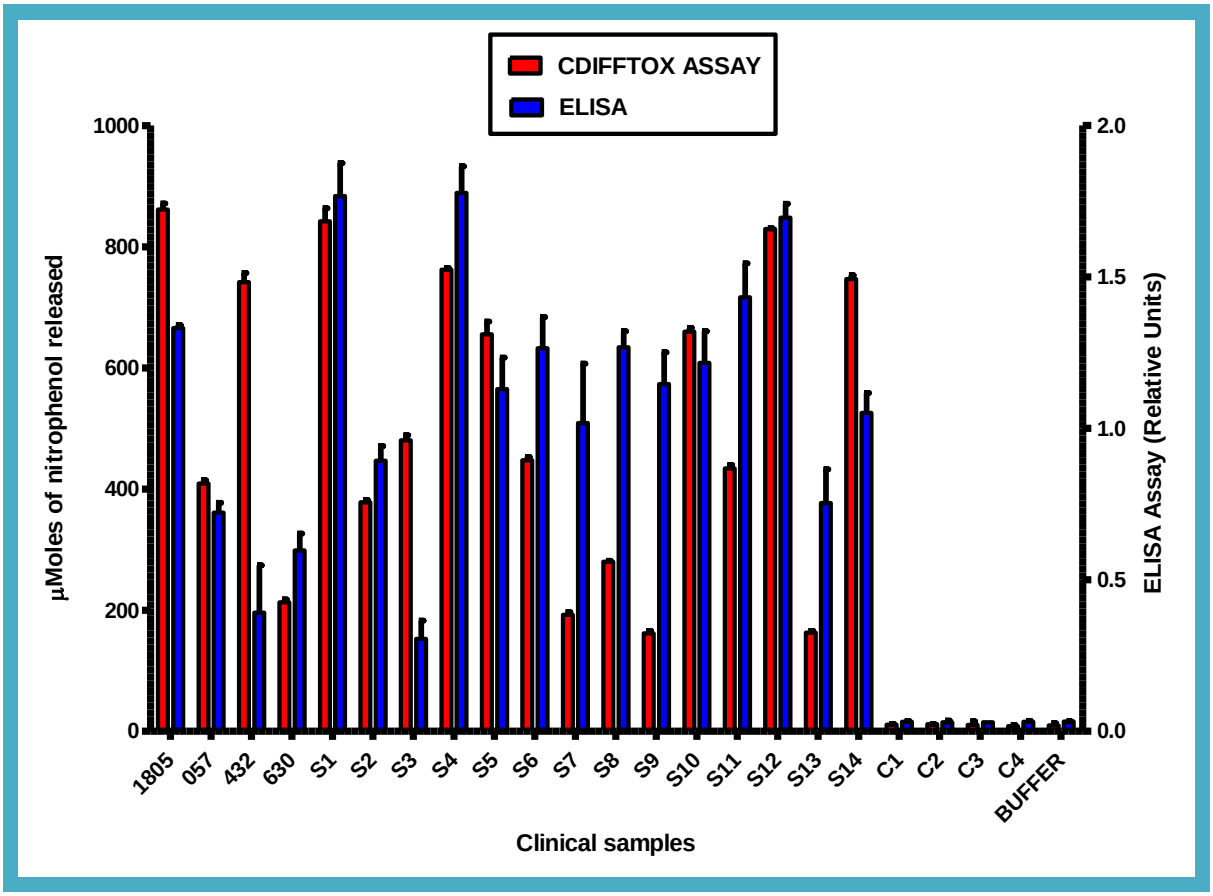

Figure 2.11: Comparison of clinical isolates for the presence of *C. difficile* toxins A and B using the Cdifftox Activity assay and ELISA assay. Supernatant (250 µl) from isolated strains cultured in BHI media was incubated with 10 mM of PNPG and incubated for 3 hrs at 37^{o}C. The assay was monitored by measuring absorbance at 410 nm. Moles of glucose released was calculated using a molar extinction coefficient for *p*-nitrophenol of ε = 17700 $M^{-1}cm^{-1}$ (192). ATCC strains: 1805 = BAA-1805 (*tcdA*+/*B*+; NAP1), 057= 700057 (*tcdA*-/*B*+), 432= 43255 (*tcdA*+/*B*+), 630= BAA-1382 (*tcdA*+/*B*+), Clinical isolates: S1-S14= Culture supernatant from independent clinical isolates obtained from different patients that were *tcdA*+ /*tcdB*+; C1-C4= Culture supernatant from independent clinical isolates that were *tcdA*-/*tcdB*-. Error bars represent the standard deviation between two replicate experiments. Paired t-test analysis showed both ELISA and Cdifftox Activity assay correlated significantly in detecting the presence of the toxins (p = 0.001).

DISCUSSION

Current methods for diagnosing *C. difficile* infection are based on detection of the organism, the toxin genes and proteins, or the effect of the cytotoxin on tissue culture cells (10, 44-46). The only method that can provide information about the activities of the toxins is the tissue culture cytotoxicity assay. Such limitations are problematic for diagnosis and studies of these toxins. The necessity for a more sensitive assay to enable detection of the toxins at early stationary phase led to the development of this cost-efficient, sensitive, and reliable assay designated the Cdifftox activity assay that utilizes PNPG as a chromogenic substrate, which is similar to the native substrate of these toxins. Perhaps as a result, the Michaelis constants (Km) obtained for the toxins with the non-native PNPG substrate (1.04 ± 0.06 mM and 0.24 ± 0.02 mM for toxins A and B, respectively) were relatively close to those reported for the native UDP-glucose substrate (0.14 mM and 0.18 mM for toxin A and B, respectively) (37).

The new assay was successfully used for the purification of *C. difficile* toxins A and B, and simultaneously evaluated by comparison to the antibody-based ELISA assay. Unlike commercial ELISA-based assays that only detect the presence of a fragment or region of the toxins, the Cdifftox activity assay detects the presence of the toxins and quantitates their substrate cleavage activities. It is important to note that the Cdifftox activity assay does not distinguish between toxins A and B, since both toxins cleave PNPG and act on the same cellular substrate *in vivo* (50, 112,

113). This lack of distinction is of little consequence since both toxins are responsible for the pathogenesis of *C. difficile* infections (64, 123, 136).

The PNPG cleavage activities of *C. difficile* toxins A and B was inhibited by sodium taurocholate in a dose-dependent manner (Fig. 2.9). Taurocholate, which is one of the major bile acids found in humans (55, 150), is formed and secreted into the lumen of the small intestine by conjugation of cholic acid with taurine. The total bile acid concentration in the small intestine varies depending on diet and other metabolic conditions (55, 159, 169). However, only about 2-5% of the bile acids secreted in normal humans enter the colon because the majority of the bile acids are reabsorbed in the ileum (55, 60). The finding of inhibition of the *C. difficile* toxins by a major bile acid may explain why the pathology of *C. difficile* infection is almost exclusively restricted to the bile acid-poor colon with relative sparing of the bile-rich small bowel.

Sodium taurocholate is known to non-competitively inhibit mammalian β-glucosidases (35, 83, 99, 167). These enzymes, including glucosyltransferases, belong to a large family of enzymes that mediate a wide variety of functions such as carbohydrate biosynthesis, metabolites storage, and cellular signaling (27). Glycosyltransferases transfer a monosaccharide from an activated nucleotide sugar donor to specific sugar residues, proteins, lipids, DNA or small molecule acceptors. This transfer may occur either by inversion or retention of the configuration of the anomeric carbon (22, 175). Inhibition of toxin A and B activities by a molecule that also inhibits glucosidases suggest that the cleavage of the PNPG substrate utilized in the Cdifftox activity assay is due to the glucosyltransferase activities of the toxins.

However, further confirmatory experiments are planned to test the activity of the toxins A and B glucosyltransferase domains. The use of the glucosyltransferase activities of the A and B toxins to identify toxigenic *C. difficile* is unique and has not previously been reported.

The Cdifftox activity assay was modified into an agar-based method for detection of toxin-producing *C. difficile* strains from stool samples. The Chapter that follows describes how this was done and the validation experiments performed, which demonstrated that the modified agar-based method may be useful for clinical detection and diagnosis of *C. difficile* directly from stool.

CHAPTER 3: A NOVEL APPROACH FOR DETECTION OF TOXIGENIC *CLOSTRIDIUM DIFFICILE* FROM STOOL SAMPLES

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INTRODUCTION

Clinical identification of *C. difficile* in fecal samples relies on a combination of at least two techniques, which may include culture isolation, PCR detection of the toxin-encoding genes, the tissue culture cytotoxicity assay, and immunological detection of the toxins (ELISA). Culture isolation is normally performed on the commercially available media, cycloserine-cefoxitin fructose agar (CCFA), which is selective but does not differentiate the toxin-producing strains from strains that do not produce the toxins. As a result, a second method is required to determine if a strain is pathogenic. PCR assays are gaining popularity for the diagnosis of CDI because of their high sensitivity in detecting the toxin-encoding genes. The tissue culture cytotoxicity method is not as sensitive as culture isolation combined with toxin testing (44, 70), although it is considered by some laboratories as the gold standard. Other approaches include the glutamate dehydrogenase screening assay (180, 211), and automated PCR-based methods such as Cepheid Xpert *Clostridium difficile* Epi Assay (7, 209), and the loop-mediated isothermal amplification test (158). However, these methods do not isolate and differentiate toxigenic from non-toxigenic strains of *C. difficile*.

This Chapter describes the development of a single-step selective and differential agar-based assay, the Cdifftox Plate assay (CDPA), which enables identification of toxin-producing *C. difficile* without the need for additional toxin-confirmatory tests. This assay was based on the finding, as described in Chapter 2, that the A and B toxins of *C. difficile* cleave a chromogenic substrate that has stereochemical characteristics similar to their natural substrate, UDP-glucose.

MATERIALS AND METHODS

3.2.1 Bacterial Strains and Growth Conditions

The toxigenic *C. difficile* strains ATCC 43255 (*tcdA*+/*B*+), ATCC BAA-1382 (*tcdA*+/*B*+), ATCC 700057 (*tcdA*-/*B*+), and the hypervirulent strain, ATCC BAA1805 (*tcdA*+/B+) were purchased from the American Type Culture Collection (Manassas, VA). Brain Heart Infusion (BHI) medium was purchased from Becton Dickinson (Cockeysville, MD). The substrates: 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside, 5-bromo-4-chloro-3-indoxyl-phosphate, 5-bromo-4-chloro-3-indoxyl butyrate, 5-bromo-4-chloro-3-indoxyl-β-D-xylopyranoside, 5-bromo-4-chloro-3-indoxyl palmitate, 5-bromo-4-chloro-3-indoxyl-α-D-maltotrioside, 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, 5-bromo-4-chloro-3-indoxyl caprylate , and 5-bromo-4-chloro-3-indoxyl choline phosphate were purchased from Biosynth International (Itasca, IL). The liquid or plate cultures were incubated anaerobically in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 at 37°C in a Controlled Atmosphere Anaerobic Chamber (PLAS LABS, Lansing, MI) (40).

3.2.2 Sample Storage Conditions

The clinical isolates were either stored short-term (less than 1 month) in chopped meat broth (BD Diagnostics, Franklin Lakes, NJ) at room temperature or long-term in 15% glycerol stocks at -80°C. The culture supernatants were stored at 4°C for a maximum of 2 weeks with no loss of toxin activity (40).

3.2.3 Clinical Stool Samples

The clinical stool samples were obtained from a study approved by the Institutional Review Boards of The University of Texas Health Science Center at Houston and St. Luke's Episcopal Hospital (Houston, Texas). All of the participating patients or their legal guardians provided written informed consent upon admission to the hospital. All the stool samples used were tissue culture cytotoxicity assay-positive, as determined by the Medical Microbiology Laboratory at the St. Luke's Episcopal Hospital (40).

3.2.4 Cdifftox Plate Assay Medium

This agar-based culture medium was developed to be selective for growth of *C. difficile* and differential, in that it simultaneously identifies colonies producing active toxins A and/or B, while inhibiting the growth of non-*C. difficile* colonies. The following components were chosen to compose this new Cdifftox Plate assay (CDPA) medium (per liter): BHI (6 g), peptic digest of animal tissue (6 g), pancreatic digest of gelatin (14.5 g), NaCl (5 g), dextrose (3 g), anhydrous Na_2HPO_4 (2.5 g), sodium taurocholate (0.1%) (Sigma-Aldrich, St. Louis, MO), D-cycloserine (300 mg) and cefoxitin (8.5 mg) (Fisher Scientific, Pittsburgh, PA), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (150 mg), 0.5 mM glycine, 4-methylphenol (0.025%), defibrinated Horse blood (6%), and agar (12 g). Alternatively, BHI (6 g), peptic digest of animal tissue (6 g), pancreatic digest of gelatin (14.5 g), NaCl (5 g), dextrose (3 g), anhydrous Na_2HPO_4 (2.5 g) can be replaced with 37 g of BBL Brain Heart Infusion medium (BD Diagnostic Systems, Franklin Lakes, NJ) (40).

3.2.5 Cdifftox Plate Assay

For the Cdifftox Plate assay, each stool sample was streaked directly onto two CDPA plates using a sterile loop. The plates were incubated anaerobically for 24-72 hr at 37^{o}C, until colonies appeared and then the plates were exposed to air at room temperature for at least 30 min to allow the chromogenic reaction to complete. The assay was initially tested using the well-characterized toxin overproducing *C. difficile* strain ATCC 43255 (*tcdA*+ *tcdB*+) and the hypervirulent strain ATCC BAA-1805 (*tcdA*+ *tcdB*+), along with two strains that produce less toxins, ATCC BAA-1382 (*tcdA*+ *tcdB*+) and ATCC 700057 (*tcdA*- *tcdB*+). The presumptive active toxin-producing *C. difficile* colonies appeared blue, while non-toxin producers remained pale white. The blue colonies were phenotypically classified as Tox^{+} (presumably *tcdA*+ and/or *tcdB*+), whereas the pale white colonies were denoted Tox^{-} (presumably *tcdA*$^{-}$ and *tcdB*$^{-}$ or mutants with genetic alterations that affect toxin production or activity) (40).

3.2.6 Isolation and PCR Identification of Toxin-Producing *C. difficile* Strains

A total of 528 single colonies consisting of 486 Tox^{+} and 42 Tox^{-} colonies were selected from the CDPA plates for further analysis. These colonies represented a total of 10-12 independent isolates from each stool sample. When possible eight Tox^{+} and two Tox^{-} colonies were selected from each sample. The presence of the toxin-encoding genes (*tcdA* or *tcdB*) in the genomes of the presumptive Tox^{+} and Tox^{-} isolates was examined by PCR amplification of a portion of each of these genes. A portion of the 16S

ribosomal RNA (rRNA) genes was also amplified. These reactions were performed by first isolating genomic DNA from 1 ml of an overnight culture of each isolated colony using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Amplification was performed using Phire Hot Start DNA Polymerase II Kit (Finnzymes, Woburn, MA). The following primers were used: toxin A (F- 5'TGATGCTAATAATGAATCTAAAATGGTAAC3'and R-5'ACCACCAGCTGCAGCCATA3'); toxin B (F-5'GTGTAGCAATGAAAGTC CAAGTTTACGC3' and R-5'CACTTAGCTCTTTGATTGCTGCACCT3') and 16S rRNA (F-5'ACACGGTCCAAACTCCTACG3' and R-5'AGGCGAGTTTC-AGCCTACAA3'). The DNA was amplified with an initial denaturation of 98^{o}C for 30 sec followed by 36 cycles of 98^{o}C for 10 sec, 60^{o}C for 10 sec and 72^{o}C for 10 sec, with a final extension of 72^{o}C for 1 min (40).

3.2.7 Toxin Assays

Preparation of Culture Supernatants from the Tox^{+} and Tox^{-} Clinical Isolates

Single colonies (486 Tox^{+} and 42 Tox^{-}) were inoculated into 10 ml of BHI medium and incubated anaerobically at 37^{o}C for 72 hrs resulting in an O.D. 600 nm of about 1.3-1.4. After centrifugation at 10,000 x g for 10 min to remove the cells, the culture supernatants were collected and stored at 4^{o}C until use (40).

ELISA Assay: The presence of toxins A and/or B in the culture supernatants from the isolates was evaluated using the Wampole *C. difficile* TOX A/B II Kit (TechLab, Blacksburg, VA). This was performed according to the protocol provided by the manufacturer (40).

Cdifftox Activity Assay: The activities of toxin A and/or toxin B produced by the *C. difficile* isolates were measured using the Cdifftox activity assay (41), described in Chapter 2. Briefly, this assay quantitatively measures the activity of toxins A and B based on the inherent properties of these toxins to cleave p-Nitrophenyl-β-D-glucopyranoside, a chromogenic substrate with stereo-chemical characteristics similar to the natural substrate of the toxins, UDP-glucose. The assay was performed on 250 µl of each sample supernatant fluid to which 50 µl of the substrate reagent (30 mM p-Nitrophenyl-β-D-glucopyranoside, 50 mM Tris-HCl, (pH 7.4), 50 mM NaCl, and 100 µM $MnCl_2$) was added in a Costar sterile polystyrene 96-well plate (Corning Inc., NY). It was incubated at 37^{o}C for 1-4 hrs. Cleavage of the substrate was monitored by measuring the absorbance at 410 nm using a SPECTRA max Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). A molar extinction coefficient for *p*-nitrophenol of ε = 17700 $M^{-1}cm^{-1}$ was used in the calculations (192) (40).

3.2.8 Evaluation of Non-*C. difficile* Bacteria on the Cdifftox Plate Assay

The growth of non-*C. difficile* bacteria on the CDPA medium under the standard conditions used for the stool samples was examined. The following strains were tested (asterisks indicate Gram-positive strains): *Alcaligenes faecalis, Bacteroides fragilis*, *B. thetaiotaomicron*, *B. uniformis, Bifidobacterium adolescentis*, Campylobacter jejuni*, *C. perfringens*, Enterobacter cloacae, Enterococcus faecalis*,* enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli* H10407, *Eubacterium lentum**, *Lactobacillus** spp, *Parabacteroides distasonis, Peptostreptococcus anaerobius*, Plesiomonas shigelloides, Prevotella melaninogenica, Proteus mirabilis, P. vulgaris, Salmonella enteritica*, *S. typhimurium, Shigella flexneri, S. sonnei, Staphylococcus aureus**, *Vibrio alginolyticus, V. parahaemolyticus,* and *Yersinia enterocolitica*. Single colonies of these bacteria were selected from agar plates and cultured in 10 ml of appropriate broth media. Pure broth cultures of each of these strains were spread directly on the CDPA plates using a sterile loop and incubated as described above (40).

3.2.9 Growth of *C. difficile* on Cycloserine-Cefoxitin Fructose Agar and BHI-Agar Media

The stool samples were also cultured on CCFA medium (54, 70), which was prepared as follows per liter: proteose peptone #2 (40 g), anhydrous Na_2HPO_4 (5 g), anhydrous KH_2PO_4 (1 g), NaCl (2 g), anhydrous $MgSO_4$ (0.1 g), fructose (6 g), neutral red (0.003%), D-cycloserine (500 mg), cefoxitin (15.5 mg), and agar (15 g). To test for the growth of other anaerobes present in the stool, the samples were also cultured on BHI-agar plates without antibiotics. All plates were incubated for 24-72 hrs under anaerobic conditions at 37^{o}C (40).

RESULTS

3.3.1 Examination of Known Active Toxin-Producing *C. difficile* Strains Using the Cdifftox Plate Assay

The Cdifftox Plate assay uses a novel selective and differential agar-based culture medium to specifically allow the growth of *C. difficile* and simultaneously identify colonies producing active toxins A and/or B, while inhibiting the growth of non-*C. difficile* colonies. The assay was initially tested using the well-characterized toxin overproducing *C. difficile* strain ATCC 43255 (*tcdA*+ *tcdB*+) and the hypervirulent strain ATCC BAA-1805 (*tcdA*+ *tcdB*+), along with two strains that produce less toxins, ATCC BAA-1382 (*tcdA*+ *tcdB*+) and ATCC 700057 (*tcdA*- *tcdB*+). After 24 hrs of incubation, colonies of these strains that produced high levels of active toxins appeared blue (Tox^+), whereas those that produced undetectable toxin levels remained pale white (Tox^-), similar to the colonies shown in Figure 3.1. By 48 hrs, all the toxin-producing colonies were blue, indicating they produced active toxins (40).

3.3.2 Examination of Clinical *C. difficile* Isolates from Stool Samples Using the Cdifftox Plate Assay

To evaluate the ability of the new Cdifftox Plate assay to detect *C. difficile* strains producing active toxins from clinical stool samples, 60 tissue culture cytotoxicity assay-positive clinical stool samples collected at the St. Luke's Episcopal Hospital (Houston, Texas) were tested. A loopful of each stool sample was spread directly onto the CDPA plates and incubated anaerobically at 37^oC for 24-72 hrs. Viable bacterial colonies were successfully isolated from 50 of the 60 tissue culture cytotoxicity assay-positive stool samples analyzed. The percentage of colonies from the 50 stool samples that appeared Tox^+ on the CDPA plates is shown in Table 2. After 24 hrs of anaerobic incubation, Tox^+ colonies were detected in 44 (88%) of the 50 CDPA culture-positive stool samples. By 48 hrs of incubation, Tox^+ colonies were detected in all (100%) of the 50 samples CDPA culture-positive stool samples. It is noteworthy that none of the stool samples required more than 48 hrs of incubation for the Tox^+ colonies to be detected. Tox^- colonies selected after 72 hrs of incubation and transferred onto new CDPA plates remained negative in the differential plate assay, suggesting that no further incubation was necessary after the initial 48 hr of incubation to confirm Tox^- strains (40).

Figure 3.1: Differentiation of toxigenic and non-toxigenic strains of *C. difficile* using the Cdifftox Plate assay. The stool sample was spread directly onto the plate and incubated anaerobically at 37^{o}C for 48 hrs. Blue colonies are toxin-producing *C. difficile* (Tox^{+}); pale white colonies are non-toxin producing *C. difficile* (Tox^{-}) (40).

To further evaluate the Cdiffitox Plate assay, 20 cytotoxicity assay-negative stool samples were tested on the CDPA plates and no colonies grew. To confirm that toxins are not present in the 30 stool samples (10 cytotoxicity assay-positive and 20 cytotoxicity assay-negative stool samples) that did not produce colonies on the CDPA plates, an ELISA assay was performed on each of the 30 samples. All of the ELISA results were negative, confirming that no detectable toxins (whole toxins or portions thereof), were present in these 30 stool samples. These data indicate that the Cdifftox Plate assay is accurate in discriminating stool samples that do not contain viable *C. difficile* strains. Furthermore, these results demonstrate that the 10 cytotoxicity assay-positive stool samples that were ELISA-negative and did not produce viable colonies on the CDPA plates should be considered false positives (40).

3.3.3 PCR Amplification of *C. difficile* Toxin-Encoding Genes

To confirm that the Tox^+ colonies isolated using the Cdifftox Plate assay possessed the genes in their genomes that encode the toxins, a total of 528 single bacterial colonies comprised of 486 Tox^+ and 42 Tox^- independent clinical isolates from the 50 CDPA culture-positive stool samples were examined by PCR amplification. All the 528 isolates tested were positive for the conserved region of the *C. difficile* 16S rRNA gene (Fig. 3.2). Of the 486 Tox^+ isolates evaluated, 485 (99.8%) were positive for either *tcdA* and/or *tcdB*. Of the 42 Tox^- isolates evaluated, 31 (74%) were PCR-positive for either *tcdA* and/or *tcdB,* whereas 11 (26%) were negative for both *tcdA* and *tcdB*. These data indicate that all the Tox^+ and Tox^- isolates that were able to grow on the CDPA plates from the stool samples were *C. difficile*. Furthermore, almost 100% (99.8%) of the Tox^+ strains encode the genes for synthesis of either *C. difficile* toxin A and/or toxin B. Interestingly, 74% of the 42 Tox^- strains selected encoded one or both of the toxin genes in their genomes (40).

Table 2: Detection of Tox^+ colonies from 50 cytotoxicity assay-positive samples on the Cdifftox Agar plates at different times. Clinical stool samples were streaked directly on the CDPA plates, incubated anaerobically for 24, 48, and 72 hrs and examined after 30 min of exposure to air (40).

PERCENT OF TOX^+ COLONIES	NUMBER OF STOOL SAMPLES		
	24 HOURS	48 HOURS	72 HOURS
76-100%	0	31	47
51-75%	1	14	-
26-50%	5	4	2
1-25%	38	1	1
0%	6	0	0

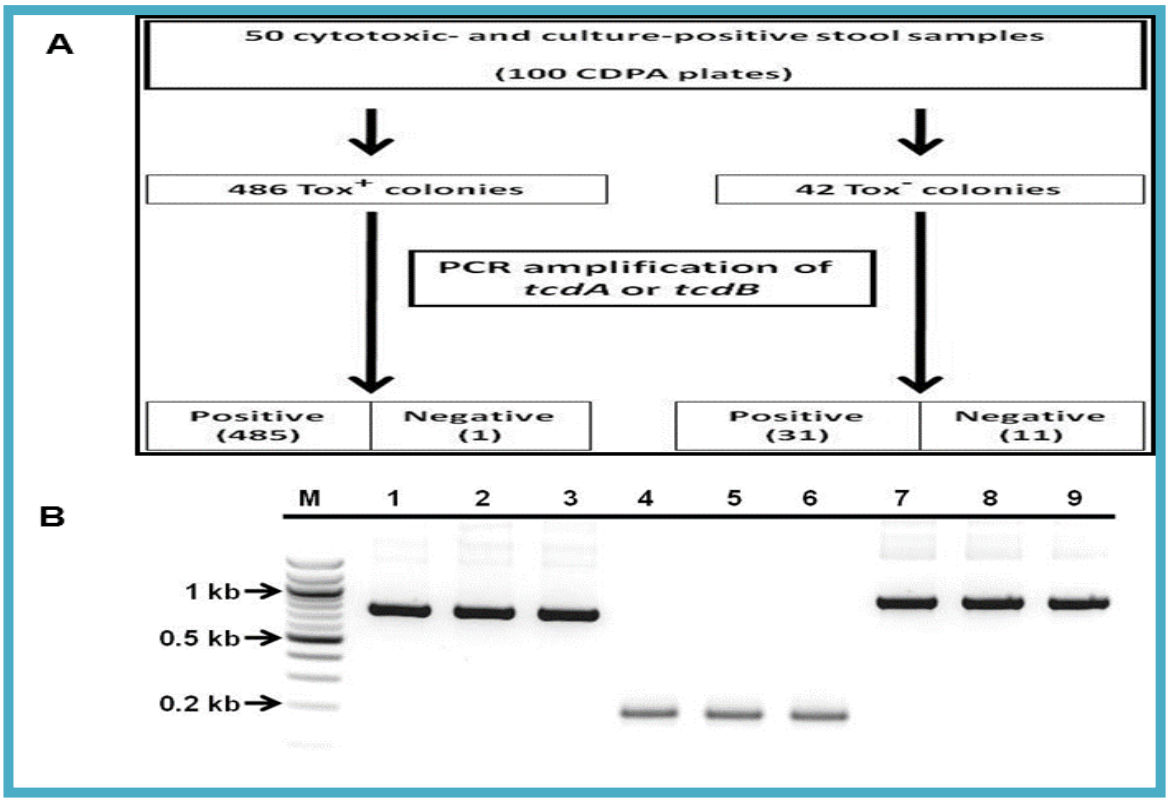

FIGURE 3.2: Schematic representation of the analysis of 50 cytotoxic- and CDPA-positive stool samples. **A.** PCR was performed using the genomic DNA isolated from the Tox^+ and Tox^- colonies to identify a portion of the genes that encode toxin A (*tcdA*) and toxin B (*tcdB*). **B.** PCR analysis of representative Tox^+ and Tox^- strains. Genomic DNA was isolated from the colonies and used as template in PCR reactions with primers specific for the genes that encode toxin A (*tcdA*) and toxin B (*tcdB*), and a conserved region of the *C. difficile* ribosomal RNA (16S rRNA) gene. 'M' represents 1Kb marker (New England BioLabs, Ipswich, MA); lanes 1-3: *tcdA* amplicons; lanes 4-6: *tcdB* amplicons; lanes 7-9: 16S rRNA amplicons. The PCR products were electrophoresed through a 1% agarose gel and the DNA was detected digitally upon exposure of the ethidium bromide-treated gel to UV light (40).

3.3.4 Analysis of Toxin Production and Toxin Activity by the Tox$^+$ and Tox$^-$ Strains

The Cdifftox Plate assay differentiates *C. difficile* strains producing active toxins from strains producing either inactive or no toxins, based on their ability to cleave a chromogenic substrate. To ensure that the Tox$^+$ *C. difficile* cells were able to secrete active toxins, the presence and activity of toxins in the culture supernatants of Tox$^+$ and Tox$^-$ isolates were evaluated. Toxin detection using the ELISA assay was performed on culture supernatants from three isolates of each stool sample. Toxin activity was also tested on all these isolates using the Cdifftox activity assay (41). The results (Fig. 3.3 shows a subset of these results) reveal that all the CPDA Tox$^+$ culture supernatants from the clinical isolates that were positive for *tcdA* and/or *tcdB* by PCR amplification also tested positive for the presence of toxin proteins by ELISA, and tested positive for toxin activity by the Cdifftox Activity assay. Remarkably, all the colonies determined by the Cdifftox Plate assay to be Tox$^-$, whether they encoded *tcdA* or *tcdB* in their genomes or not, were negative for the presence and activity of the toxins in both the ELISA and Cdifftox Activity assays. These data support the results that all but one of the 486 Tox$^+$ colonies selected on the Cdifftox Plate assay were active toxin-producing (toxigenic) *C. difficile* and the 42 Tox$^-$ colonies selected were non-toxin producing (non-toxigenic) *C. difficile* (40).

3.3.5 Selectivity of the Cdifftox Agar Medium

To evaluate the specificity of the Cdifftox Plate assay, non-*Clostridium difficile* bacteria were tested for growth on the CDPA agar under the same culture conditions as the clinical stool samples. No viable colonies were observed when pure cultures of the following strains were streaked on the CDPA plates: *Alcaligenes faecalis, Bacteroides fragilis, B. thetaiotaomicron, B. uniformis, Bifidobacterium adolescentis, Campylobacter jejuni, C. perfringens, Enterobacter cloacae, Enterococcus faecalis, enteropathogenic Escherichia coli, enterotoxigenic E. coli H10407, Eubacterium lentum, Lactobacillus spp, Parabacteroides distasonis, Peptostreptococcus anaerobius, Plesiomonas shigelloides, Prevotella melaninogenica, Proteus mirabilis, P. vulgaris, Salmonella enteritica, S. typhimurium, Shigella flexneri, S. sonnei, Staphylococcus aureus, Vibrio alginolyticus, V. parahaemolyticus, and Yersinia enterocolitica*. These data suggest that the Cdifftox Plate assay medium is selective against non-*C. difficile* bacteria (40).

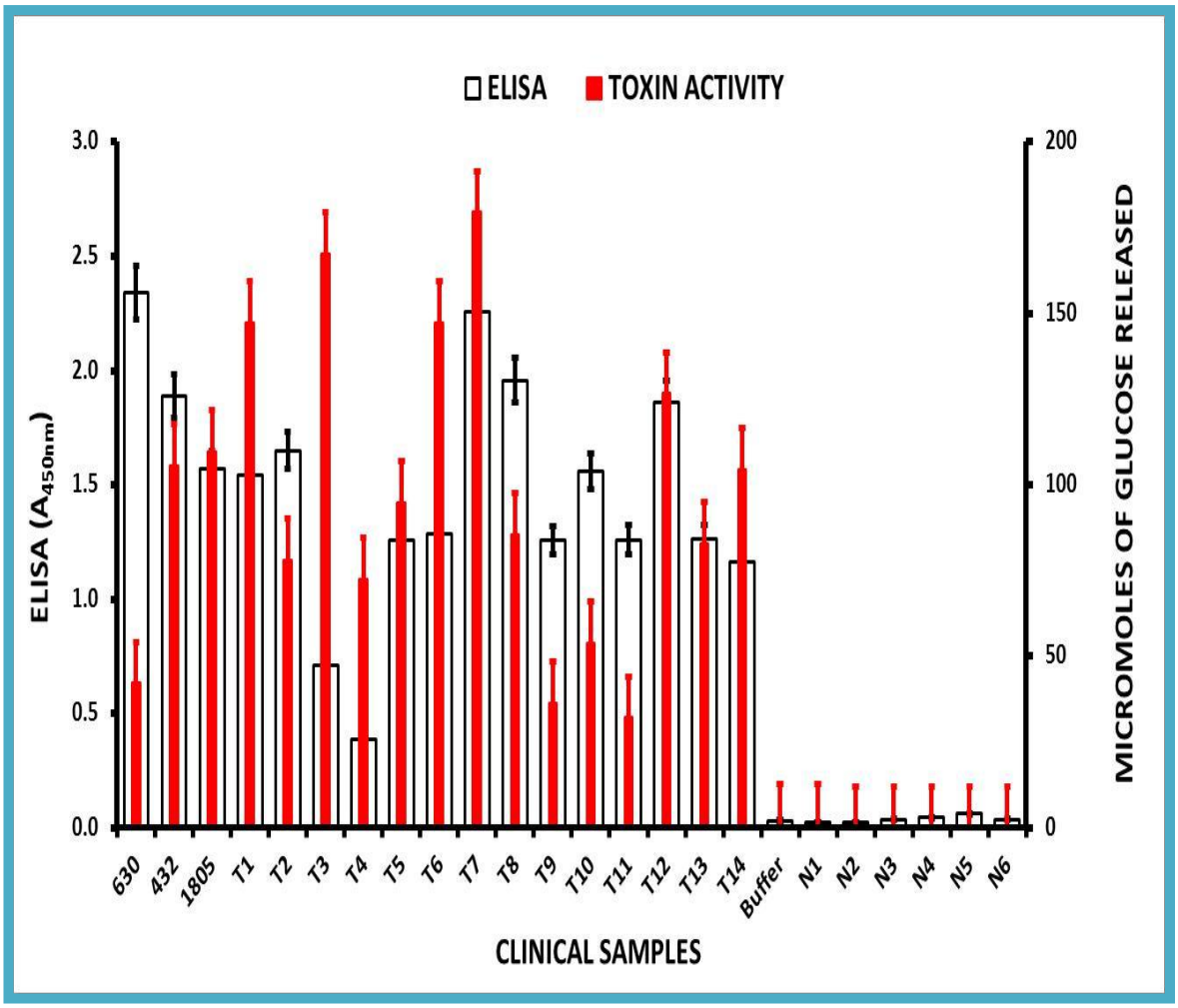

FIGURE 3.3: Analysis of *C. difficile* toxin production in culture supernatants of representative Tox$^+$ and Tox$^-$ clinical isolates and defined ATCC strains. Toxin detection was performed by ELISA and the Cdifftox Activity Assay, as described in the Materials and Methods. ATCC strains: 630 is historical strain BAA-1382 (*tcdA*+/*B*+), 432 represents 43255 strain (*tcdA*+/*B*+), and 1805 represents BAA-1805 strain (*tcdA*+/*B*+; NAP1). Clinical isolates: T1-T14 represent Tox$^+$; N1-N6 are Tox$^-$ (*tcdA*- and *tcdB*-). Error bars represent the standard deviation from two replicate experiments.

To more directly compare bacterial growth on the CDPA medium with CCFA medium (54), which is another *C. difficile* selective medium, and BHI-agar, a non-selective medium, all 60 cytotoxic-positive stool samples were spread directly onto the pre-reduced plates of CCFA- and BHI-agar media and incubated anaerobically at 37^{o}C for 24-72 hr. The CCFA and BHI-agar media allowed the growth of colonies from the same 50 stool samples that were CDPA-culture positive. Overall, approximately 15% more bacterial colonies that appeared morphologically different grew on the CCFA-agar plates and approximately 40% more bacterial colonies grew on the BHI-agar plates compared to the CDPA-agar plates. It appears that the additional colonies observed on the CCFA-agar and BHI-agar plates represent non-*C. difficile* anaerobic bacteria in the stool samples (40).

DISCUSSION

Strains of *C. difficile* are broadly classified as being either toxin-producing or non-toxin producing. It has been established that only the toxin-producing strains cause disease and that toxins A and B play critical roles in the pathogenesis of *C. difficile* (123, 135-138). The alarming emergence of hypervirulent strains of *C. difficile* with increased toxin production, severity of disease, and mortality (59, 78, 79, 132) emphasizes the need for a sensitive diagnostic method that can simultaneously isolate and identify toxin-producing strains. The current available culture methods do not differentiate toxin-producing and non-toxin-producing strains of *C. difficile*. The Cdifftox Plate assay described here advances and improves the culture approach by combining the isolation of *C. difficile* strains on a selective medium with the detection of active toxins in a single step, such that only *C. difficile* strains will grow on this agar and virulent strains producing active toxins can be differentiated from non-virulent strains that do not produce active toxins and as a result do not cause disease. This new assay drastically reduces the time and effort required to isolate and confirm an infection resulting from toxin-producing *C. difficile* strains.

This new CDPA assay was evaluated by testing 60 tissue culture cytotoxicity-positive and 20 cytotoxicity-negative clinical stool samples. After 24 hrs of incubation, the assay detected toxin-producing *C. difficile* colonies in 88% (44/50) of the CDPA culture-positive samples tested (Table 2). Toxin-producing *C. difficile*

colonies were detected from 100% of the 50 CDPA culture-positive stool samples evaluated within 48 hrs of incubation. No viable bacterial colonies grew on the CDPA plates when the 20 cytotoxicity assay-negative, ELISA-negative stool samples and the 10 cytotoxicity assay-positive, ELISA-negative stool samples were tested. These results reveal the high accuracy and specificity of this method.

The Cdifftox Plate assay identifies toxin-producing *C. difficile* colonies by their ability to cleave a chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside, into a distinct insoluble blue product that precipitates around the toxin-producing cells. This substrate cleavage by the toxins was confirmed by the examination of 528 independent *C. difficile* colonies isolated from 50 stool samples from different patients suffering from *C. difficile* infection. Although, non-toxin-producing strains of *C. difficile* can also grow on the CDPA plates, they are differentiated by their inability to cleave the substrate and appear as white colonies. Furthermore, none of the non-*C. difficile* bacterial pathogens tested could grow on the CDPA plates under the culture conditions used to grow *C. difficile*. The CDPA plate medium appeared to be more selective than the CCFA agar (54), in that the CCFA agar allowed the growth of morphologically different colonies from the 50 culture-positive, cytotoxicity-positive stool samples.

Surprisingly, 10 stool samples evaluated as positive by the tissue culture cytotoxicity assay did not result in colony growth on any of the culture media utilized (selective and non-selective) under anaerobic conditions. Furthermore, the ELISA assay that utilizes antibodies specific for toxins A and B did not detect any toxin in all the 10 cytotoxic-positive samples. These results suggest that the tissue culture

cytotoxicity assay misdiagnosed 10 (16%) of the 60 samples as toxin-positive. This could have been a result of mishandling of the samples, absence of viable bacteria in the 10 samples, or misinterpretation of the initial cytotoxicity assay results. As a result, we agree with others (44, 70) who suggest that it is necessary to culture a toxin-producing *C. difficile* bacterium from the stool to confirm a diagnosis of an active infection or colonization.

It is important to note that whereas almost all Tox^+ colonies (99.8%) on the CDPA plates encoded the toxin genes, 74% of the Tox^- colonies also encoded the *tcdA* or *tcdB* genes in their genomes. This suggests that the genomes of some *C. difficile* strains may encode the toxin genes, but do not secrete detectable amount of toxins. It is possible that these active proteins are not produced as a consequence of mutations in the toxin coding sequences. It is also possible that these toxins are not produced due to alternations in the regulatory elements necessary for transcription, translation, or secretion. Alternatively, the bacterial cells may not have been exposed to the necessary conditions to activate toxin gene expression (82, 89, 90, 103, 149). Factors that have been suggested to influence toxin production are cell density, exposure to antibiotics, phage lysogeny, growth medium composition, and nutrient limitation (51, 82, 91, 182, 231). Furthermore, *C. difficile* cells in stool samples may exist as either vegetative cells at different growth stages or as spores. Perhaps, variations in cell physiology may explain why some colonies became Tox^+ later than others. In any case, our results indicate that this heterogeneity did not lead to false negative interpretations of any of the samples analyzed by the Cdifftox Plate assay.

To our knowledge, the use of the glucosyltransferase activities of the A and B toxins to identify toxin-producing *C. difficile* is unique and has not been reported in the literature or used commercially. The Cdifftox Plate assay represents a novel detection method with potentially improved sensitivity and efficiency compared to current diagnostic methods.

In Chapter 2, it was demonstrated that taurocholate inhibited the substrate cleavage of *C. difficile* toxins A and B. The implication of this finding was that taurocholate-mediated inhibition of these toxins may be exploited to treat *C. difficile* infections. Chapter 4 extends this finding by investigating whether taurocholate could protect colonic epithelial cells from the damaging effects of these toxins.

CHAPTER 4: BILE SALT INHIBITION OF HOST CELL DAMAGE BY *CLOSTRIDIUM DIFFICILE* TOXINS

INTRODUCTION

Primary bile salts (cholate and chenodeoxycholate) are biosynthesized from cholesterol in the liver and are conjugated with either glycine or taurine prior to their release into the gall bladder (15, 151, 181). Conjugation makes bile salts less hydrophobic, more soluble, and prevents passive re-absorption as they traverse the gastrointestinal tract (97). In addition to their role in fat digestion and absorption, bile salts also inhibit bacterial overgrowth in the small intestine (104, 204), a major site of absorption of nutrients and other metabolites. Various bacteria synthesize hydrolases that modify conjugated bile salts by deconjugation. Further alterations can occur through dehydroxylation, dehydrogenation, and sulfation, resulting in the generation of secondary and tertiary bile salts (109, 131, 181, 206). These bacterial modifications render bile salts essentially insoluble, resulting in decreased aqueous concentrations and bacteriostatic effects. Moreover, the release of amino acids as a result of these modifications may also act as alternative electron acceptors in this anaerobic environment (43, 102, 215), improving bacterial growth. Bile-salt hydrolases are produced by several genera of enteric bacteria including *Clostridium*, *Bifidobacterium, Bacteroides*, *Lactobacillus,* and *Enterococcus* (109). We suggest that modifications to bile salts have evolved to enable the intestinal microflora to gain survival advantage by counteracting this host defense mechanism.

A large number of *C. difficile* isolates have shown an alarming pattern of resistance to the majority of antibiotics currently used in hospitals and outpatient settings (6, 16, 164, 193). As a result of the dwindling number of antibiotics available to effectively clear this infection, there is renewed interest in exploring non-antibiotic treatment approaches to CDI, especially in cases of recurrent CDI. Non-antibiotic treatment options that have been reported include infusion of stools from healthy donors (1), adjunctive use of monoclonal antibodies specific to the toxins (134), probiotics (145), and the use of non-toxigenic *C. difficile* strains to out-compete the toxigenic strains. As the toxins play an essential role in *C. difficile* pathogenesis, inhibition of either toxin production or toxin activity is another promising approach.

Taurocholate, a major human conjugated bile salt, was previously reported by our laboratory to inhibit the *in vitro* substrate cleavage activity of the *C. difficile* toxins A and B in a dose-dependent manner (41). This Chapter demonstrates that physiologic concentration of taurocholate protects Caco-2 colonic epithelial cells in an *ex vivo* assay from the damaging effects of *C. difficile* toxin B. Furthermore, in a dose-dependent manner toxin B induces production of caspase-3, a protease that commits cells to apoptosis. However, taurocholate treatment of Caco-2 cells reduced caspase-3 induction in the presence of lethal concentrations of toxin B. These findings suggest that in addition to their established physiological role in metabolism, protection of the small intestine from microbial proliferation, and neutralization of deleterious microbial products, bile salts may be effective in protecting the gut epithelium from *C. difficile* toxin damage. It is possible that

delivery of physiologic amounts of taurocholate to the colon may be developed into a novel treatment approach for CDI.

MATERIALS AND METHODS

4.2.1 Bacterial Strains and Growth Conditions

C. difficile strains VPI 10463 [ATCC (American Type Culture Collection) 43255 (*tcdA*+ *tcdB*+)], 630 [ATCC BAA-1382 (*tcdA*+ *tcdB*+)], 5325 [ATCC BAA-1875 (*tcdA*+ *tcdB*+)], VPI 11186 [ATCC 700057 (*tcdA*- *tcdB*+)], and the hypervirulent strain ATCC BAA-1805 (*tcdA*+ *tcd*B+)], which all produce active toxins were purchased from the ATCC (Manassas, VA). Brain Heart Infusion (BHI) medium was purchased from Becton Dickinson (Cockeysville, MD). The bacteria were grown in either liquid culture in BHI medium or on Cdifftox Agar plates (40) and incubated anaerobically in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 at 37°C in a Controlled Atmosphere Anaerobic Chamber (PLAS LABS, Lansing, MI). Sodium taurocholate and sodium cholate were purchased from Sigma-Aldrich (St. Louis, MO). The *C. difficile* toxins were purified as described in Chapter 2.

4.2.2 Growth of *C. difficile* Strains in BHI Medium Containing Taurocholate

Overnight cultures (O.D. 600 nm= 1.4) of each strain of *C. difficile* were diluted 1:100 in 30 ml of fresh BHI broth medium in the presence or absence of 5 mM taurocholate and incubated anaerobically at 37^{o}C. Portions of the cultures were removed every 2 hrs to monitor cell growth by optical density measurements at 600 nm. After a 48 hrs incubation period, 1.5 ml of respective cultures were centrifuged

at 10,000 x g and the supernatants were tested for the presence of toxins A and B using the enzyme-linked immunosorbent (ELISA)-based Wampole *C. difficile* TOX A/B II assay (TechLab, Blacksburg, VA) assay. The culture supernatants were also tested for toxin activity using the Cdifftox activity assay (41). Briefly, 250 µl of the culture supernatants were incubated with 5 mM of p-Nitrophenyl-β-D-glucopyranoside at 37^{o}C for 4 hrs. The cleavage of the substrate by the toxins was measured spectrophometrically at 410 nm wavelength.

4.2.3 Growth and Maintenance of Caco-2 Cells

The Caco-2 cell line (ATCC HTB-37), a human colonic epithelial cell line was purchased from ATCC. The cells were cultured and maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum in a humidified incubator with 5% CO_2. No antibiotics were used in the preparation of the media. Cells were grown to confluence in 24-well culture plates (Corning, Corning, NY) in a final volume of 2 ml as described (24), prior to adding taurocholate in the presence or absence of either *C. difficile* toxin A or B. Sodium cholate was used as a control. Cells were visualized using light microscopy every 24 hrs over a 5-day period. Cells in the different treatment groups were evaluated for morphological changes including rounding, cytoskeleton disruption, and cell death resulting from exposure to *C. difficile* toxins using an EVIS XL microscope (Advanced Microscopy Group, Bothell, WA).

4.2.4 Capase-3 Activity Assay

The caspase-3 assay required that a total protein lysate of Caco-2 cells was prepared from harvested cells. First, the treated cells were removed from the 24-well plates using the tip of a 1-ml pipette to scrape the attached cells from the bottom of the wells. Then the cells were transferred to microcentrifuge tubes and centrifuged at 6,000 x g to separate the cells from the medium. Caspase-3 detection was performed as described by the manufacturer of the Caspase-3 Colorimetric Kit (Invitrogen, Carlsbad, CA). Briefly, the harvested cells were lysed on ice for 10 min using 50 µl of cell lysis buffer and centrifuged at 10,000 x g. For this assay, 75 µg of protein was incubated with the DEVD-NA substrate reagent for 8 hrs at 37^{o}C and the absorbance at 410 nm was measured using the Spectramax Plus spectrophotomer (Molecular Devices, Sunnyvale, CA). A molar extinction coefficient for *p*-nitrophenol of ε = 17700 $M^{-1}cm^{-1}$ was used for the calculations (192). Total lysate protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.).

4.2.5 Data Analysis

All the data were analyzed and plotted using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, California). The Student's *t*-test was used to compare differences between the samples. In all cases, statistical significance was defined as having a *P* value of <0.05.

RESULTS

4.3.1 Taurocholate and Toxin Titration Assays

The total concentration of bile salts in the human small bowel ranges from 2 mM to 30 mM, depending on diet and other metabolic conditions (159). To determine the amount of taurocholate that could be tolerated by the Caco-2 cells, cells were cultured in the presence of 1-25 mM taurocholate as described in the Materials and Methods. From this analysis, 5 mM of taurocholate was determined to be the optimum concentration tolerated based on cell viability (data not shown). Cells incubated with and without 5 mM taurocholate were indistinguishable over a 5-day period (Fig. 4.1, A-B). Caco-2 cells were not viable at taurocholate concentrations above 20 mM.

Confluent Caco-2 cells were also cultured in the presence of increasing amounts of each toxin (0, 4, 8, 12, 16, and 24 µg) to determine the amount needed to elicit visible cytopathic changes. Cytopathological effects were observed in cells cultured in the presence of toxin B within 24 hrs of incubation. The cells appeared rounded, spindle-like, detached from the plate surface, and presented with an altered cytoskeleton; all of these phenotypes were consistent with previous reports describing the cytopathic and cytotoxic effects of these toxins (69, 101). The addition of toxin A resulted in mild cytopathic effects that required longer incubation times of more than 48 hrs before cytopathic changes were observed (data not shown). These results confirmed that toxin B is more potent than toxin A (111, 217,

219). As a consequence, only toxin B was tested in the Caco-2 protection experiments described.

4.3.2 Taurocholate Protects Caco-2 Cells from Toxin B-Mediated Toxicity

Taurocholate was shown previously in our laboratory to inhibit the substrate cleavage activity of toxins A and B *in vitro* in a dose-dependent manner (41). To test whether taurocholate could protect Caco-2 cells from toxin B-mediated toxicity *ex vivo*, Caco-2 cells were incubated in the presence or absence of 5 mM taurocholate (Fig. 4.1, A-B). When lethal doses of toxin B (8 µg and 16 µg) were added to Caco-2 monolayers in the presence of taurocholate, no detectable cytopathic damage to Caco-2 cells was apparent (Fig. 4.1, C-F). These data revealed that taurocholate protected cells from toxin-mediated damage and supported our previous report demonstrating that taurocholate inhibits toxin B activity.

4.3.3 Taurocholate Decreases *C. difficile* Toxin B-Mediated Induction of Host Cell Caspase-3 Production

During *C. difficile* infection, apoptosis is an important downstream effect resulting from receptor-mediated toxin endocytosis into the host cell cytoplasm and subsequent inactivation of the host GTPases. Previous reports showed that toxin A induces cell death in human epithelial cells *ex vivo* via the activation of caspases (23, 29, 71).

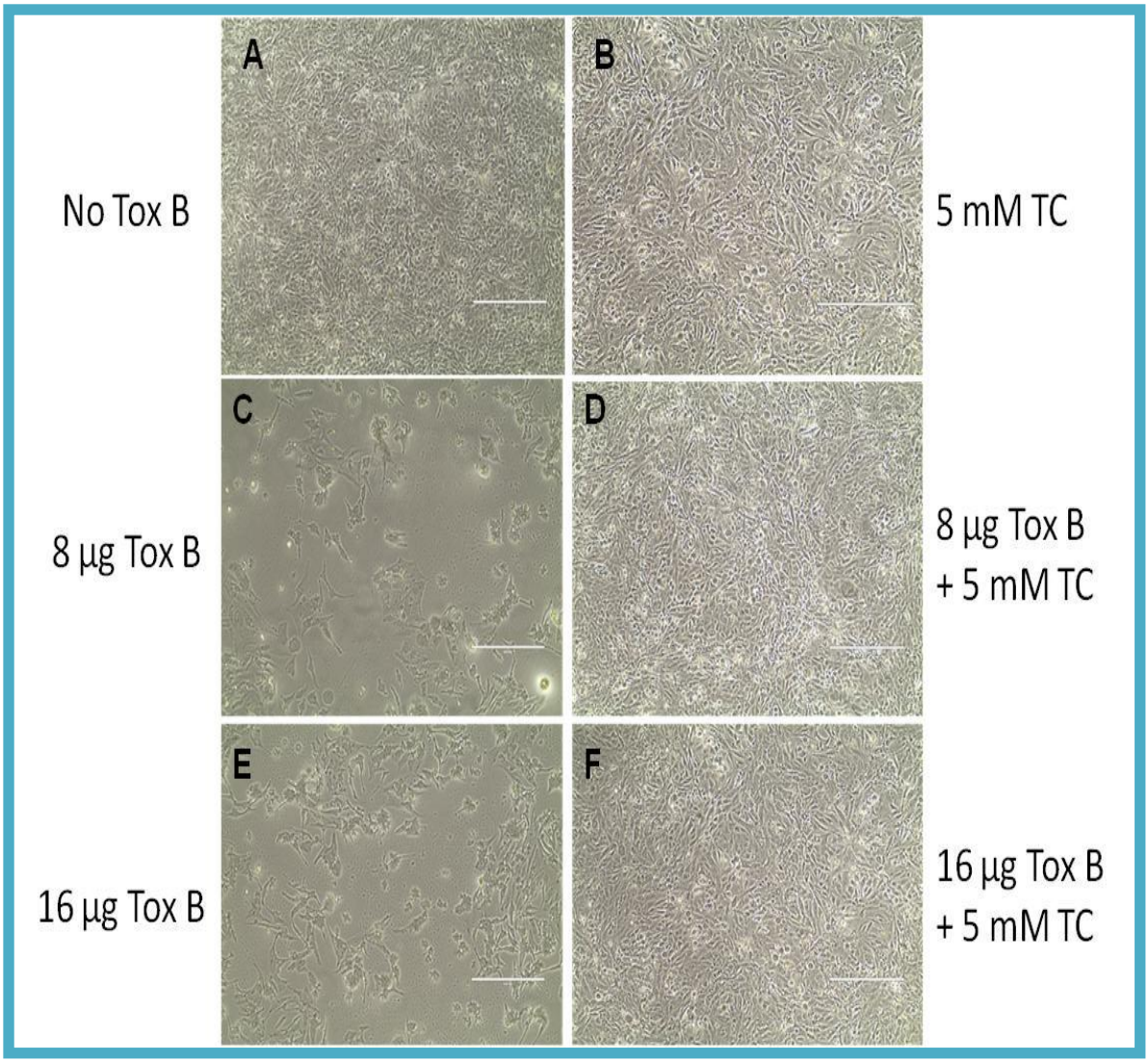

Figure 4.1: Effect of *C. difficile* toxin B and taurocholate on Caco-2 Cells. Confluent Caco-2 cell monolayers were incubated with 8 and 16 µg of toxin B in the presence or absence of 5 mM taurocholate in a total medium volume of 2 ml in 24-well plates for 24 hrs. Images were captured using an EVIS XL microscope. Magnification 10X. Tox B, purified toxin B; TC, taurocholate.

Caspase-3 plays a crucial role in the apoptotic pathway by catalyzing the specific cleavage of key apoptotic mediators essential to chromatin condensation, DNA fragmentation, dismantling of the cell, and formation of apoptotic bodies (127, 170, 234). Figure 4.2 show that various amounts (4, 8, 12, and 24 µg) of toxin B induced elevated levels of caspase-3 activity. To determine whether taurocholate protected Caco-2 cells from toxin B damage by preventing apoptosis, caspase-3 activity was assessed in taurocholate-treated and untreated cells. Crude protein lysates prepared from Caco-2 cells cultured with various amounts of toxin B in the presence or absence of taurocholate were tested for caspase-3 activity. No caspase-3 activity was detected either in the absence of toxin B or in cells cultured with 5 mM taurocholate as a sole additive. In the presence of toxin B, however, caspase-3 activity was detected in a dose-dependent manner (Fig. 4.2).

Cells treated with 4-24 µg of toxin B showed a 2.5-7.5-fold increase in caspase-3 activity. In contrast, Caco-2 cells cultured in the presence of toxin B and taurocholate had significantly ($p= 0.002$) reduced caspase-3 activity. The addition of 5 mM taurocholate reduced caspase-3 activity in cells treated with 4, 8, 12, and 24 µg of toxin B by 99%, 68%, 68%, and 54%, respectively. These data demonstrated that taurocholate protected Caco-2 cells from the damaging effects of toxin B as evidenced by the decreased levels of the pro-apoptotic protease, caspase-3.

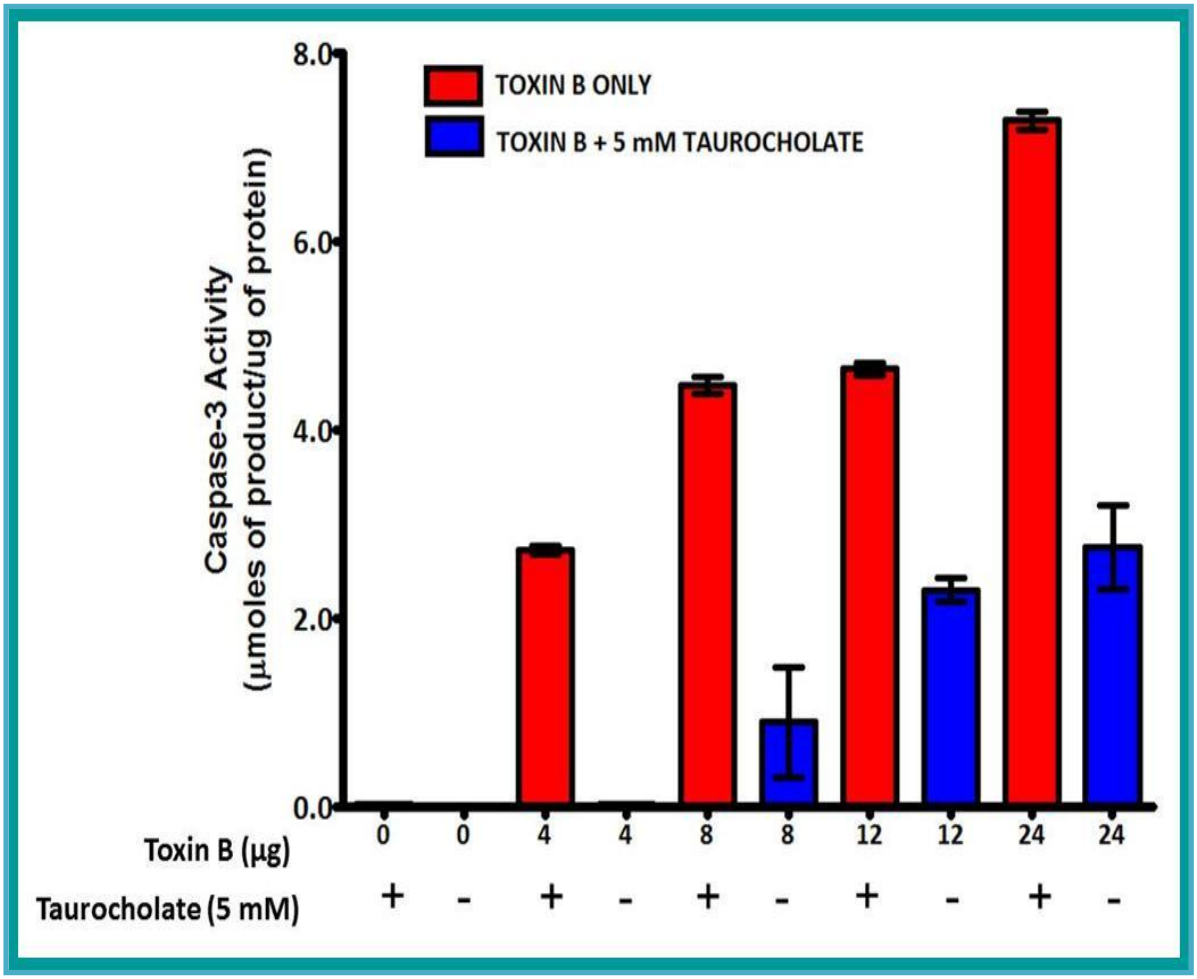

Figure 4.2: Effect of *C. difficile* toxin B and taurocholate on caspase-3 activity in Caco-2 cells. Caco-2 cells were incubated for 48 hrs with 0, 4, 8, 12 and 24 µg of toxin B in the presence or absence of 5 mM taurocholate. Cell monolayers were scraped from the bottoms of wells of a 24-well plate and lysed to obtain crude protein lysates. Crude protein lysates (75 µg) were incubated with the substrate reagent for 8 hrs at 37^{o}C and absorbance at 410 nm was measured. A molar extinction coefficient for *p*-nitrophenol of ε = 17700 $M^{-1}cm^{-1}$ was used in the calculations (192). The error bars represent the standard error from two different experiments.

4.3.4 Taurocholate Does Not Inhibit *C. difficile* Growth or Toxin Production

In addition to their role in fat digestion and absorption, bile salts also inhibit bacterial overgrowth in the small intestine (104, 204), which is a major site of nutrient and metabolite absorption. Interestingly, some enteric bacteria such as *E. coli* and *Salmonella* produce various bile salt hydrolases capable of modifying bile salts and rendering them non-toxic to bacteria. Analysis of the *C. difficile* strain 630 genome revealed the presence of a homologue of bile salt hydrolase similar to those characterized in classic enteric bacteria. To examine whether *C. difficile* could grow in the presence of the physiologic taurocholate concentration used, different toxigenic *C. difficile* strains were cultured in the presence or absence of 5 mM taurocholate. Although, some strains appeared to grow better than others in the presence of taurocholate, none of the strains tested were negatively affected (Fig. 4.3).

To assess the effect of taurocholate on toxin production and toxin activity, an ELISA-based assay was used to analyze toxin production whereas toxin activity was determined using the Cdifftox activity assay (41). As shown in Figure 4.4A, the presence of taurocholate did not affect toxin production. However, toxin activity was significantly lower ($P < 0.05$) for all the strains cultured in the presence of taurocholate compared to the activity observed in the absence of taurocholate (Fig. 4.4B).

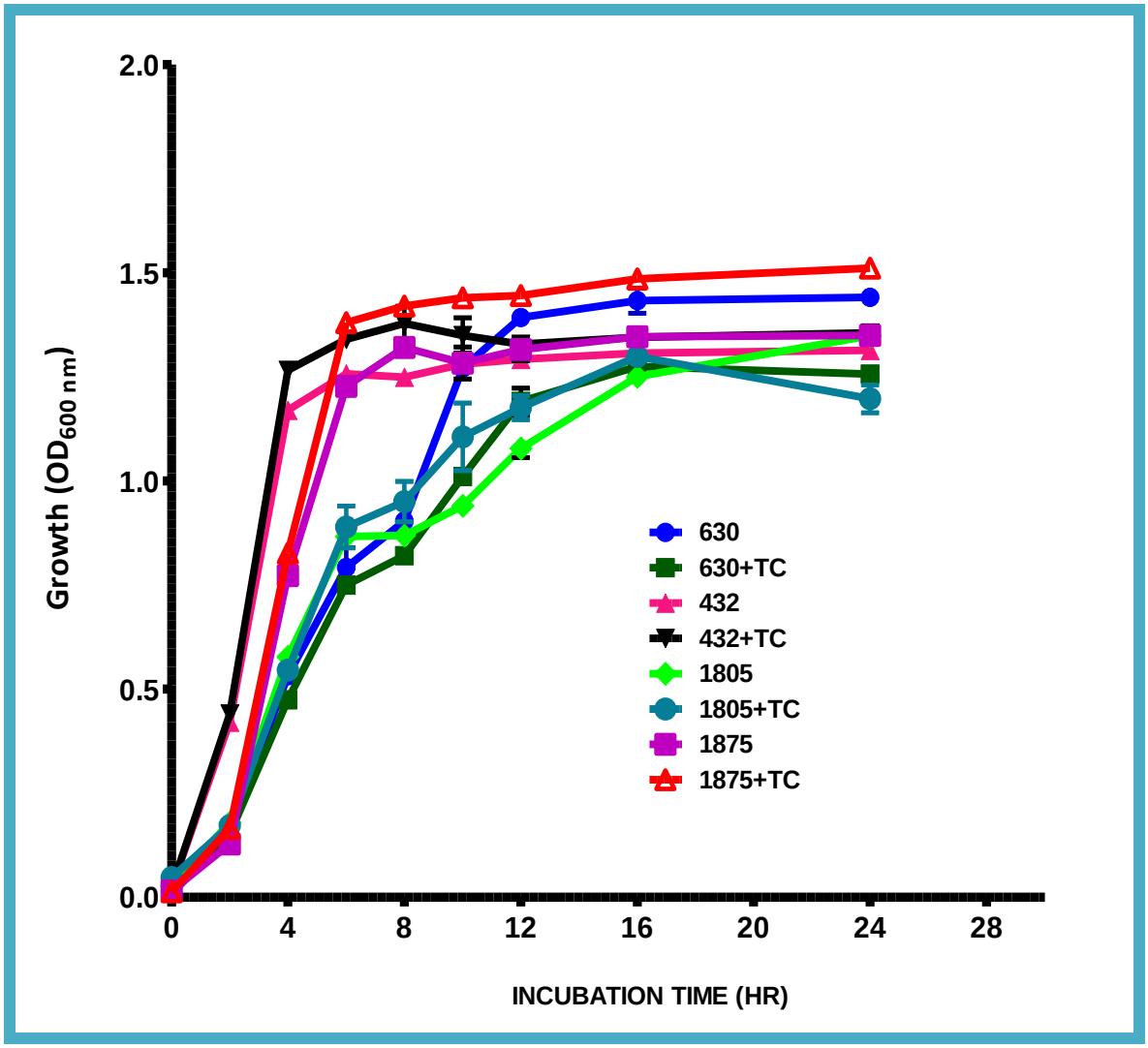

Figure 4.3: Growth of toxin A- and B-producing strains of *C. difficile* in the presence of sodium taurocholate. Overnight cultures (O.D. 600 nm = 1.4) of each strain of *C. difficile* tested were diluted 1:100 in 30 ml of fresh medium with or without 5 mM of taurocholate (TC) and incubated anaerobically at 37^{o}C for 48 hrs. Portions (2 ml) of the cultures were removed for optical density measurement at 600 nm. Strain designations: 630, ATCC BAA-1382; 432, ATCC 43255; 1805, ATCC BAA-1805; 1875, ATCC BAA-1875. The error bars represent the standard deviation from two different experiments.

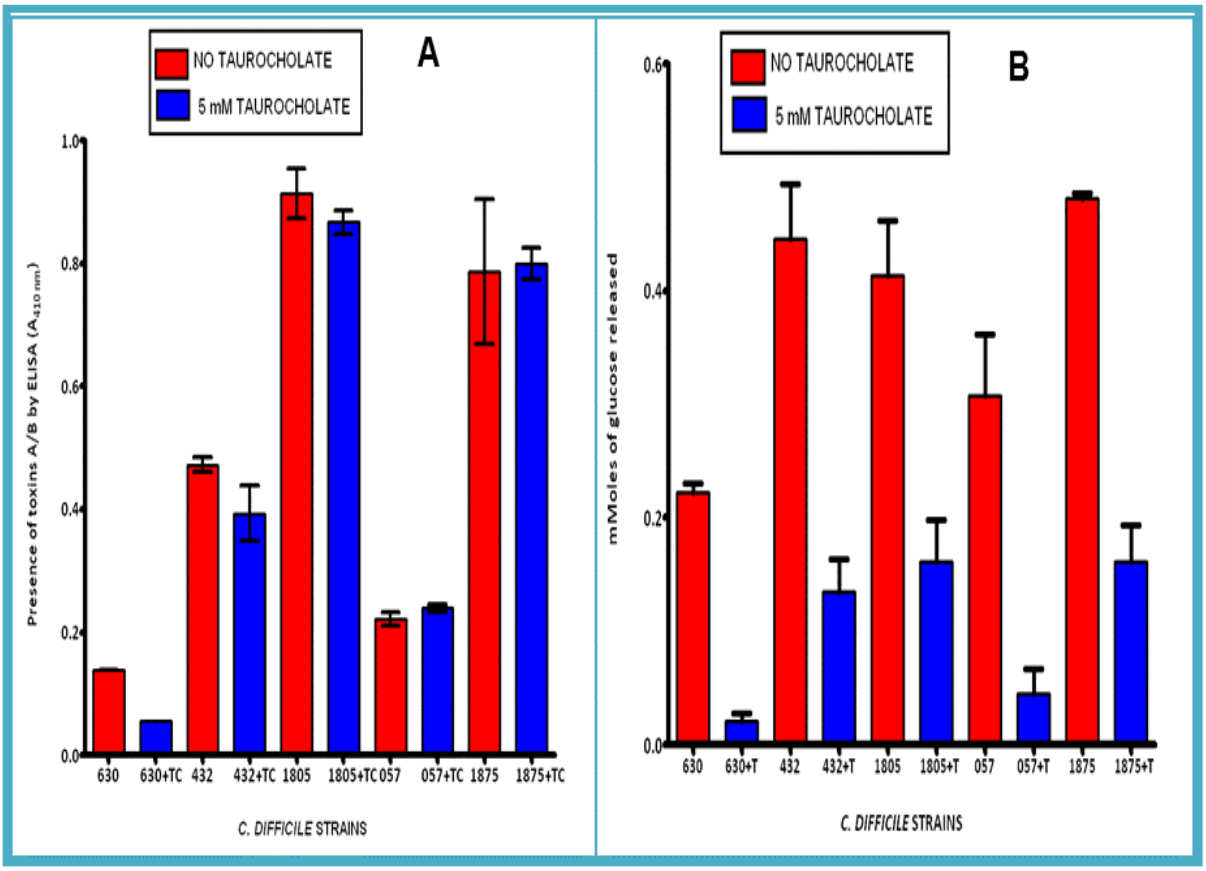

Figure 4.4: Effect of taurocholate on *C. difficile* toxin production (**A**) and activity (**B**). Overnight cultures (O.D. 600 nm = 1.4) of each strain of *C. difficile* tested were diluted 1:100 in 30 ml of fresh medium with or without 5 mM of taurocholate and incubated anaerobically at 37^{o}C for 48 hrs. The ELISA assay was performed using the Wampole *C. difficile* TOX A/B II assay to monitor toxin production. Toxin activity was tested using the Cdifftox activity assay (41). Strain designations: 630, ATCC BAA-1382; 432, ATCC 43255; 1805, ATCC BAA-1805; 057, ATCC 700057; 1875, ATCC BAA-1875. The error bars represent the standard deviation from two different experiments.

DISCUSSION

Treatment of CDI has been hampered by recurrent infections, multi-drug resistance, and the lack of drug concentration or functional activity in the colon to successfully treat CDI and prevent recurrence of the infection. As a result, there is renewed interest in finding alternative treatments, either as stand-alone therapies or therapies designed to augment the efficacy of currently used antibiotic regimens. An important potential treatment approach is to inhibit the activities of toxins A and B, which are directly responsible for intestinal damage and the subsequent inflammation associated with CDI. An approach that targets the toxins without affecting cell growth may be ideal since it is unlikely to impose selective pressure on *C. difficile*, thereby minimizing the risk of developing resistance. In our search for compounds that inhibit toxin activity, the Cdifftox assay was developed (41), which uses the ability of toxins A and B to cleave a chromogenic substrate that is stereochemically similar to their native substrate (UDP-glucose). Using this Cdifftox assay, taurocholate was identified as a compound that inhibits the substrate cleavage activity of these toxins (41).

In this Chapter, it was demonstrated that a physiologic concentration of taurocholate (5 mM) was able to protect human colonic epithelial Caco-2 cells from *C. difficile* toxin B-mediated damage. When taurocholate and toxin B (8 and 16 µg) were added simultaneously to confluent Caco-2 cell monolayers, toxin-mediated cytopathic effects were prevented (Fig. 4.1). One of the mechanisms by which *C. difficile* toxins mediate cell damage is by inducing apoptosis. Specifically, toxin A

has been reported to induce cell death in human epithelial cells *ex vivo* by activating caspases (23, 29, 71). The data presented demonstrate for the first time that toxin B increases caspase-3 activity in Caco-2 cells in a dose-dependent manner (Fig. 4.2). Moreover, the data presented established that taurocholate reduced caspase-3 activity in the presence of lethal toxin B concentrations.

The concentration of taurocholate used in this study did not affect either the growth or toxin production of *C. difficile* strains tested. However, it significantly decreased total toxin activity in all the strains tested. It is important to note that the lowest percent inhibition of toxin activity was observed in strains that produce high levels of the toxins, suggesting that more taurocholate may be necessary to neutralize the toxins.

The mechanism of taurocholate-mediated inhibition of *C. difficile* toxin activity remains to be determined. Brandes et al. (20) reported that tauroursodeoxycholic acid, a modified conjugated bile acid, affected the host cell by inducing phosphorylation of Rac1/Cdc42 that inhibited *C. difficile* toxin B-mediated monoglucosylation of this GTPase. Taurocholate may function through hydrophobic interactions to saturate the Caco-2 cell membranes, thereby inhibiting toxin entry and/or toxin activity. Other inhibitory mechanisms may involve direct effects of taurocholate on toxins such as alterations to toxin structure leading to loss of activity, or binding of taurocholate to the toxins leading to the prevention of entry into the host cell. Further research is on-going to identify the mechanism of taurocholate action.

One limitation of this *ex vivo* study was the inability to co-culture *C. difficile* with the Caco-2 cells in the presence of taurocholate; *C. difficile* does not grow under aerobic conditions, and Caco-2 cells do not grow under anaerobic conditions. Thus, an *in vivo C. difficile* animal model is required to provide more insight into these findings.

It is important to note that the majority of nutrient absorption in the gastrointestinal tract occurs in the small intestine where bile salts are at much higher concentrations compared to the colon. This difference in bile salts concentration is due to the reabsorption of more than 95% of the total human bile via the enterohepatic circulation in the ileum (55), which is directly proximal to the colon. Clearly, only a small amount of bile salts enter the colon where *C. difficile* most frequently colonizes. An intriguing explanation for the CDI pathology being mostly limited to the bile salt-deficient colon and the associated sparing of the bile salt-rich small intestine is that toxin activity may be inhibited in the small intestine by the high bile salt concentrations. Our data suggest that these toxins are active in the colon because of its low bile salt concentrations. These results highlight the point that the bile salt concentration represents a host-mediated mechanism that naturally protects the absorptive surfaces of the small intestine from deleterious microbial products produced by pathogens such as *C. difficile* and acts to inhibit bacterial growth. Moreover, the lack of bile salts in the small intestine in disease states (such as cirrhosis of the liver) may lead to bacterial overgrowth and result in a competition for the essential nutrients required for normal human growth and function. The therapeutic benefits of bile salts are well documented; they prevent hepatocyte

injury and cholestasis (93, 94, 172), drug-induced cholestasis (174), and endotoxin absorption (9, 80). We suggest that uncovering a mechanism to deliver higher concentrations of bile salts and/or their derivatives, perhaps in conjunction with antibiotics into the colon of individuals suffering from recurrent CDI may help protect the colon from the damaging effects of the *C. difficile* toxins and facilitate clearance of the pathogen. This line of research may result in a novel treatment of CDIs that can produce recurrent and chronic disease or death.

I have demonstrated in Chapters 2-4 that a more sensitive and specific *C. difficile* toxin assay that was required to perform the experiments necessary to confirm the hypothesis set forth in this study has been successfully developed. The next chapter describes how the new assay was utilized to provide insight into the regulation of the *C. difficile* toxins.

CHAPTER 5: QUORUM SIGNALING-MEDIATED REGULATION OF *CLOSTRIDIUM DIFFICILE* TOXIN SYNTHESIS

INTRODUCTION

The prevalence of *C. difficile* infections (CDI) has been increasing exponentially within the last decade, causing between 1-3.2 billion U.S. dollars annually in healthcare cost (124, 226). The morbidity and mortality resulting from *C. difficile*-associated diseases have also increased significantly as a result of changes in the virulence of the causative strains, antibiotic usage patterns, increases in the number of the elderly and immune-compromised patients, and the lack of proper infection control (143, 160, 177). Treatment of CDI is hampered by recurrence, multi-drug resistance, and the limited number of antibiotics with functional activity in the colon. In addition, a large number of *C. difficile* isolates are resistance to the majority of antibiotics currently used in hospitals and outpatient settings (6, 16, 164, 193). As a result of the dwindling number of antibiotics that are able to effectively clear this infection and prevent recurrence, there is a renewed interest in exploring a non-antibiotic treatment approach to CDI.

The range of disease symptoms caused by *C. difficile* is variable and the severity depends on the level of toxins produced (3). This indicates that toxin production is a critical and significant element in *C. difficile* pathogenesis. Therefore, the inhibition of toxin synthesis is a promising approach to combating CDI. Furthermore, targeting the regulatory mechanisms that control toxin production and other virulence factors that do not affect growth would be ideal. They are unlikely to generate a selective pressure on the bacterium and as a result, the risk of

developing resistance would be minimized. However, currently little is known about the mechanisms and factors that regulate *C. difficile* toxin synthesis.

Bacterial cell-to-cell communication has been shown to control the pathogenesis of a variety of bacteria by regulating virulence factor synthesis (31). Most forms of intercellular communication involve the extracellular generation of small soluble diffusible signals that accumulate in the environment and reach a critical threshold when the cell density reaches a specific level. This cell density-dependent regulation was first identified in *Vibrio fisheri* more than 30 years ago and is now termed quorum signaling (67). Bacterial quorum signaling regulates processes that require the co-operation of a population of bacterial cells in order to be effective; individual cells in the group benefit from the activity of the entire assembly (42, 148, 188). The Gram-negative bacteria produce various types of N-acyl-homoserine lactones that are synthesized by enzymes encoded by the *luxI* family of genes. Another signaling molecule designated as autoinducer-2 (AI-2), which is synthesized by enzymes encoded by the *luxS* family of genes, is involved in quorum signaling in both Gram-negative and Gram-positive bacteria (53, 216). AI-2 appears to mediate both intraspecies and interspecies communication in bacteria (189, 230). AI-2/*luxS*-mediated regulation is important in controlling different activities in a variety of bacterial species (230). These activities include biofilm production in *Streptococcus mutans*, *Salmonella enterica serovar typhimurium*, and *Vibrio cholera* (88, 147, 173, 232); motility in *Campylobacter jejuni*, enterohemorrhagic *Escherichia coli* (EHEC), and enteropathogenic *E. coli* (62, 75, 197); and expression of virulence

factors in *Aggregatibacter actinomycetemcomitans*, *EHEC*, *Porphyromonas gingivalis, V. cholerae*, and *Clostridium perfringens* (36, 66, 161, 196, 236).

Another Gram-positive-specific quorum signaling system, the accessory gene regulator (*agr*) system, has been shown to regulate virulence in *Staphylococcus aureus* and other Gram-positive bacteria. The *agr* quorum signaling system is specific to Gram-positive bacteria. It is transcribed as an operon and consists of 5 genes: *agrA, B, C, D*, and *RNAIII*. The *agrD* encodes an autoinducer pre-peptide, which is processed by the transmembrane protein AgrB, leading to release of the autoinducer peptide (AIP) into the extracellular *milieu*. The AgrC (sensor histidine kinase) protein senses the AIP, which activates its ATPase activity leading to phosphorylation of AgrA (the response regulator). Phosphorylated AgrA either binds to the promoter region of the *agr* operon to produce more of the *agr* system components or the RNAIII promoter to regulate the transcription of its target genes.

The genome of *C. difficile* encodes both the *luxS* gene and *agrB* and *agrD* genes of the *agr* system. However, neither of these quorum signaling systems has been unquestionably shown to be involved in toxin synthesis regulation. In *C. difficile*, toxin synthesis occurs during the stationary phase of growth, which also corresponds to high cell density. To elucidate the processes that govern the production of these toxins, the *C. difficile* stationary phase culture supernatant fluid was analyzed for the presence of factors or molecules that induce toxin production. This Chapter describes and discusses the results from the analyses of the stationary phase culture supernatant fluid, which was based on the hypothesis that the synthesis of *C. difficile* toxins A and B is cell density-dependent and involves a

signaling pathway that responds to extracellular cues for regulation. The goal was to understand the regulation of *C. difficile* toxins A and B synthesis, to enable the identification of the key players involved, with the hope of discovering potential novel genes, molecules, or pathways that could be targeted therapeutically for the prevention and treatment of *C. difficile* infections.

MATERIALS AND METHODS

5.2.1 Bacterial Strains and Growth Conditions

Toxigenic *C. difficile* strains ATCC BAA-1805 (*tcdA*+/*B*+; NAP1/027), ATCC BAA-1875 (*tcdA*+/*B*+, NAP7) and ATCC BAA-1382 (*tcdA*+/*B*+, strain 630) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The clinical isolates were kindly provided by Dr. Kevin Garey (University of Houston, Houston, Texas). These isolates are: R20291 (NAP1), CD196 (NAP1), BI-9 (NAP2), TL176 (NAP4), CF5 (NAP9), and Liv022 (NAP11). The strain BAA-1382 was used for all the experiments, unless stated, because no regulatory mutations, such as a *tcdC* deletion or a *tcdC* open reading frame shift have been reported in this strain. The bacterial cultures were grown in BBL Brain Heart Infusion (BHI) medium (Becton Dickinson and Company, Cockeysville, MD) or single colonies were isolated on the Cdifftox agar (40) plates. Cultures were incubated anaerobically in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 at 37°C in a Controlled Atmosphere Anaerobic Chamber (PLAS LABS, Lansing, MI). The BHI medium used in all the experiments was reduced by placing it in the anaerobic chamber for at least 4 hrs prior to use. The substrate for the Cdifftox activity assay (41), p-Nitrophenyl-β-D-glucopyranoside, was purchased from Biosynth International (Itasca, IL).

5.2.2 Sample Storage Conditions for the Bacterial Stocks

Bacterial stocks were stored short-term in chopped meat broth (BD Diagnostics, NJ) at room temperature or long-term in either 10% dimethyl sulfoxide or 15% glycerol stocks at -80°C.

5.2.3 Toxin Assay

C. difficile toxins A and B were detected in the culture supernatant fluid using the Cdifftox activity assay (41), as described in Chapter 2. Briefly, the culture was centrifuged for 15 min at 3200 x g at 4^{o}C, 240 µl of the supernatant fluid was incubated with 30 µl of Cdifftox substrate reagent containing 45 mM p-Nitrophenyl-β-D-glucopyranoside and incubated at 37^{o}C for 4 hrs. The assay was quantitated spectrophotometrically at an absorbance of 410 nm. A molar extinction coefficient for *p*-nitrophenol of ε = 17700 $M^{-1}cm^{-1}$ was used for the calculations (192).

5.2.4 *C. difficile* Quorum Signaling Bioassay

The *C. difficile* quorum signaling bioassay was performed by incubating low-density log-phase cells with culture supernatant fluid collected from stationary-phase cells. This assay was based on the premise that the presence of toxin-inducing activity in the stationary-phase culture supernatant should induce low-density log-phase cells to produce the toxins. These toxins are normally produced during the stationary phase of the growth cycle. Thus, log-phase cells when incubated with stationary phase supernatant fluid must behave as though they are in stationary-phase and produce the toxins. The scheme of how this assay was

performed is shown in Figure 5.1. Briefly, the culture supernatant fluid was collected at various growth phases: 4 hrs (log phase), 8 hrs (late-log phase to early-stationary phase), 16 hrs (mid-stationary phase) and 26 hrs (late-stationary phase). For the assay, 100 µl of washed low-density log-phase tester cells (prepared as described below) was added to a medium containing 3 ml of the stationary phase supernatant fluid (prepared as described below) and 2 ml of fresh reduced BHI medium. The medium was incubated for 4-8 hrs anaerobically at 37^{o}C. As a control, the low-density log-phase tester cells were added to 5 ml of fresh reduced BHI medium and incubated under the same conditions as the treated cells. At the end of the incubation period, the culture was centrifuged at 3200 x g for 15 min and the supernatant fluid was tested for the presence of the toxins using the Cdifftox activity assay (41). After the boiling experiment (described in section 5.2.7), all the subsequent experiments were performed using boiled supernatant fluid collected at 72 hrs instead of a 26-hr culture supernatant fluid.

5.2.5 Collection of Culture Supernatant Fluid for *C. difficile* Quorum Signaling Bioassay

An overnight culture (O.D. 600 nm of 1.5) was generated from a single colony of *C. difficile* strain 630. The culture was diluted 1:100 with fresh reduced BHI medium and incubated anaerobically at 37^{o}C. Aliquots of the culture were collected at 4 hrs (log phase), 8 hrs (late-log phase to early-stationary phase), 16 hrs (mid-stationary phase) and 26 hrs (late-stationary phase). The culture was centrifuged at 3200 x g for 20 min and the supernatant fluid was filtered using 0.2 µm SFCA filter (Corning

Incorporated, Corning, NY). The filtered supernatant fluid was stored at 4^{o}C until use.

5.2.6 Preparation of Tester *C. difficile* Cells for Quorum Signaling Bioassay

A single colony of *C. difficile* strain 630 was selected from a Cdifftox agar plate (40) and cultured in 10 ml of fresh BHI medium overnight (14-16 hrs). The overnight culture (O.D. 600 nm = 1.4-1.5) was centrifuged at 3200 x g for 20 min and washed twice with fresh reduced BHI medium. The washed culture was diluted 1:100 with fresh BHI medium and incubated for 4 hrs (mid-log phase). For the quorum signaling experiment, 100 µl or 1:50 dilution of the tester cells was used.

5.2.7 The Effect of Boiling of the Stationary Phase Culture Supernatant Fluid on *C. difficile* Toxin Synthesis

Culture supernatant fluid (45 ml) was collected from the late-stationary phase (26 hrs) and prepared as described above. The supernatant fluid was boiled for 20 min and allowed to cool at room temperature. The boiled supernatant fluid was centrifuged at 3200 x g for 20 min and sterilized with a 0.2 µm SFCA filter. For the bioassay, the boiled supernatant fluid (3 ml) was diluted with 2 ml of fresh reduced BHI medium and added to 100 µl of washed low-density log-phase tester cells. The culture was incubated for 8 hrs anaerobically at 37^{o}C and the supernatant fluid was tested for toxin-inducing activity.

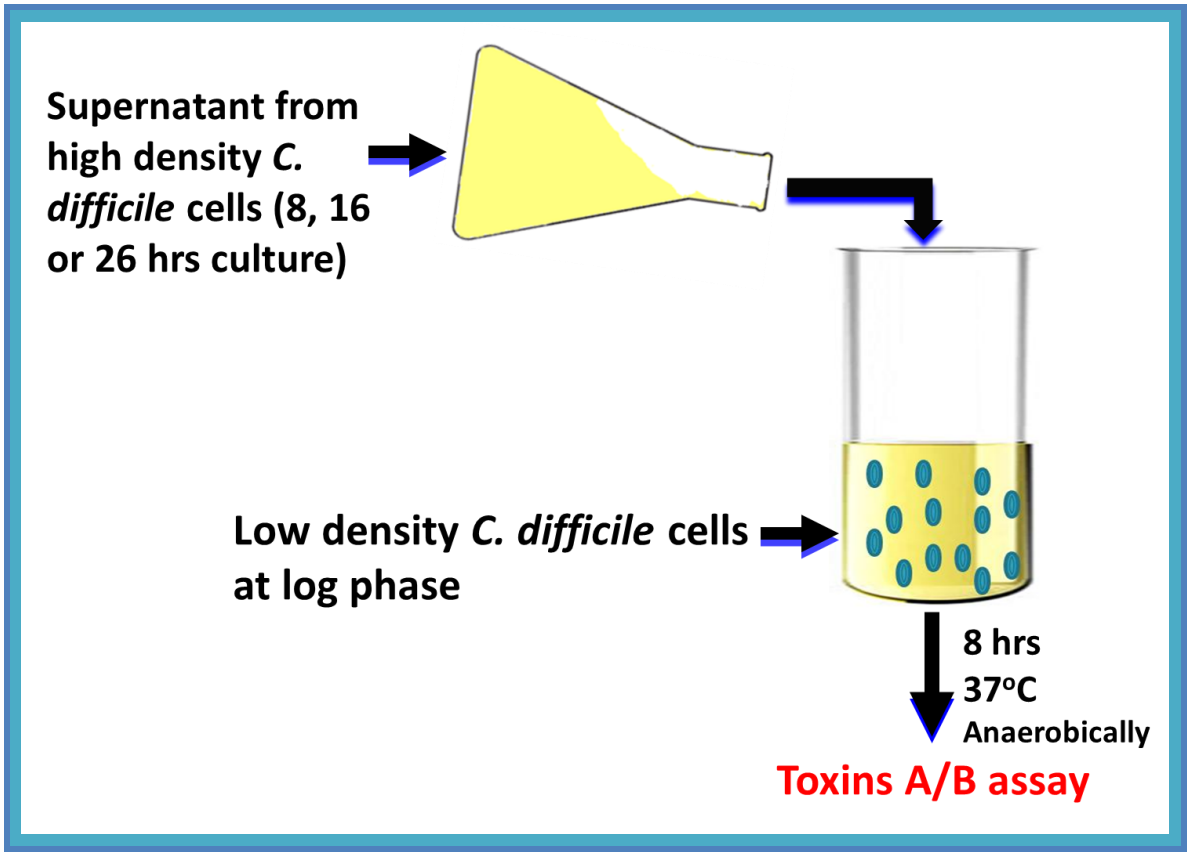

Figure 5.1: The *C. difficile* quorum signaling bioassay. Low density *C. difficile* cells at log phase (tester cells) were suspended in stationary phase culture supernatant fluid and incubated for 4-8 hrs anaerobically at 37^{o}C. The culture supernatant fluid was tested for the presence of toxins using the Cdifftox activity assay.

5.2.8 Dialysis of the Stationary Phase Culture Supernatant Fluid

Culture supernatant fluid collected after 72 hrs of incubation and boiled as described above, was dialyzed in fresh BHI medium for 18 hrs. The culture supernatant fluid was placed into a seamless cellulose dialysis membrane (Sigma-Aldrich, St. Louis, MO) with different molecular weight cut-off values (1-, 3.5-, and 12- kDa MWCO). The dialysis membranes containing the sample (15 ml) were placed in a flask containing 500 ml of fresh BHI medium and incubated at 4^{o}C on a magnetic stirrer. The fluids in the dialysis membranes were tested for toxin-inducing activity using the quorum signaling bioassay. The bioassay was performed by adding low-density log-phase cells (100 µl) to 1 ml of the fluid in the dialysis membranes and 4 ml of fresh reduced BHI medium. The culture was incubated for 4 hrs anaerobically at 37^{o}C and tested for toxin-inducing activity.

5.2.9 Effect of Cell Density on Induction of *C. difficile* Toxin Synthesis

An artificially high cell density was created by culturing the cells in a dialysis membrane with a 12 kDa MWCO. This experiment was set up in three flasks as shown in Figure 5.2. Flask 1 contained 220 ml of fresh reduced BHI medium and 10 ml of low-density log-phase cells at O.D. 600 nm of 0.05. Flask 2 contained 220 ml of fresh reduced BHI medium and a 12-kDa MWCO dialysis membrane containing 10 ml of low-density log-phase cells at O.D. 600 nm of 0.05. Flask 3 was similar to Flask 2, but the BHI medium was removed and replaced with fresh medium after every 4 hrs. The three flasks were incubated for 8 hrs anaerobically at 37^{o}C.

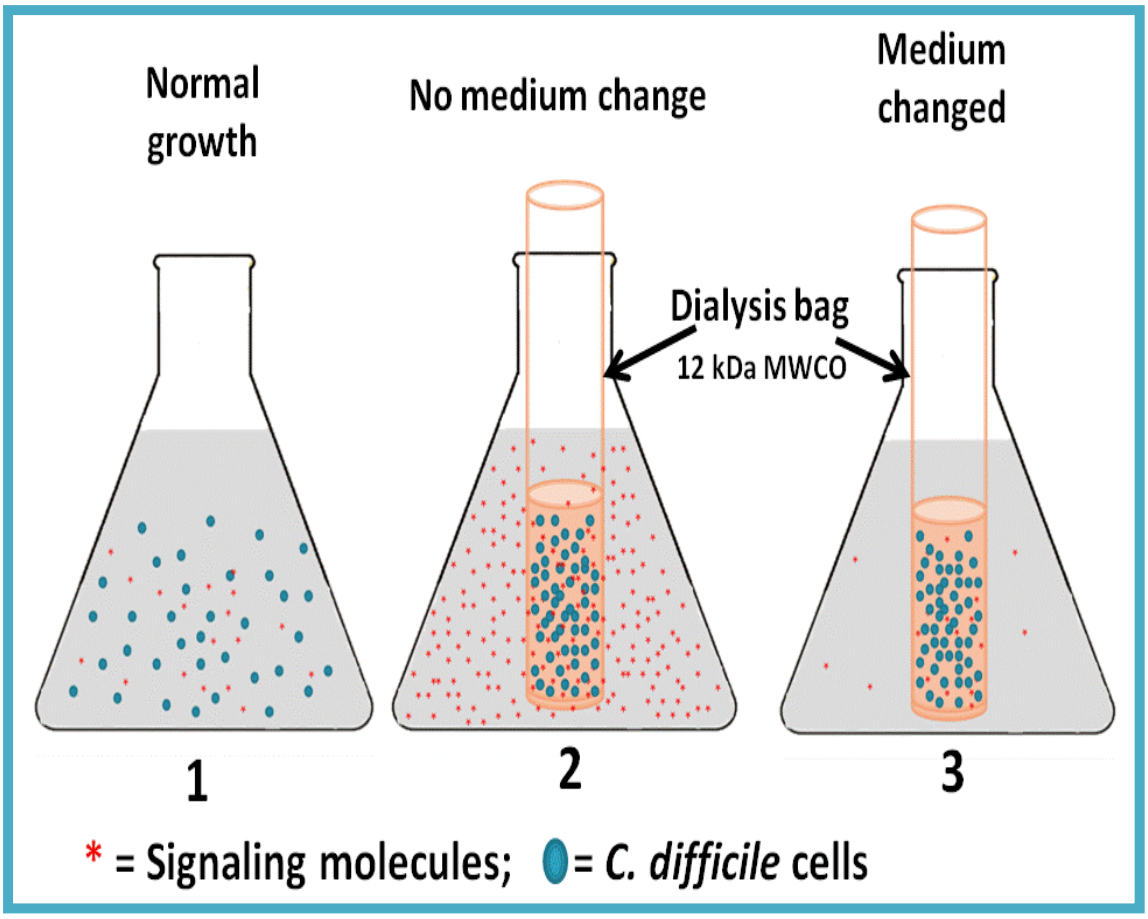

Figure 5.2: Effect of cell density on induction of toxin synthesis in *C. difficile*. An artificially high cell density was created by growing the cells in 12-kDa MWCO dialysis membranes. In Flask 1, cells were cultured as normally performed using 10 ml of low-density log-phase cells. In Flasks 2 and 3, the same amount of cells as in Flask 1 were added to dialysis membranes and placed in a flask containing fresh reduced BHI medium. The BHI medium in Flask 3 was removed and replaced with fresh BHI medium after 4 hrs whereas, the medium in Flask 2 remained unchanged. Culture supernatant fluid in the dialysis membranes and Flask 1 were tested for toxin activity using the Cdifftox assay.

Culture supernatant fluids in the dialysis membranes (for Flasks 2 and 3) as well as the culture in Flask 1 were tested for the toxin activity after the incubation period.

5.2.10 Effect of Known Autoinducers on *C. difficile* Toxin Synthesis

Autoinducers from the LuxS and *agr* quorum signaling systems were tested to evaluate their effect on *C. difficile* toxin synthesis. For the LuxS autoinducer, 4, 5-Dihydroxy-2, 3-pentanedione (DPD) was purchased from OMM Scientific (Dallas, Texas). The predicted putative *C. difficile agr* autoinducer cyclic peptide (NSTCPWII) was synthesized and kindly provided by Dr. Weng Chan and Dr. Paul Williams (Centre for Biomolecular Sciences, University of Nottingham, U.K.). Different concentrations of DPD (1 nM, 10 nM, 100 nM, 1µM, 10 µM, 50 µM, and 200 µM) and 10-50 µg/ml of the cyclic peptide NSTCPWII were incubated with low-density log-phase tester cells for 8 hrs and the culture supernatant fluid was tested for toxin activity.

5.2.11 Multiple Sequence Alignment of the *agrD* Gene in Gram-Positive Bacteria

To compare the similarities of the *agrD* gene sequence among Gram-positive bacteria, the amino acid sequences of this gene from various Gram-positive bacteria were analyzed using multiple sequence alignment. This analysis was performed using BioEdit (Ibis, Biosciences, Carlsbad, CA). The sequences from following bacteria were analyzed: *Clostridium difficile, Clostridium botulinum, Clostridium sporogenes, Eubacterium ventriosum, Eubacterium rectale,*

Staphylococcus epidermidis, Staphylococcus simiae, Staphylococcus intermedius, and *Staphylococcus aureus.*

5.2.12 Purification of the *C. difficile* Toxin-Inducing Activity From Stationary-Phase Culture Supernatant Fluid

The toxin-inducing activity in the stationary-phase supernatant was purified from boiled supernatant using the purification scheme outlined in Figure 5.3. Briefly, a 48-hr culture (500 ml) from *C. difficile* strain 630 was prepared as described above. The supernatant was boiled for 20 min to inactivate the toxins. The sample was cooled, and centrifuged for 20 min at 3200 x g to remove the denatured toxins and other high molecular weight proteins. The clear supernatant fluid was precipitated in 60% ice-cold acetone and incubated overnight at -20^{o}C. The precipitate was centrifuged at 3200 x g for 10 mins and the pellet was carefully washed with milliQ water. The washed pellet was re-suspended in 20 ml of milliQ water and incubated at 4^{o}C overnight. A white crystal-like precipitate was observed at the bottom of the tube after the overnight incubation (Fig. 5.3). The top liquid portion was decanted and the crystal-like material (CRYS) was re-dissolved in milliQ water and sterilized with a 0.2 µm SFCA filter. To test for the presence of toxin-inducing activity in CRYS, low-density log-phase tester cells (100 µl) were added to fresh reduced BHI medium (5 ml) containing 200 µl of CRYS and incubated anaerobically for 4 hrs at 37^{o}C. The culture supernatant fluid was tested for toxin activity. To evaluate whether CRYS could induce toxin synthesis in a dose-dependent manner, different amounts of CRYS (100, 200, 300, and 400 µl) were tested as described above.

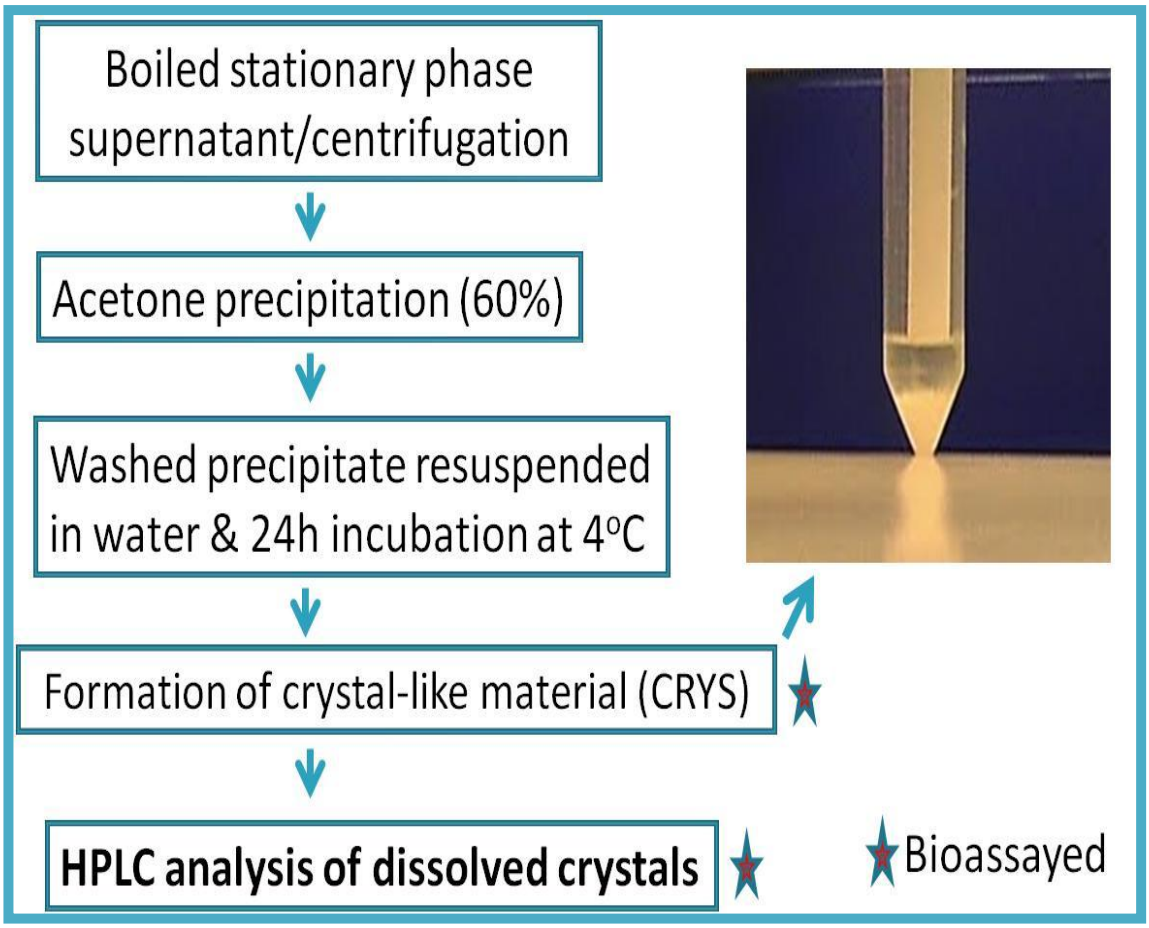

Figure 5.3: Scheme for the purification of *C. difficile* toxin-inducing activity from stationary-phase culture supernatant fluid. Culture supernatant was boiled and centrifuged to inactivate the toxins and to eliminate high molecular weight proteins. The supernatant fluid was precipitated in 60% ice-cold acetone overnight. The precipitate was washed with water, re-suspended, and incubated at 4^{o}C overnight. Crystal-like material that formed after the overnight incubation was re-dissolved in water and tested for toxin-inducing activity, followed by high performance liquid chromatography (HPLC) purification.

5.2.13 Effect of the Partially Purified Toxin-Inducing Activity (CRYS) on Growth of *C. difficile* and Transcription of the *tcdA* and *tcdC* Genes

Tester *C. difficile* strain 630 cells at O.D. 600 nm of 0.6 were diluted 1:100 in fresh reduced BHI medium (35 ml) containing 2 ml of the partially purified toxin-inducing material (CRYS) and incubated anaerobically at 37^{o}C for 16 hrs. As negative control, the tester cells were cultured in fresh BHI medium without CRYS. Aliquots of the culture were taken every 2 hrs for a measurement of growth at O.D. 600 nm, toxin testing, and mRNA analysis.

5.2.14 Analysis of *tcdA* and *tcdC* Transcription

Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's directions. The mRNA was converted to cDNA by reverse transcription using ProtoScript AMV First Strand cDNA Synthesis kit (New England bioLabs, Ipswich, MA) according to the manufacturer's instructions. For reverse transcription, 1 µg of total RNA was used. The primers used for the PCR were: *tcdA*: F-5-TGCCAGAAGCTCGCTCCACA3', R-5'TGCACTTGCTTGATCAAAGCTCCA3'; *tcdC*: F-5'GAGCACAAAGGGTATTGCT-CTACTGGC3', R-5'CCAGACACAGCTAATCTTATTTGC-ACCT3'. The PCR was performed using OneTaq HS Quick-Load 2X master mix (New England BioLabs) with the following conditions: initial denaturation of 94^{o}C for 30 sec and 36 cycles of 94^{o}C for 30 sec, 56^{o}C for 30 sec, and 68^{o}C for 30 sec. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

5.2.15 Effect of the Partially Purified Toxin-Inducing Activity on Toxin Synthesis in High Density Cells

To gain insight into the sensitivity of cells to the presence of the toxin-inducing activity, tester cells at different cell densities were evaluated with the bioassay. Tester cells were grown to O.D. 600 nm of 1.5 and washed twice with fresh reduced BHI medium. A 100 µl (1X), 500 µl (5X) and 1000 µl (10X) of the tester cells were added to fresh reduced BHI (5 ml) containing 200 µl of CRYS. The culture was incubated for 90 min anaerobically at 37^{o}C and the supernatant fluid was tested for toxin activity.

5.2.16 Effect of the Partially Purified Toxin-Inducing Activity on Epidemic Strains of *C. difficile*

The effect CRYS on toxin synthesis in clinical epidemic hypervirulent strains of *C. difficile* was evaluated. The following strains were tested: ATCC BAA-1832 (non-epidemic historical strain), ATCC BAA-1805 (NAP1/027), R20291 (NAP1), CD196 (NAP1), BI-9 (NAP2), TL176 (NAP4), ATCC BAA-1875 (NAP7), CF5 (NAP9), and Liv022 (NAP11). The standard quorum signaling bioassay was performed on these strains, as previously described. The tester cells (100 µl) were added to fresh reduced BHI medium (5 ml) containing 200 µl of CRYS and incubated anaerobically for 4 hrs at 37^{o}C. For a negative control, the tester cells were incubated in fresh reduced BHI without CRYS. Culture supernatant fluid was tested for toxin activity at the end of the 4-hr incubation period using the Cdifftox activity assay.

5.2.17 Purification of the Partially Purified Toxin-Inducing Activity by High Performance Liquid Chromatography

CRYS was further purified by high performance liquid chromatography (HPLC) using a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Columbia, MD) with a Phenomenex Jupiter 4µ Proteo 90A (250 mm x 4.6 mm) C18 column (Phenomenex, Torrance, CA). CRYS was diluted 5-fold with HPLC-grade water and 100 µl was injected onto the column. The purification was performed with HPLC-grade water as buffer A and acetonitrile as buffer B. A gradient of acetonitrile from 0-95% in 15 min at a flow rate of 0.5 ml/min was used. Fractions (0.5 ml) were collected and dried using a SPD111 speedvac (Thermo Scientific). To identify fractions that contained the toxin-inducing activity, the dried fractions were re-suspended in 200 µl of milliQ water and 100 µl was added to 3 ml of fresh reduced BHI medium containing 50 µl of the tester *C. difficile* strain 630 cells. The culture was incubated for 4 hrs anaerobically at 37^{o}C and the supernatant fluid was tested for toxin activity.

5.2.18 Mass Spectrometry Analysis

The fraction (Fraction #6, which eluted at 5.2 min) from the HPLC purification that induced toxin synthesis in the tester cells was analyzed by MALDI-ToF mass spectrometry by the Rice University Shared Equipment Facility (Rice University, Houston, TX) and the Institute for Biomolecular Design (University of Alberta, Canada).

5.3.1 *C. difficile* Stationary Phase Culture Supernatant Fluid Induces Toxin Synthesis in Low-Density Log-Phase Cells

Toxin synthesis in *C. difficile* occurs during the stationary-phase of growth. The toxins were detected in the culture supernatant fluid by the Cdifftox activity assay between 14-16 hrs during normal liquid growth. To test whether the stationary-phase culture supernatant fluid contained an activity that could induce toxin synthesis, supernatant fluids were collected at 4 hrs (log phase), 8 hrs (late-log phase to early-stationary phase), 16 hrs (mid-stationary phase) and 26 hrs (late-stationary phase) from *C. difficile* strain 630 during normal liquid growth. These supernatant fluids were added to uninduced low-density log-phase cells (tester cells), incubated for 8 hrs and the resulting supernatant was tested for induction of toxin activity. Under these conditions, no toxin activity was detected in the culture supernatant fluid when the tester cells were incubated with either fresh reduced BHI medium or 4-hr culture supernatant fluid (Fig. 5.4). In contrast, toxin activity was detected in the culture supernatant fluid when the tester cells were incubated with spent supernatant fluids collected from 8, 16, and 26 hrs cultures. These data suggest that the stationary-phase culture supernatant fluid contained an activity that induced toxin synthesis.

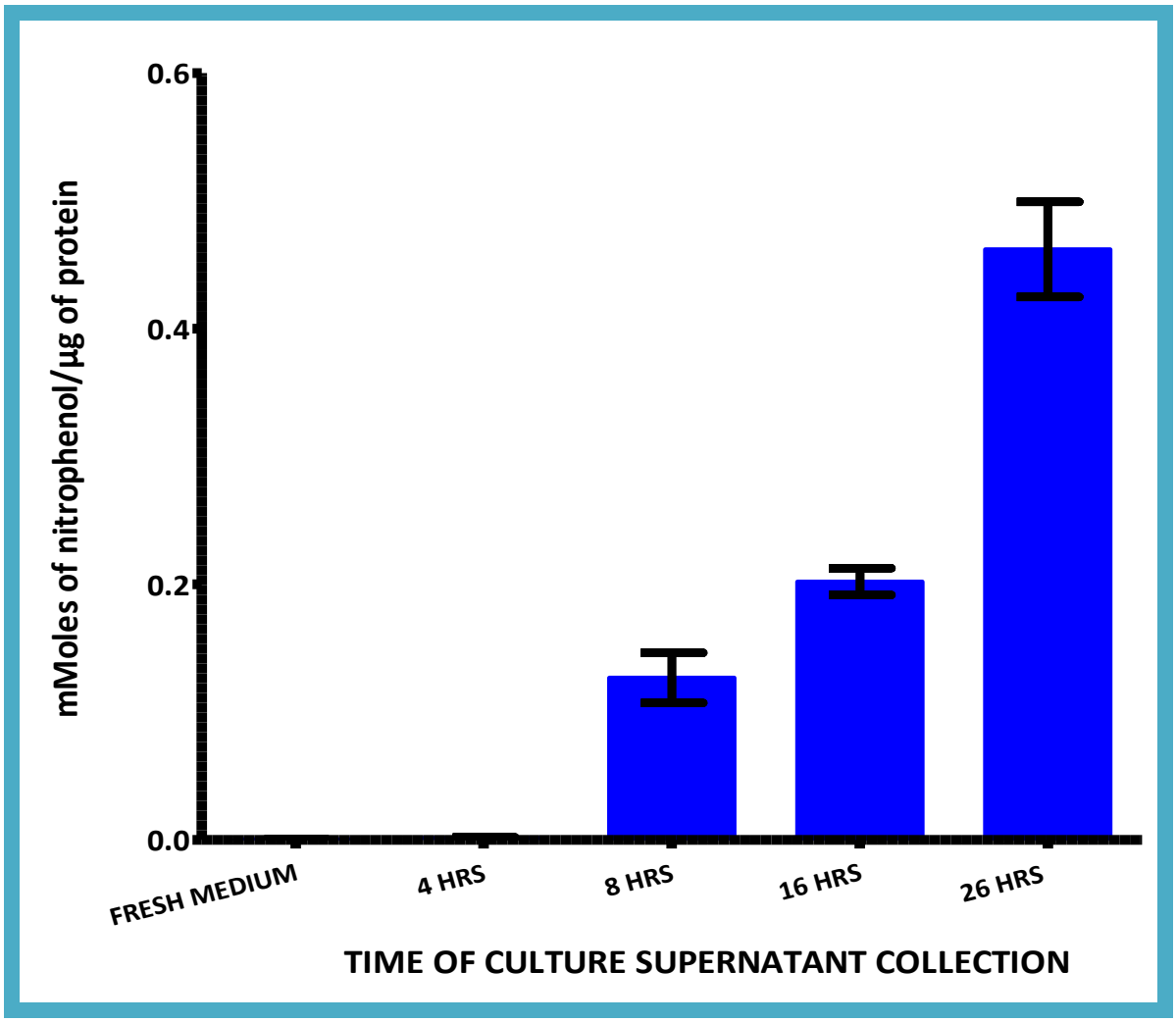

Figure 5.4: The effect of *C. difficile* stationary-phase spent culture supernatant fluid on toxin synthesis. Log-phase low-density cells were incubated for 8 hrs anaerobically at 37^{o}C with spent culture supernatant fluid collected from the exponential phase (4 hrs), early-stationary phase (8 hrs), mid-stationary phase (16 hrs), and late-stationary phase (26 hrs). The resulting culture supernatant fluid was tested for toxin activity using the Cdifftox activity assay.

During bacterial growth, cell density increases with incubation after the initial lag phase through the exponential phase and into the stationary phase. During stationary phase, the cell density remains relatively constant. At this stage, equilibrium is reached between the rate of cell division and the rate of cell death. This equilibrium is eliminated when the growth medium cannot sustain the burgeoning cell density. At late-stationary phase, the essential nutrients in the medium become limited, metabolic products and metabolic wastes build up, cell division slows down, and more bacterial cells die. The stationary phase in *C. difficile* is unusually long, lasting approximately 30 hrs (Fig. 2.10); it is during this time that the toxins are produced. Our data reveal that the 4-hr exponential phase culture supernatant collected from cells at low density did not induce toxin synthesis. Furthermore, there was an increase in the stimulation of toxin activity with spent supernatant fluids that were from later stages of growth (Fig. 5.4). These results imply that the culture supernatant fluid collected at late-stationary phase contained a greater amount of the toxin-inducing activity than the supernatant fluid collected from the early stationary phase. Moreover, the cell density at 26 hrs was higher than the cell density at 8 hrs. These results suggest that the production of toxin-inducing activity was cell density-dependent and accumulated during *C. difficile* growth.

5.3.2 Characterization of the Toxin-Inducing Activity in *C. difficile* Stationary-Phase Culture Supernatant Fluid

To assess the nature and stability of the toxin-inducing activity, the late-stationary-phase culture supernatant fluid was boiled for 20 min and tested using

the quorum signaling bioassay for induction of toxin synthesis. Boiling the spent culture supernatant fluid reduced the toxin-inducing activity by only 25% (Fig. 5.5A). This result demonstrates that the majority of the toxin-inducing activity is heat stable. As a result, all the subsequent experiments were performed using boiled supernatant fluid collected at 72 hrs instead of a 26-hr culture supernatant fluid. The boiling of the supernatant fluid inactivated the toxins present and thus, eliminated the effect of “carry-over toxins” in the subsequent assays. Moreover, using a 72-hr culture supernatant fluid reduced the incubation time required for induction of toxin synthesis from 8 hrs to 4 hrs, because there was a greater amount of the toxin-inducing activity present in the 72-hr spent supernatant fluid.

To establish the putative size of the toxin-inducing activity, the culture supernatant fluid was dialyzed with membranes of different molecular weight cut-off sizes. This was performed by suspending the dialysis membranes containing the supernatant fluid in fresh BHI medium overnight at 4^{o}C and testing for either retention or loss of the toxin-inducing activity. Induction of toxin synthesis was lost when the spent culture supernatant fluid was dialyzed with all of the dialysis membranes (1, 3.5, 12 kDa MWCO). These data suggest that a small molecule that is probably less than 1 kDa is responsible for the toxin-inducing activity.

All together, these results established that during growth *C. difficile* generates a small (< 1 kDa), heat-stable, toxin-inducing factor into the extracellular *milieu* that accumulates and subsequently activates toxin synthesis.

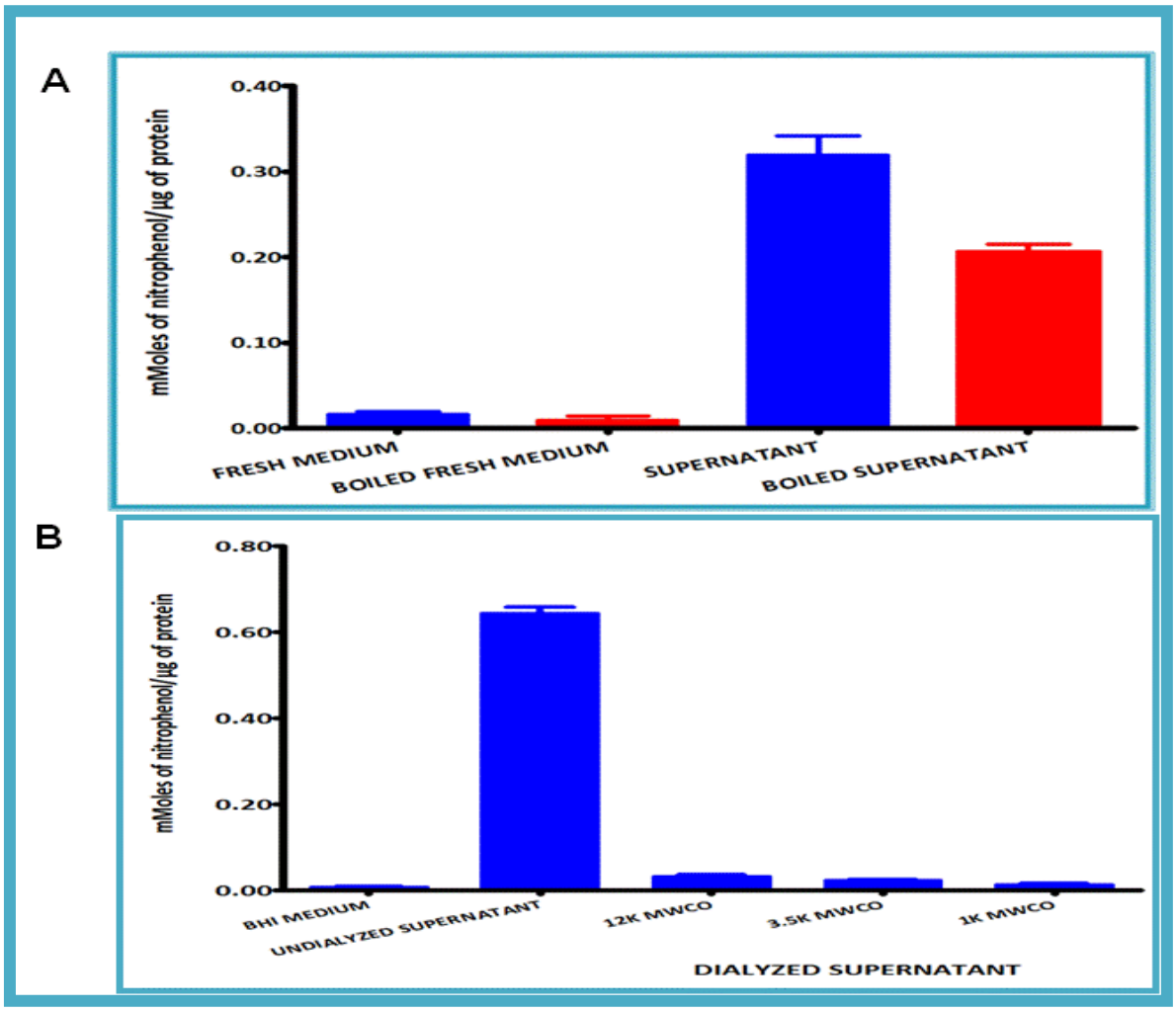

Figure 5.5: Toxin-inducing activity in boiled (**A**) and dialyzed (**B**) stationary-phase spent culture supernatant fluid. **A**, Cdifftox Activity assay was performed on culture supernatant collected from low-density log-phase tester cells incubated with fresh BHI medium, boiled, and unboiled 26-hr culture supernatant fluid and incubated for 8 hrs anaerobically at 37^{o}C. **B**, Cdifftox Activity assay was performed on culture supernatant fluid collected from log-phase low-density tester cells incubated for 4 hrs anaerobically at 37^{o}C with boiled 72-hr spent culture supernatant fluid dialyzed with membranes of various molecular weight cut-offs (12 KDa, 3.5 KDa, 1 KDa).

5.3.3 Artificially-Created High Cell Density Induces Premature Toxin Synthesis in *C. difficile*

The stationary phase at which *C. difficile* produces the toxins corresponds to high cell density. It was postulated that accumulation of the toxin-inducing activity present in the spent stationary-phase supernatant increases with cell density. To investigate this hypothesis, the *C. difficile* tester cells were cultured in a 12-kDa MWCO dialysis membrane. The membrane was placed in a flask containing fresh reduced BHI medium and incubated anaerobically for 8 hrs at 37^{o}C. The culture medium surrounding the dialysis membrane was either changed or remained unchanged during the incubation period. Under this condition, the toxins were retained in the dialysis membrane due to their large size (toxin A [308 kDa] and toxin B [269 kDa]), whereas the small toxin-inducing activity diffused out of the membrane into the surrounding BHI medium. The optical density of the cells that were cultured in the dialysis membrane was approximately 3 times higher than cells cultured normally in a flask. The resulting supernatant fluid in the dialysis membrane was tested for the toxin activity. Under this condition, the tester cells that were grown normally in a flask for 8 hrs were not induced and as expected, no toxin activity was detected (Fig. 5.6). Toxin synthesis was induced in the tester cells that were cultured in the 12-kDa dialysis membrane in which, the medium was not changed. However, no toxin activity was detected by the tester cells that were cultured in the 12-kDa dialysis membrane, in which the spent growth medium was removed and replaced with fresh medium, despite the high cell density. Changing the spent growth medium is expected to have removed the toxin-inducing activity

from the medium, thus, prohibiting the induction of toxin synthesis. These results support the idea that toxin synthesis in *C. difficile* is cell density-dependent and involves accumulation of an inducing factor, which increases with cell density.

5.3.4 The LuxS and Agr quorum Signaling Systems do not Influence Toxin Synthesis in *C. difficile*

The genome of *C. difficile* encodes two known quorum signaling systems; the LuxS and the accessory gene regulator (*agr*) systems. The signaling molecule utilized by the LuxS quorum system (AI-2) is a by-product of the activated methyl cycle, which recycles S-adenosyl-L-methionine (SAM). SAM is the main methyl donor in archaea, prokaryotes, and eukaryotes that supplies methyl groups to biomolecules such as DNA and proteins. In the activated methyl cycle, SAM is converted to S-adenosyl-L-homocysteine (SAH), which is toxic to cells. In eukaryotes, SAH is converted to non-toxic homocysteine in a single step (228). However, in archaea and prokaryotes, SAH is detoxified in a two-step process, first by the Pfs enzyme (S-adenosylhomocysteine nucleosidase) to generate adenine and S-ribosylhomocysteine (SRH). SRH is finally converted by LuxS (S-ribosylhomocysteinase) to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (188, 228). DPD undergoes spontaneous cyclization and it can be sensed and modified by different bacterial species as a signaling molecule that controls gene expression.

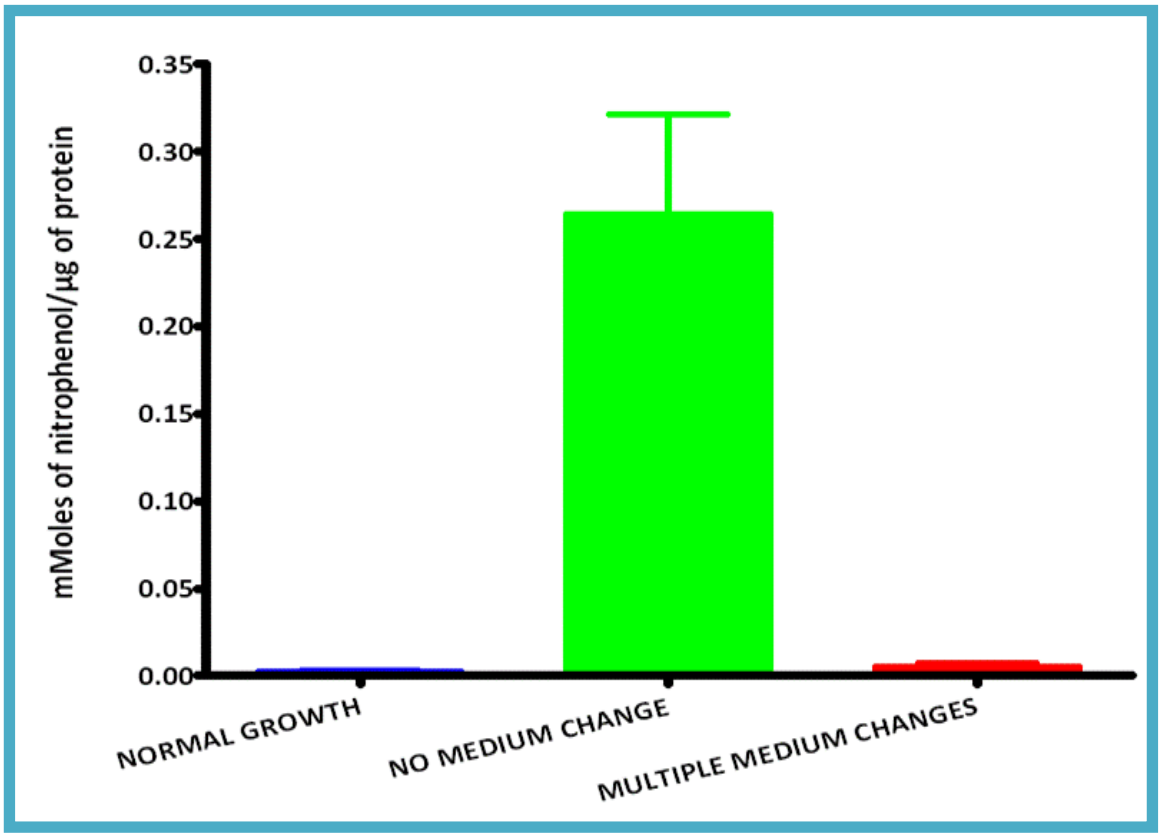

Figure 5.6: Early induction of toxin production under artificially high cell density conditions. Cells were cultured in 12-kDa MWCO dialysis membranes and placed in a flask containing fresh BHI medium. The spent BHI medium was either changed at 4 hrs or remained unchanged during an 8-hr incubation period and samples were taken from the dialysis membranes for toxin assay using the Cdifftox activity assay.

To investigate whether the LuxS autoinducer influences toxin production in *C. difficile*, different concentrations of the precursor DPD were provided exogenously to the log-phase tester cells and incubated for 8 hrs. DPD did not activate toxin synthesis (Fig. 5.7), suggesting that the LuxS system is not involved in the regulation of *C. difficile* toxin production. This result is consistent with the work reported by Carter et al. (31), which also found no association between *C. difficile* toxin production and the LuxS system.

Analysis of the genome of *C. difficile* revealed the presence of *agrB* and *agrD*, but putative homologues of *agrC* and *agrD* were not identified either around the vicinity of the *agr* locus or elsewhere in the genome. However, the genome does encode at least 37 two-component signal transduction systems. It is unusual to find *agrB* and *agrD* homologues without *agrA* and *agrC* homologues, because the *agr* components form an operon in other Gram-positives such as *Staphylococcus aureus* and are normally transcribed together. Multiple sequence alignment of the *agrD* locus from sequenced *C. difficile* strains showed that *agrD* is highly conserved in *C. difficile* (Fig. 5.8A), unlike the *agrD* in *S. aureus,* which is less conserved. In fact, four different autoinducer peptides (AIPs) have been identified in *S. aureus* that each control different effector targets. From the multiple sequence alignment, a cysteine (Cys-28) was found to be conserved in all of the different *agrD* sequences analyzed. In *S. aureus*, the AIPs contain a thioester bond between Cys-28 and the fourth amino acid towrds the C-terminus.

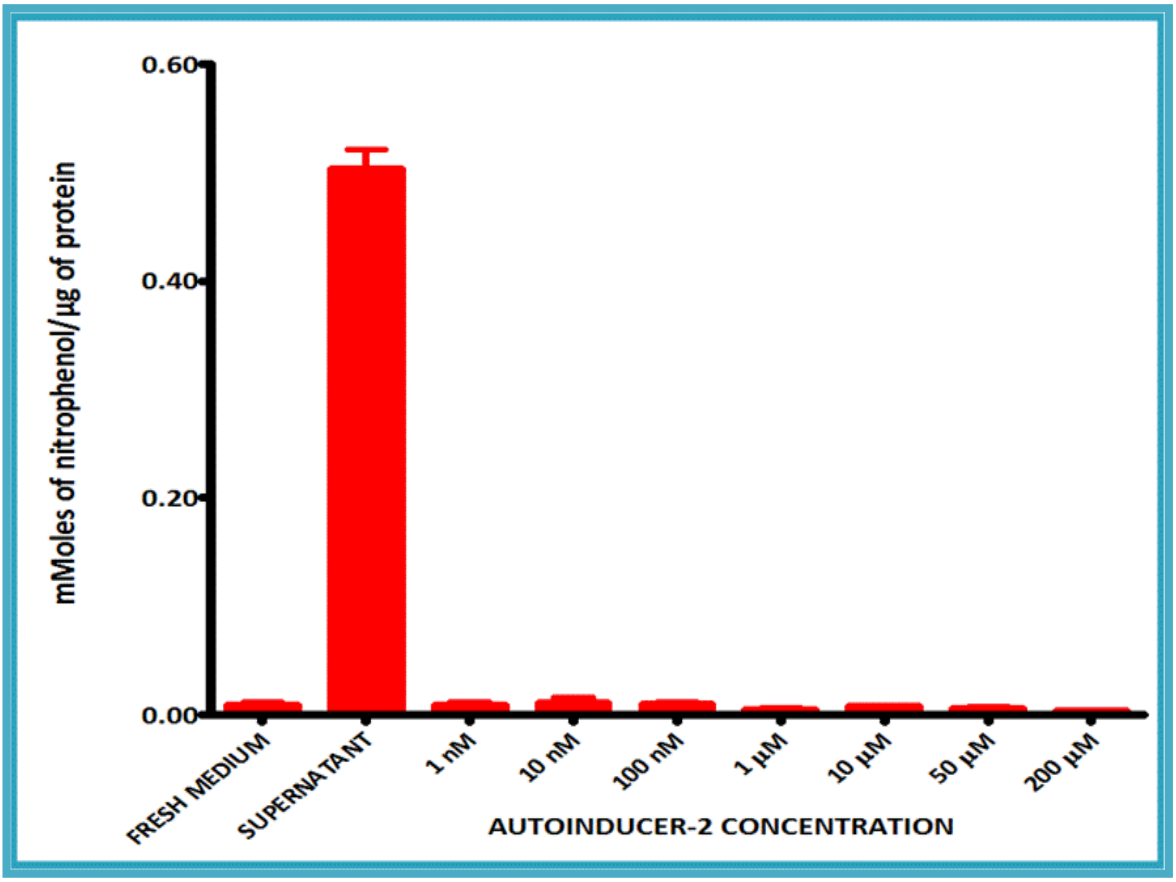

Figure 5.7: LuxS quorum signaling molecule (AI-2) does not influence toxin production in *C. difficile*. Log-phase *C. difficile* strain 630 tester cells were incubated for 8 hrs anaerobically at 37^{o}C with different concentrations of 4,5-dihydroxy-2,3-pentanedione. The culture supernatant fluid was tested for toxins using the Cdifftox activity assay.

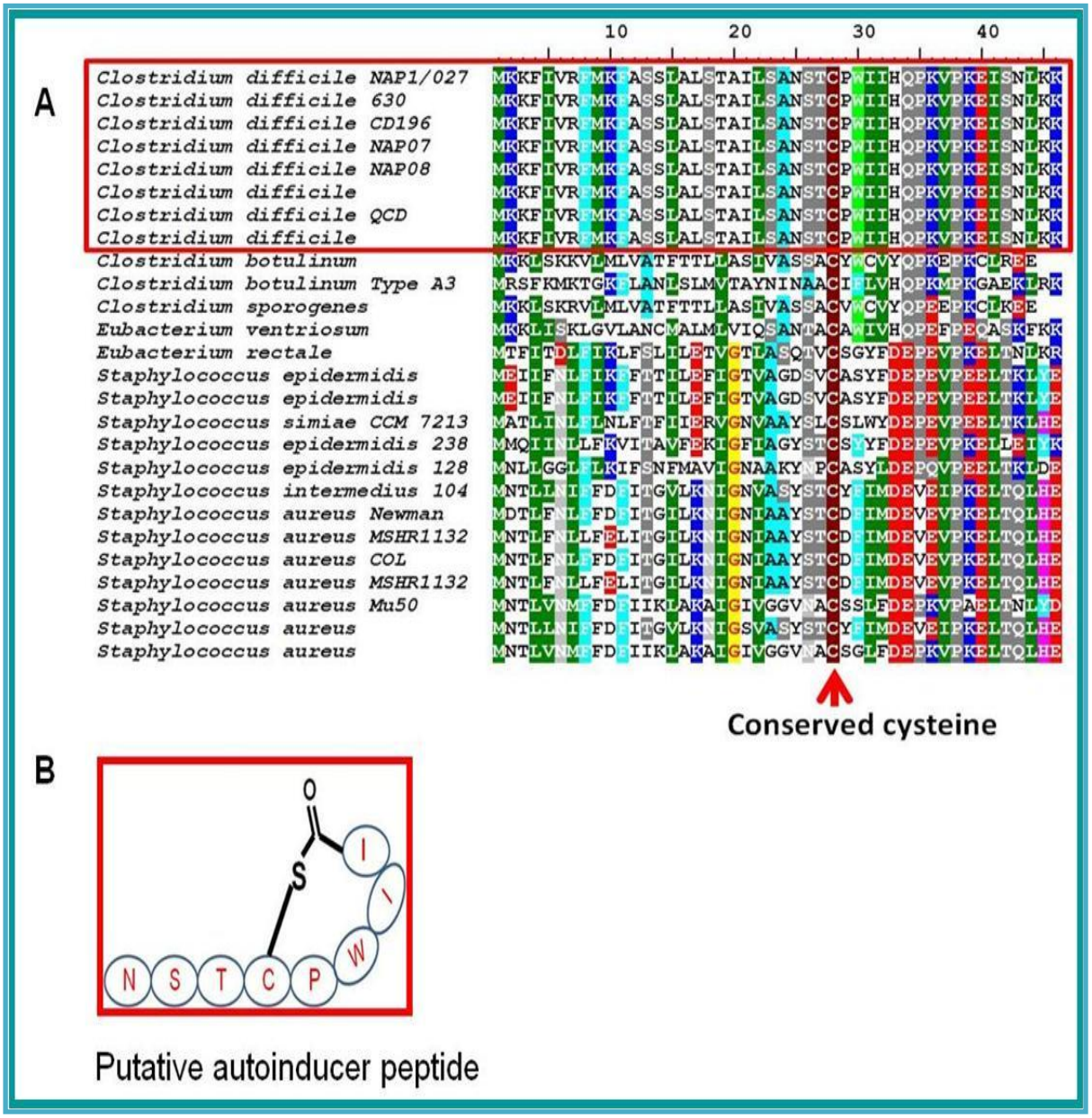

Figure 5.8: Analysis of the *agrD* locus in a subset of the Gram-positive bacteria. **A**. Multiple sequence alignment of the AgrD amino acid sequences from different Gram-positive bacteria. **B.** Putative *C. difficile* AIP showing the expected thioester bond between Cys-28 and isoleucine (Ile-32) at the fourth position toward the C-terminus.

Based on this observation, a predicted *C. difficile* AIP (Fig 5.8B) was synthesized and evaluated for its effect on toxin synthesis. The synthesized predicted *C. difficile* AIP did not influence toxin synthesis regardless of the concentration assayed (10 and 50 µg/ml) (Fig. 5.9). These results demonstrate that the predicted *C. difficile* AIP synthesized was not involved in the regulation of toxin synthesis in *C. difficile.* However, it would be best to test mutants in the *agr* system to conclusively judge the involvement of the *agr* system in *C. difficile* toxin synthesis.

5.3.5 Purification of the Toxin-Inducing Activity from Stationary-Phase Culture Supernatant Fluid

Having tested all the autoinducers associated with the known quorum signaling systems in *C. difficile* and found no association with toxin production, the toxin-inducing activity was purified from the stationary-phase culture supernatant. Based on the initial finding that the inducing activity is heat-stable, the culture supernatant fluid was first boiled to denature all high molecular weight proteins including the toxins and centrifuged to eliminate the debris. Next, the supernatant fluid was precipitated in 60% acetone. A crystal-like material (CRYS) appeared at the bottom of the tube when the acetone precipitate was washed, resuspended in water, and incubated overnight at 4^{o}C (Fig. 5.10, insert). CRYS induced toxin production when incubated for 4 hrs with the tester cells.

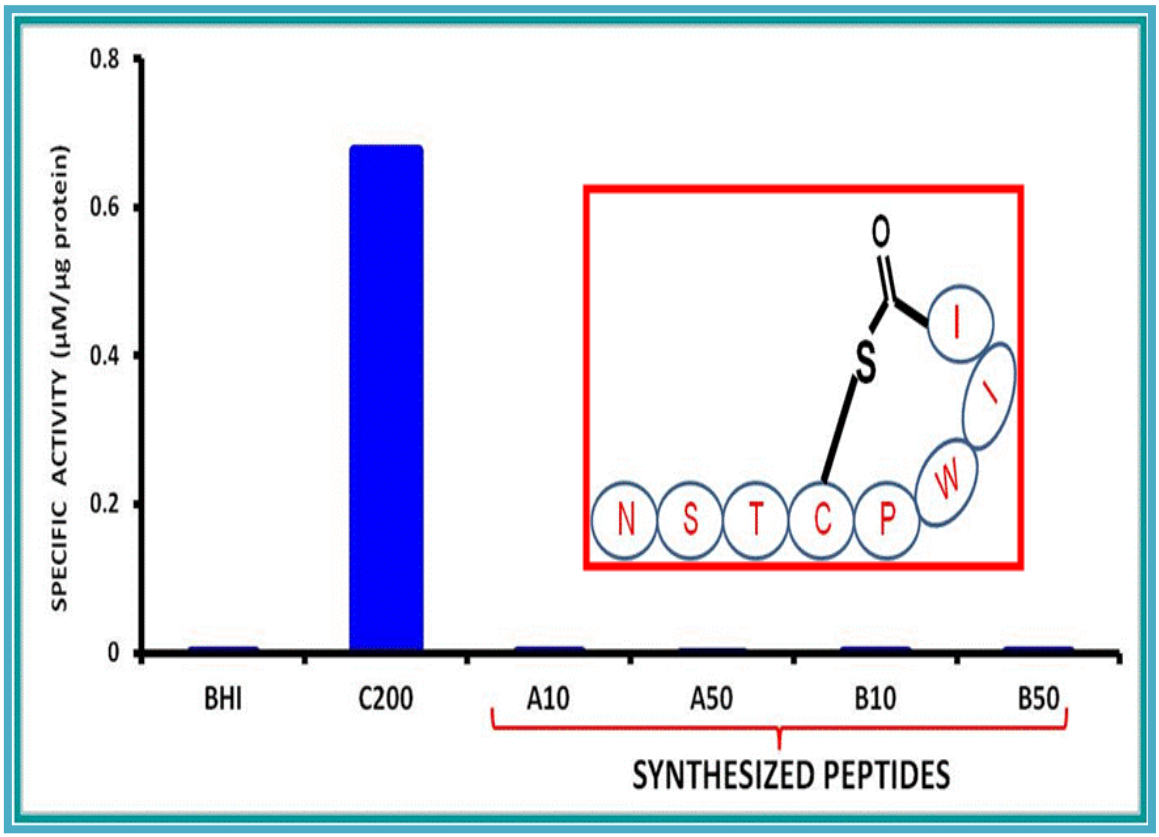

Figure 5.9: Effect of synthesized putative *C. difficile agr* autoinducer peptide (AIP) on toxin synthesis. Two isomers eluted (A and B) during the purification of the synthesized AIP, which were both collected and tested. *C. difficile* low-density log-phase tester cells were incubated in fresh reduced BHI medium containing the indicated amount of the synthesized peptides and incubated for 8 hrs anaerobically. The toxins were tested using the Cdifftox activity assay. BHI= Brain heart infusion medium, A10, A50 = 10, 50 µg/ml of isomer A, respectively; B10, B50 = 10, 50 µg/ml of isomer B, respectively. C200 = 200 µl of partially purified toxin-inducing activity from *C. difficile* culture supernatant. The structure of the predictive *C. difficile* AIP is shown in the insert.

CRYS induced toxin production in a dose-dependent manner when different amounts (100, 200, 300, and 400 µl) were tested (Fig 5.10). These results indicated that the toxin-inducing activity present in the stationary-phase spent culture supernatant fluid was precipitated and concentrated by the purification method.

5.3.6 Partially Purified Toxin-Inducing Activity (CRYS) Does not Affect Growth but Induced Early Toxin Synthesis

To assess whether the presence of CRYS affects *C. difficile* cell growth, tester cells were monitored for growth in the presence and absence of CRYS for 16 hrs. Aliquots of the culture were taken every 2 hrs for optical density determination and toxin testing. The data showed that CRYS did not affect the growth of the tester cells (Fig. 5.11). The growth kinetics of tester cells cultured in the presence of CRYS was not significantly different from cells cultured without CRYS. Furthermore, toxin activity was detected between 2 to 4 hrs in tester cells cultured in the presence of CRYS compared to 14 to 16 hrs for tester cells cultured without CRYS. These data revealed that only a small amount of CRYS is required to induce toxin synthesis. The amount of the toxins produced increased with incubation period. These results demonstrate that the toxin-inducing activity present in the spent stationary-phase culture supernatant fluid had been isolated, partially purified, and concentrated.

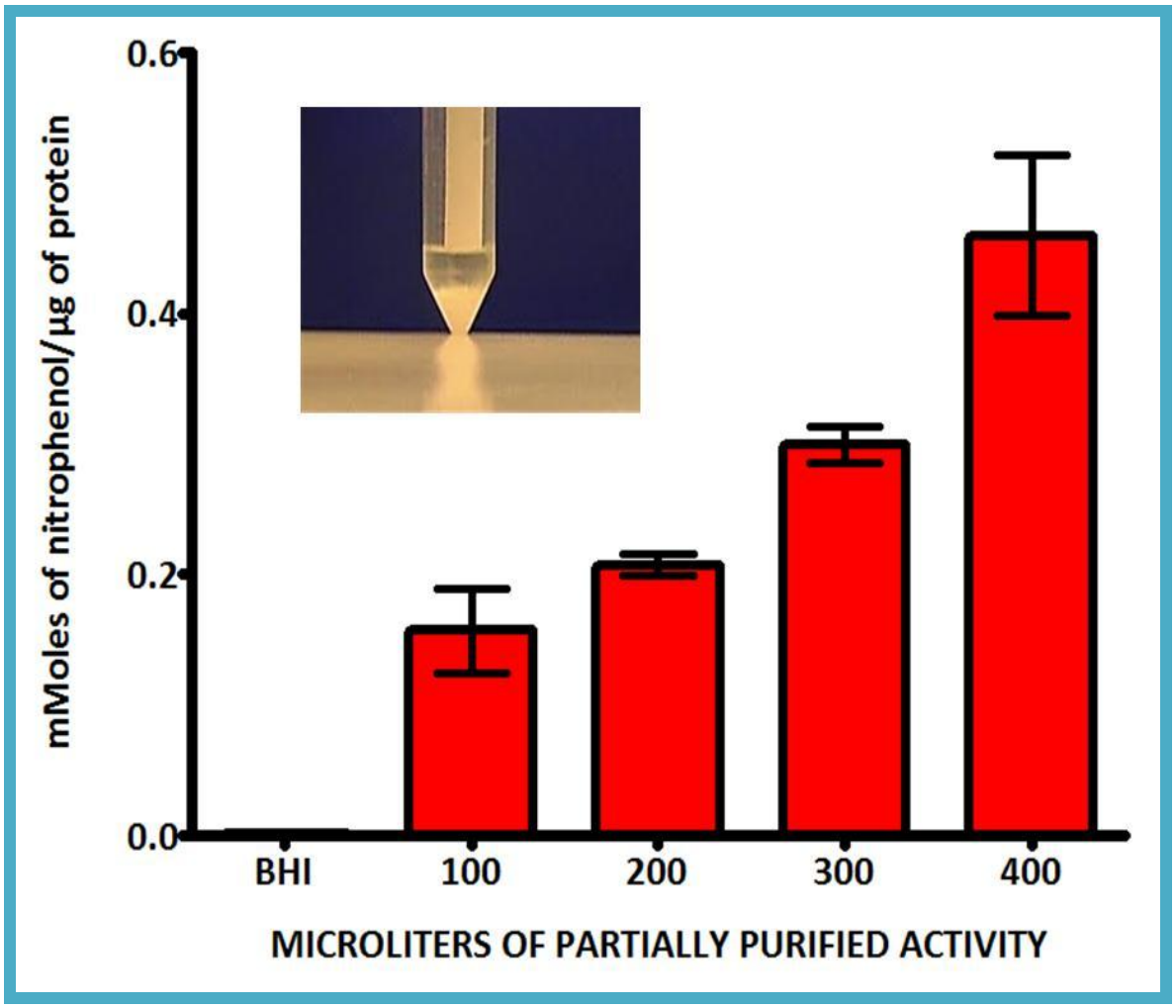

Figure 5.10: Effect of the partially purified inducing activity on *C. difficile* toxin synthesis. Different amounts of the partially purified toxin-inducing activity were added to tester *C. difficile* strain 630 cells and incubated anaerobically for 4 hrs at 37^{o}C. The resulting supernatant fluid was tested for toxin activity using the Cdifftox activity assay. The insert is a picture of the crystal-like material at the bottom of the tube.

5.3.7 Partially Purified Toxin-Inducing Activity Stimulates Transcription of the Toxin Genes

The effect of the toxin-inducing activity on transcription of *tcdA* and *tcdC* was evaluated. The tester cells were cultured with and without CRYS for 8 hrs. Aliquots of the culture were collected every 2 hrs for total RNA isolation, and subsequent cDNA synthesis by reverse transcription. PCR was performed using primers specific for *tcdA* and the gene encoding *tcdC*. In tester cells grown in the absence of CRYS, no mRNA transcript of toxin A was observed until after 8 hrs of growth (Fig. 5.12A). In the presence of CRYS, *tcdA* mRNA was detected within 2 hrs of incubation. No significant difference was detected in the mRNA transcripts of *tcdC* between cells cultured with and without CRYS (Fig. 5.12B). However, a gradual decrease in the *tcdC* mRNA transcripts was apparent in both CRYS-treated and untreated cells from 2-8 hrs. By eight hrs of incubation, the level of *tcdC* mRNA transcripts had decreased to an almost undetectable level. The mRNA level corresponding to *tcdC* has been shown to be inversely proportional to that of the toxins genes (30, 39, 103, 139, 142, 143, 222). However, this inverse relationship between *tcdC* and *tcdA* transcripts was not observed in CRYS-treated cells, suggesting that the induction of toxin synthesis by CRYS is independent of TcdC.

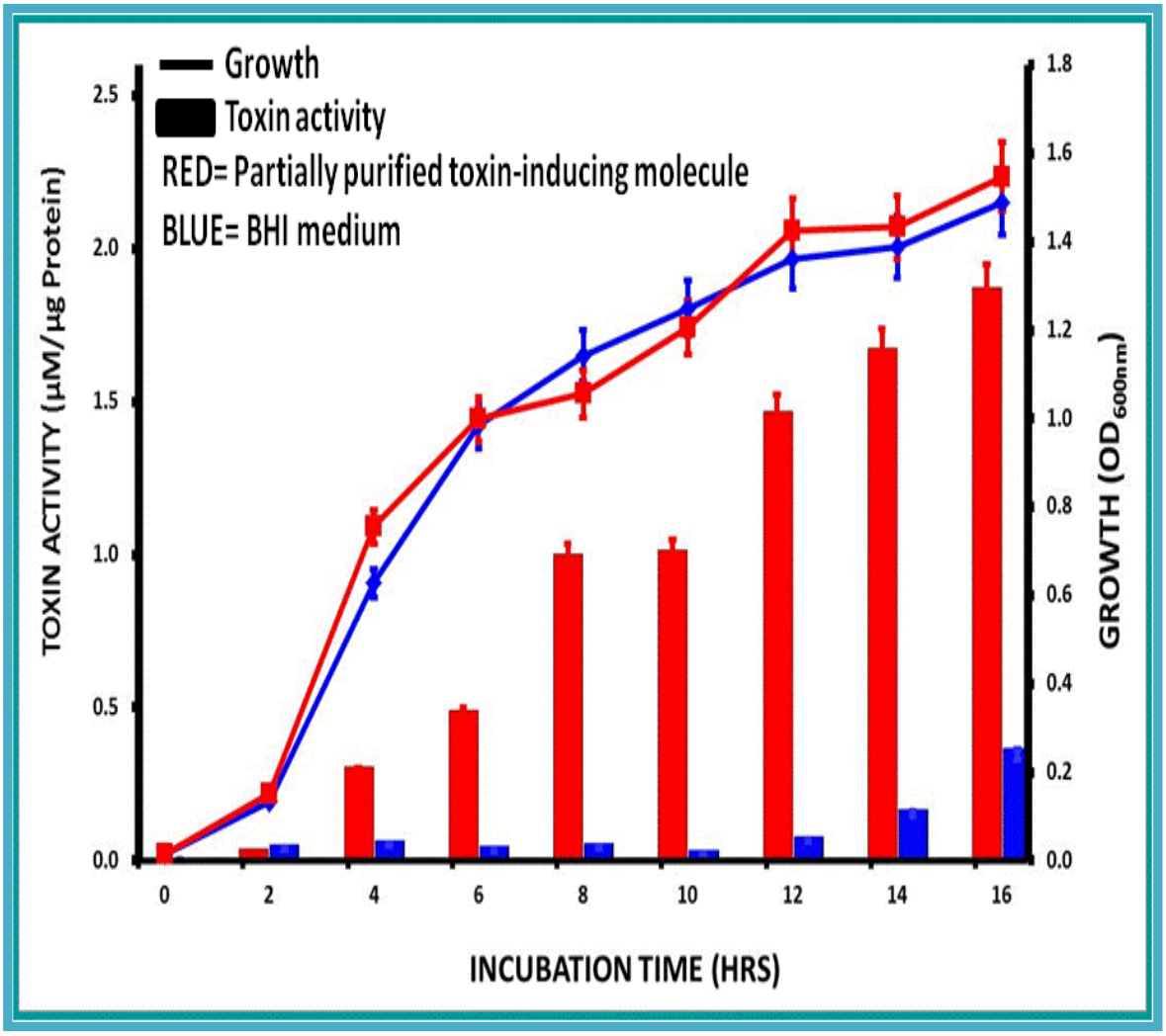

Figure 5.11: *C. difficile* growth and toxin production in the presence of the partially purified toxin-inducing activity. Tester cells (100 µl) were added to fresh reduced BHI medium (35 ml) containing 2 ml of the partially purified toxin-inducing activity (CRYS) and incubated anaerobically for 4 hrs at 37^{o}C. The resulting culture supernatant fluid was tested for toxin activity using the Cdifftox activity assay.

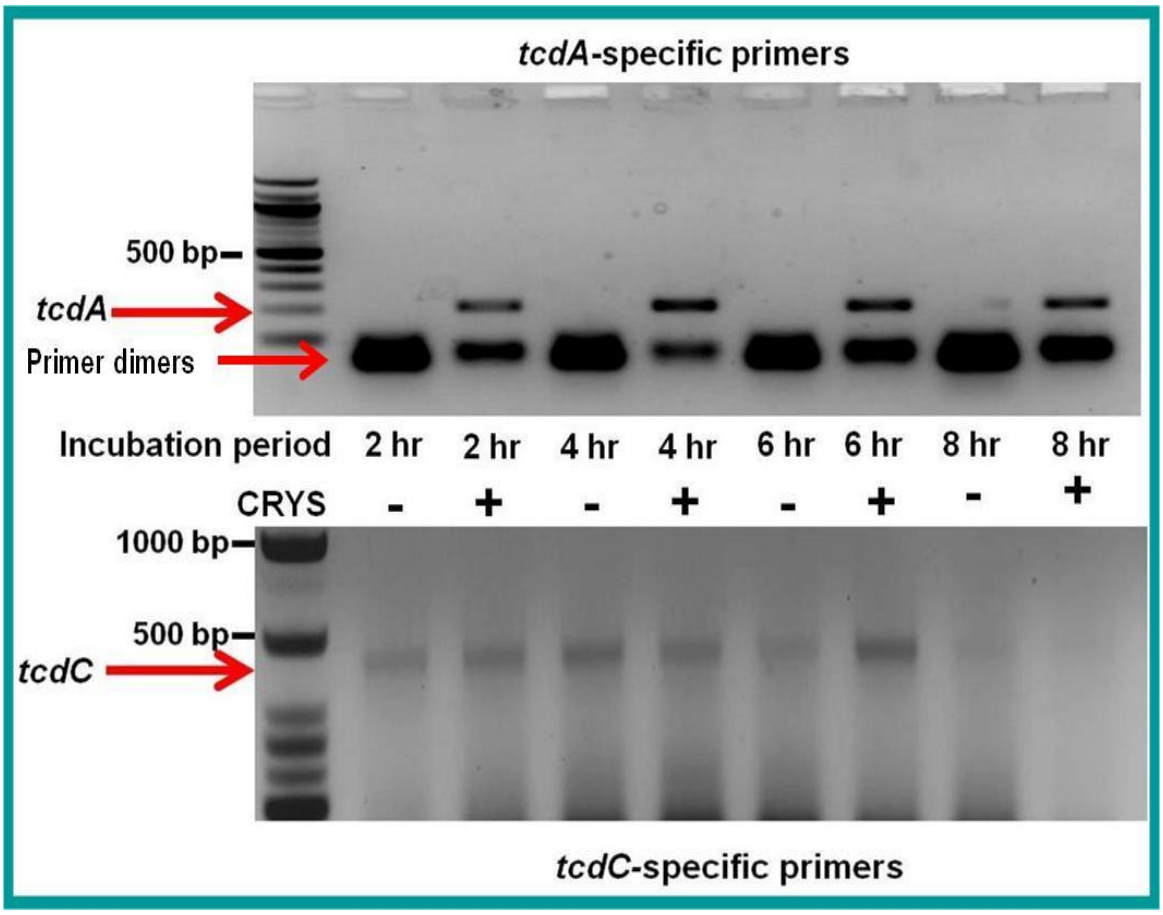

Figure 5.12: Toxin-inducing activity stimulates premature transcription of *tcdA*. Tester cells were cultured in the presence and absent of CRYS and aliquots were collected every 2 hrs for total RNA isolation, followed by cDNA synthesis by reverse transcription. PCR was performed using primers specific for *tcdA* **(A)** and *tcdC* **(B).** 'M' represents 1 Kb marker (New England BioLabs, Ipswich, MA); (-) = *tcdA* and *tcdC* amplicons from cDNA synthesized from cells incubated without CRYS; (+) = *tcdA* and *tcdC* amplicons from cDNA synthesized from cells treated with CRYS. The PCR products were electrophoresed through a 1% agarose gel and the DNA was detected digitally upon exposure of the ethidium bromide-treated gel to UV light.

5.3.8 Partially Purified Toxin-Inducing Activity Stimulates Toxin Synthesis in a Cell Density-Dependent Manner

The effect of the toxin-inducing activity on toxin synthesis in high-density cells was evaluated to gain insight into the sensitivity of cells to the presence of the toxin-inducing activity. Tester cells at different densities were incubated with CRYS and toxin synthesis was assessed after 90 min of incubation. The toxins were not induced during the 90-min incubation period when the tester cells at different cell densities were incubated in fresh reduced BHI medium without CRYS (Fig. 5.13). In the presence of CRYS, toxin synthesis occurred during the 90-min incubation period in a cell density-dependent manner. These data indicate that the transcription of the toxin genes commenced shortly after the inducing activity was added.

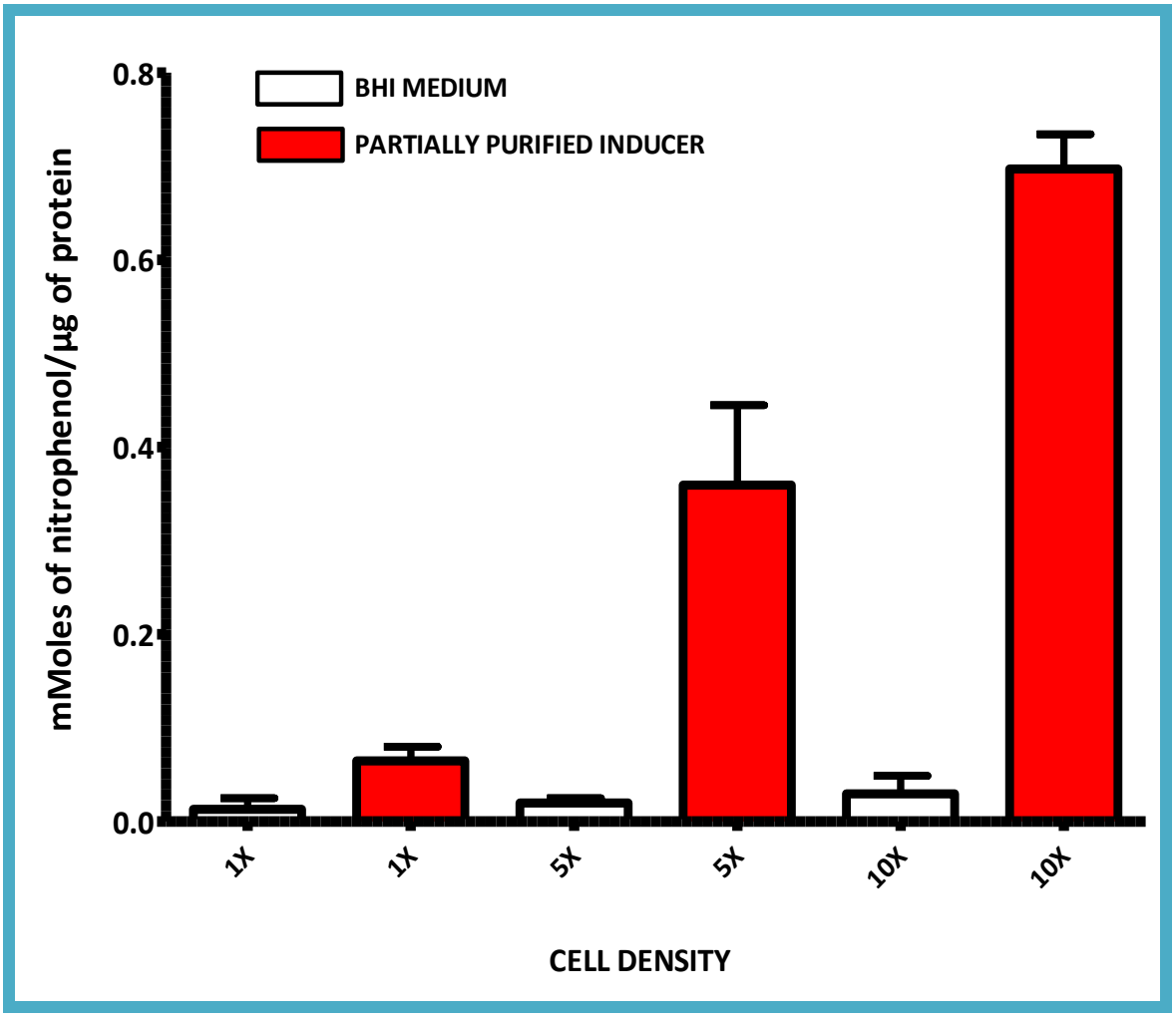

Figure 5.13: Effect of the partially purified toxin-inducing activity at different cell densities. Tester cells were grown to an O.D. 600 nm of 1.5 and washed twice with fresh reduced BHI medium. Different amounts of the tester cells (100 µl [1X], 500 µl [5X] and 1000 µl [10X]) were added to fresh reduced BHI (5 ml) containing 200 µl of CRYS. The cultures were incubated for 90 min anaerobically at 37^{o}C and the supernatant fluids were tested for toxin activity. Toxin detection was performed using the Cdifftox activity assay.

5.3.9 Partially Purified Toxin-Inducing Activity Stimulates more Toxin Synthesis in Epidemic Hypervirulent Strains of *C. difficile*

Some strains of *C. difficile* such as the NAP1/027 strains produce high levels of the toxins and this has been proposed to be due to a deletion or frame-shift mutation in the *tcdC* gene, which allows for unregulated transcription of *tcdA* and *tcdB*. CRYS was tested on clinical hypervirulent epidemic and non-epidemic strains to assess its effects on toxin synthesis in these strains. Low-density log-phase cells of the following strains: ATCC BAA-1382 (630 strain), ATCC BAA-1805 (NAP1/027 epidemic strain), R20291 (NAP1 epidemic strain), CD196 (NAP1), BI-9 (NAP2), TL176 (NAP4), BAA-1875 (NAP7), CF5 (NAP9), and Liv022 (NAP11) were incubated with and without CRYS anaerobically for 4 hrs and their supernatants were tested for the presence of toxin with the Cdifftox Activity assay. In the absence of CRYS, high basal level of toxin activity was detected in the NAP1 epidemic strains compared to the non-epidemic strains (Fig. 5.14). A significant difference ($p = 0.004$) was found between the amount of toxin activity produced by the strains with normal wild-type *tcdC* [BI-9 (NAP2), TL176 (NAP4), BAA-1875 (NAP7), CF5 (NAP9), and Liv022 (NAP11)] and the strains with mutant *tcdC* (epidemic NAP1 strains) in the absence of CRYS. All the strains produced elevated levels of the toxins when incubated with CRYS with no significant difference ($p = 0.318$) in the amount toxins produced between the strains. These results indicate that the inducing activity stimulated toxin synthesis via a common pathway present in all the toxin-producing strains tested. Furthermore, this induction of toxin synthesis appears to be independent of TcdC-mediated regulation.

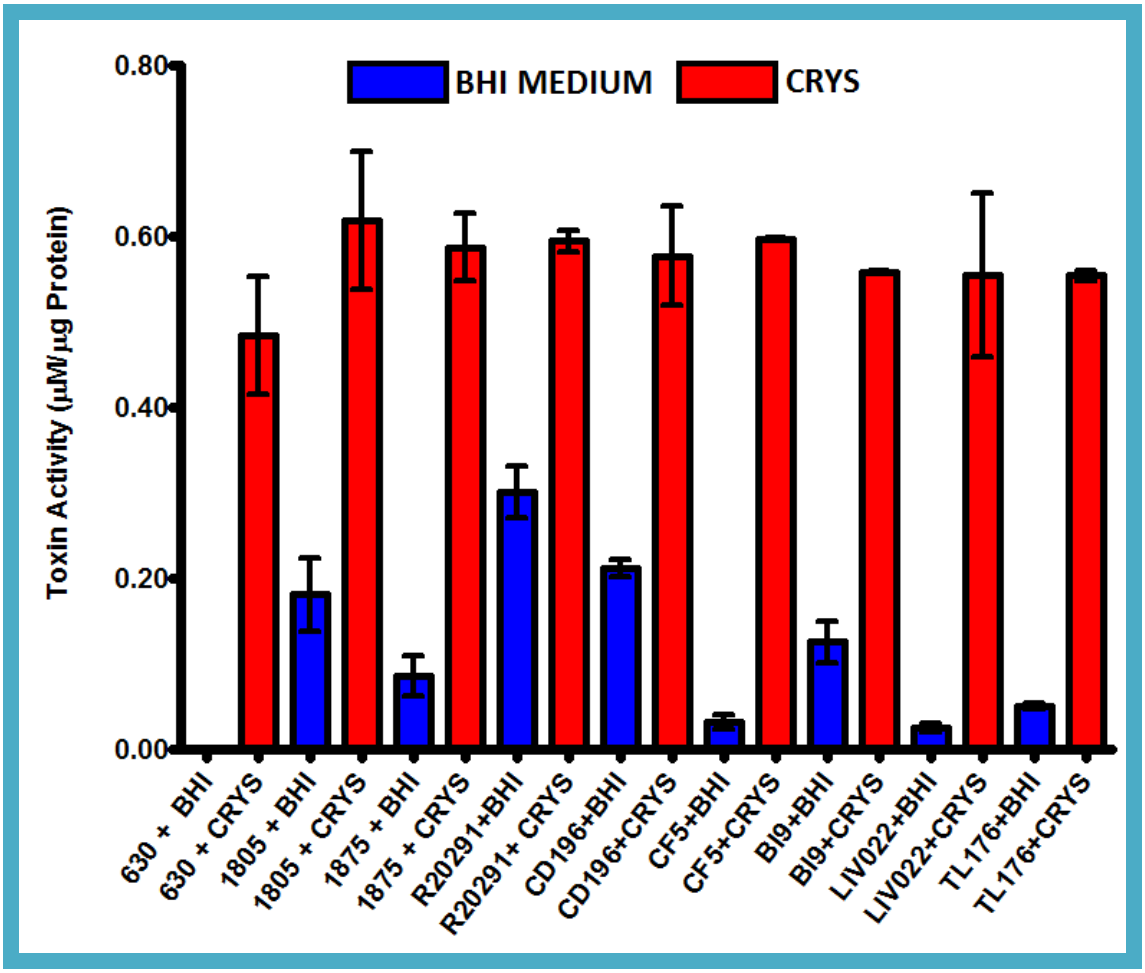

Figure 5.14: Effect of the partially purified toxin-inducing activity on epidemic hypervirulent and non-epidemic strains of *C. difficile*. Log phase tester cells were incubated with and without CRYS for 4 hrs at 37^{o}C anaerobically. The presence of toxins was tested using the Cdifftox activity assay. CRYS= Partially purified toxin-inducing activity; BHI= Reduced brain heart infusion medium; 630 = *C. difficile* historical strain ATCC BAA-1382 (non-epidemic historical strain), 1805= Hypervirulent strain ATCC BAA-1805 (NAP1/027), 1875= ATCC BAA-1875 (NAP7), R20291 (NAP1), CD196 (NAP1), BI-9 (NAP2), TL176 (NAP4), BAA-1875 (NAP7), CF5 (NAP9), and Liv022 (NAP11).

5.3.10 HPLC-Based Purification and Mass Spectrometry Analysis of the Toxin-Inducing Activity

The partially purified toxin-inducing activity, which was acetone-precipitated and concentrated from the spent stationary-phase culture supernatant fluid, was further purified by HPLC. The purification was performed in water with a gradient of acetonitrile (0-95%). The chromatogram shows a total of 8 major peaks (Fig. 5.15A). Real-time monitoring of the purification process showed several minor peaks that disappeared after the chromatogram was normalized. A total of 29 fractions (500 µl each) were collected and tested for induction of toxin synthesis. Only fraction 6 was determined to contain the toxin-inducing activity (Fig. 5.15B). The total activity obtained from fraction 6 was approximately equal to the total activity of the partially purified sample injected onto the HPLC column. This demonstrated that the entire toxin inducing activity, which was applied to the column, had been collected in a single fraction. However, fraction 6 eluted very close to the void volume at 6 min, indicating that the toxin-inducing activity interacted weakly with the C-18 HPLC column used. Fraction 6 was sent for mass spectrometry analysis and the results showed that there are other small contaminants co-eluting with the toxin-inducing activity (Fig. 5.16A). Based on MALDI-ToF mass spectrometry analysis, the molecular weight of the major compound present in fraction 6 was 655.8 Da. This molecular weight was found to be consistent with a cyclic compound containing a thioester linkage (Fig. 5.16B). Further confirmatory analysis is pending, which will be followed by synthesis of this cyclic compound to confirm its toxin-inducing activity.

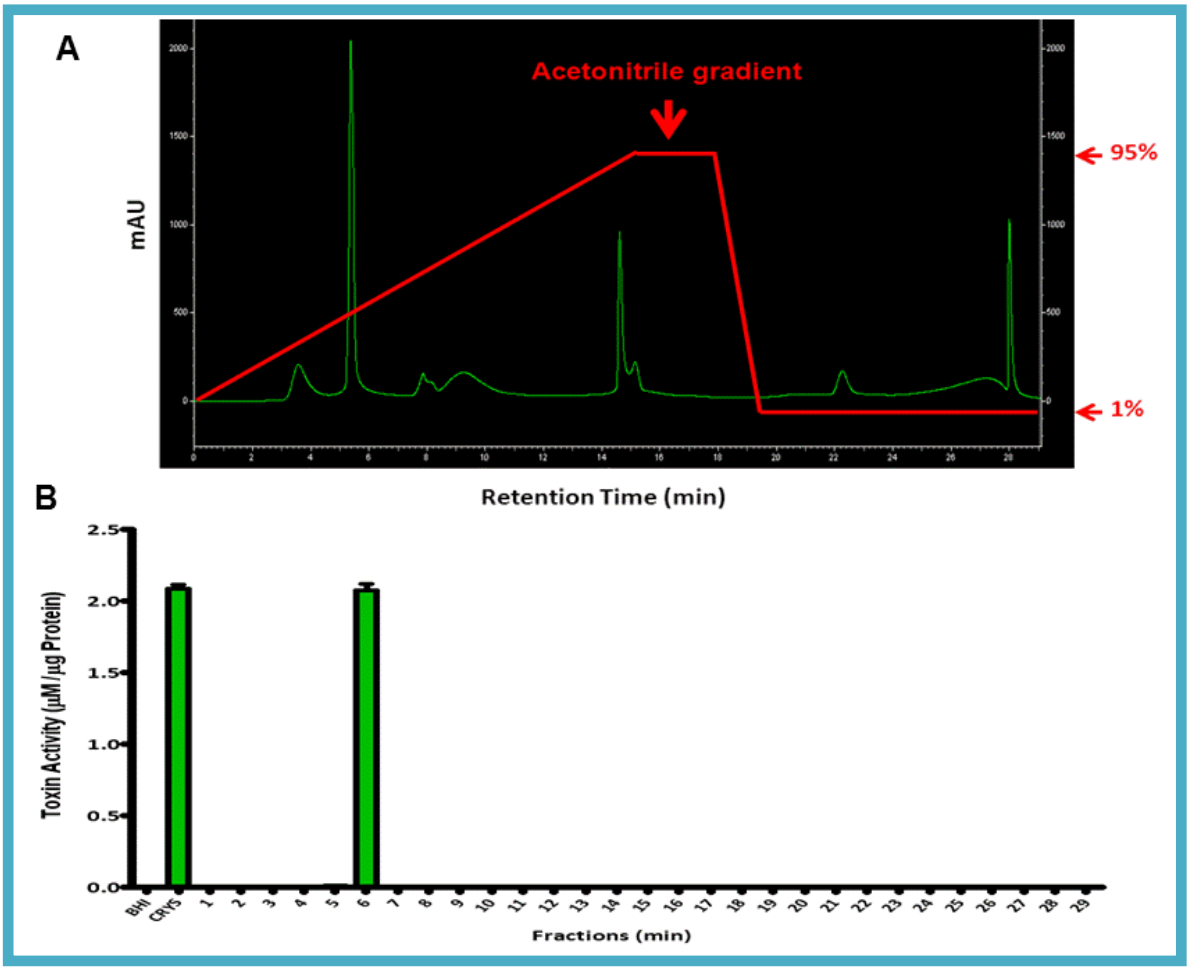

Figure 5.15: HPLC analysis of the toxin-inducing activity. **A.** Chromatogram from the HPLC purification showing the normalized peaks. CRYS (100 µl) was injected onto a Phenomenex Jupiter 4µ Proteo 90A (250 mm x 4.6 mm) C18 column (Phenomenex, Torrance, CA). The purification was performed with water as buffer A and acetonitrile as buffer B. A gradient of acetonitrile from 0-95% over 15 min at a flow rate of 0.5 ml/min was used. Fractions (0.5 ml) were collected and dried using a SPD111 speedvac (Thermo Scientific). Each fraction was resuspended in 200 µl of water and tested for toxin-inducing activity using the quorum signaling bioassay. **B.** Cdifftox activity assay showing the toxin-inducing activities of the fractions collected. Only fraction #6 was active.

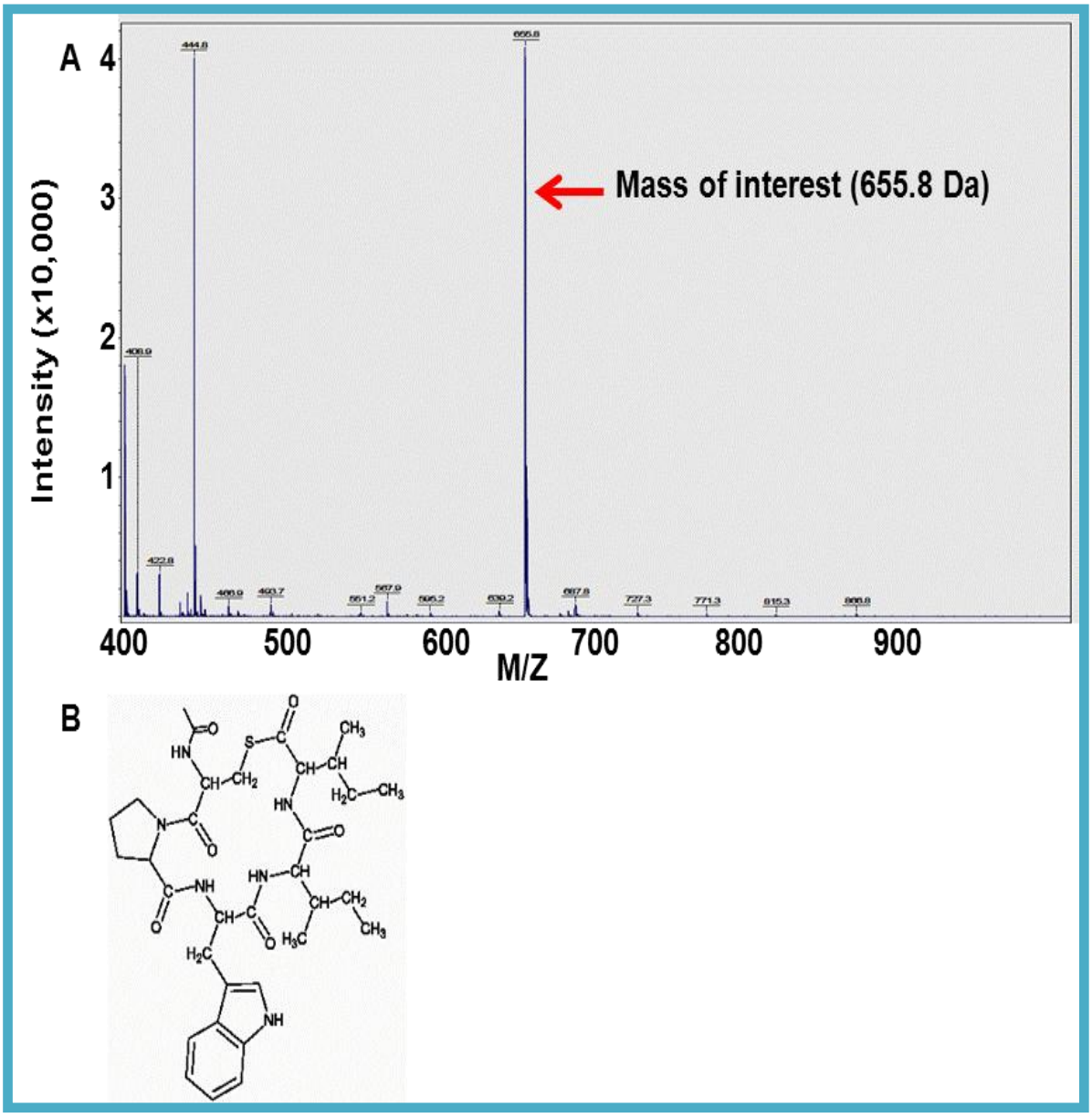

Figure 5.16: Mass spectrometry analysis of the toxin-inducing activity (fraction # 6). **A**. MALDI-ToF analysis showing the molecular weights of different compounds present in fraction #6. **B**. Predicted structure of the major compound in fraction #6 based on MS/MS spectrometry analysis.

DISCUSSION

The incidence of *C. difficile* infection (CDI) has been increasing at an alarming rate to the extent that it is now the leading definable cause of nosocomial and antibiotic-associated diarrhea (13). The major factors that have contributed to this prevalence are the increasing use of intestinal flora-altering antibiotics, the propensity of *C. difficile* to produce spores, which are refractory to disinfectants and antibiotics, sub-optimal infection control practices, and the appearance of high toxin-producing mutant strains with a more potent activity (128, 132, 143, 153). Efficient treatment of CDI has been mired by recurrent infections, multi-drug resistance, difficulties in delivering appropriate drug concentrations to the colon, and the lack of drugs with potent functional activity in the colon. As a consequence, there is renewed interest in finding alternative treatments for CDI, either as stand-alone therapies or therapies designed to improve the efficacy of currently used antibiotic regimens. A promising treatment approach is to block the synthesis or activity of toxins A and B, which are directly responsible for the CDI-associated diseases. This line of attack, which targets the toxins without affecting cell growth, may be ideal since it is unlikely to impose selective pressure on *C. difficile*, thereby minimizing the risk of developing resistance. However, finding an appropriate specific target to block toxin synthesis requires understanding the key players and the mechanisms that are involved.

Under normal growth conditions, *C. difficile* toxin production occurs during stationary phase. This study demonstrates for the first time that a small (< 1 kDa),

heat-stable, cell density-dependent factor is released into the extracellular *milieu* by *C. difficile* during growth, and activates toxin synthesis. Late-stationary-phase culture supernatant fluid, when incubated with low-density log-phase cells, induced higher toxin synthesis than early-stationary phase culture supernatant fluid. In contrast, culture supernatant fluid collected from the exponential phase of growth did not activate toxin synthesis. These results suggest that the toxin-inducing activity accumulates during growth and peaks at stationary phase leading to activation of toxin synthesis. This is consistent with the transcription of the toxin genes, which begins during early stationary phase (103, 119). The hallmark of quorum signaling is the extracellular accumulation of a small, diffusible, heat-stable, cell density-dependent signaling molecule that induces gene expression upon reaching a critical threshold concentration. The data presented here fulfill these criteria and therefore, directly establishes that *C. difficile* toxin synthesis is regulated by quorum signaling.

The standard signaling molecules associated with the two well-studied quorum signaling systems (AI-2 and AIP) were tested by direct addition of the purified molecules or their precursors in the quorum system bioassay. However, no toxin synthesis was observed. Lee, et al. reported that cell-free supernatants from mid-log phase of *C. difficile* and *E. coli* DH5α expressing recombinant *C. difficile* LuxS/AI-2 increased the transcript levels of *tcdA*, *tcdB*, and *tcdE* (130). However, Carter, et al. found no significant effect of LuxS/AI-2 on either the timing of toxin production or the amount of toxins produced by *C. difficile* (31). The results

presented here support the conclusion of Carter, et al. that the LuxS system is not involved in *C. difficile* toxin synthesis.

The role of the *agr* quorum signaling system in *C. difficile* has not been investigated previously. The putative autoinducer peptide derived from the *C. difficile agrD* component of the *agr* system suggested a complex peptide containing a linear tail and a cyclic head consisting of a thioester linkage of cysteine with isoleucine. This unusual peptide was difficult to synthesize due to its complex structure (Personal communication with Dr. W. Chan). Mass spectrometry analysis confirmed the structure and actual mass of the synthesized predicted peptide to be accurate. However, the exact nature, structure, and characteristics of the native *C. difficile* AIP are not known. The *C. difficile* AIP was predicted based on the structure of *S. aureus* AIP. Thus, the association of the *agr* system with *C. difficile* toxin synthesis cannot be ruled out, based on the data presented in this study. It is interesting to note that the *agrD* amino acid sequence is 100% conserved in all the *C. difficile* strains whose genomes have been sequenced (Fig. 5.8). This suggests that the *agr* AIP may be utilized by *C. difficile* to regulate a common process present in all of these strains. Identification of the toxin-inducing factor or generation of *C. difficile agrD* or *agrB* mutants may help clarify the role of the *agr* system in *C. difficile* toxin synthesis.

The toxin-inducing activity was precipitated and concentrated from the culture supernatant fluid. It was further purified by HPLC after confirming its activity using the quorum signaling bioassay. By HPLC analysis, the toxin-inducing activity was isolated in a single fraction, which was analyzed by mass spectrometry for structure

and actual mass determination. The results from mass spectrometry indicated that there are other compounds in the HPLC fraction that co-eluted with the toxin-inducing activity. It is important to note that the active fraction eluted from the HPLC column was very close to the void volume, suggesting a weak interaction with the C-18 column. Small molecules have traditionally been purified using C-18 columns. Hence, the lack of strong interaction of the toxin-inducing activity with the C-18 HPLC column suggested an unusual hydrophilic small molecule. The molecular weight of the major compound in the toxin-inducing fraction was consistent with a 655.8 Da cyclic compound. Further confirmatory analysis of this cyclic compound is pending, which will be followed by synthesis of the compound to confirm its role in toxin induction.

The toxin-inducing activity induced elevated levels of the toxins in different non-epidemic and epidemic strains of *C. difficile*, including the hypervirulent *tcdC* mutant strains that naturally overexpress the toxins. The TcdC protein reportedly binds to the TcdR transcription factor to prevent transcription of the toxin genes (30, 39, 103, 139, 142, 143, 222). Thus, a deletion or mutation in the *tcdC* gene would eliminate its inhibition leading to a loss of regulation and constitutive expression of *tcdA* and *tcdB*, which encode the toxins. Induction of elevated toxin synthesis in strains with intact *tcdC* and strains with defective *tcdC* imply that the level of control by the toxin-inducing activity is independent of TcdC-mediated regulation. The finding that another factor is involved in *C. difficile* toxin synthesis is novel, since TcdR and TcdC have traditionally been accepted as the only key players in *C. difficile* toxin regulation.

Based on these results, I propose that *C. difficile* cells produce and release a small (<1 kDa), diffusible, heat-stable factor into their extracellular *milieu* that accumulates during growth as the cell density increases (Fig. 5.16). This factor is sensed by *C. difficile* cells as a measure of the population cell number. When the factor reaches a certain critical threshold concentration, which directly reflects the number of cells in a population, it activates a common pathway leading to a coordinated activation of transcription of the toxin-encoding genes.

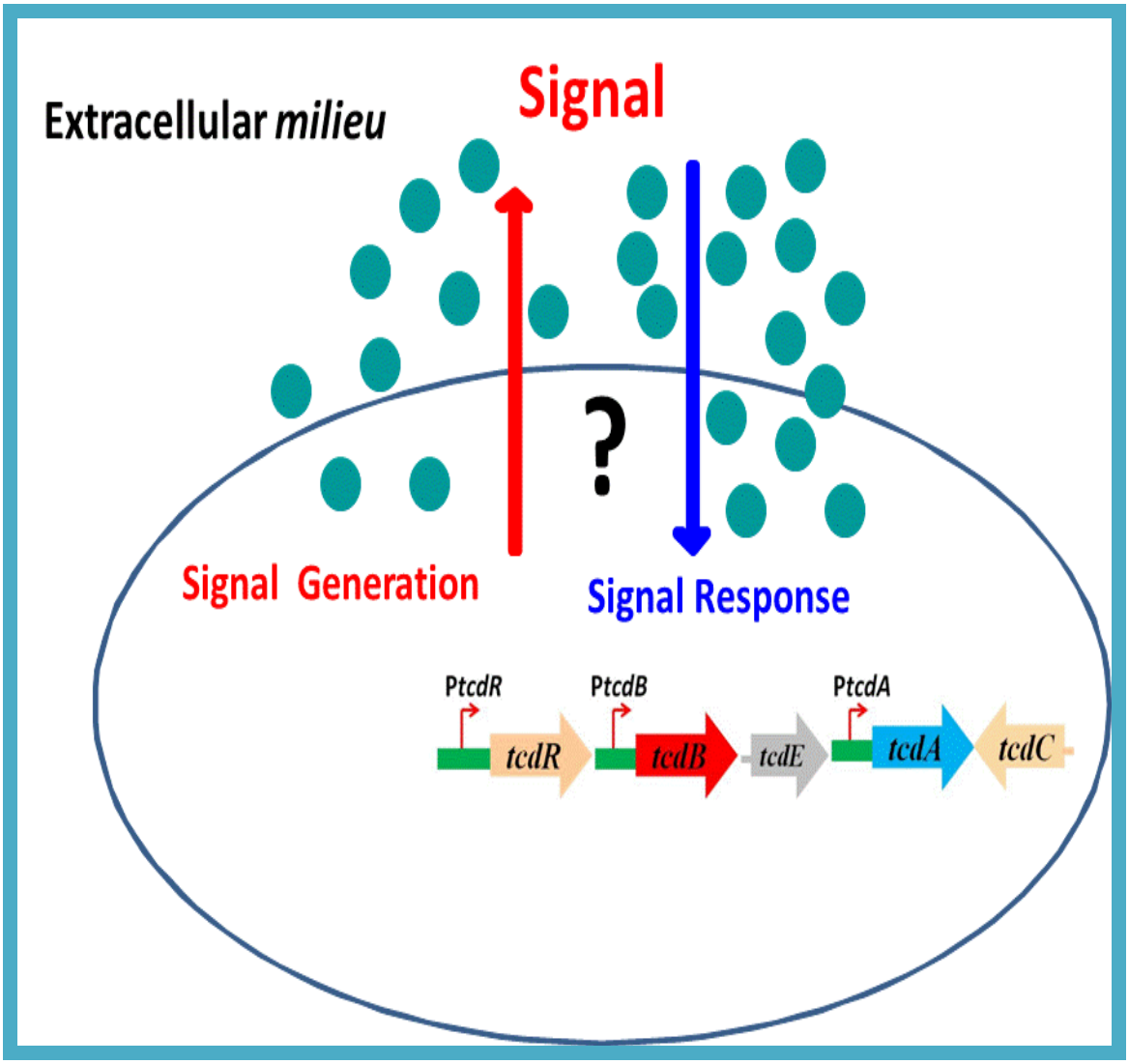

Figure 5.17: A model for the regulation of *C. difficile* toxin synthesis. A signaling factor is released extracellularly during *C. difficile* growth via a signal-generation pathway that accumulates in the extracellular *milieu*. This signaling factor is sensed and processed by a signaling-response pathway, thereby activating a transcription factor that leads to the transcription of the toxins genes.

CHAPTER 6: PERSPECTIVES AND FUTURE DIRECTIONS

The alarming emergence and increasing incidence of *C. difficile* infections (CDI) pose a public health threat and a burden to healthcare systems worldwide. The estimated number of cases of CDI occurring in U.S. hospitals exceeds 250,000 per year (226), with a total additional healthcare cost between 1-3.2 billion U.S. dollars annually (124). The number of CDI cases occurring in the community and in nursing homes, long term care facilities and skilled nursing facilities may increase the number of annual cases in the U.S. to as many as 3 million. Morbidity and mortality resulting from *C. difficile*-associated diseases in recent years have also increased significantly. This is mainly a result of changes in the virulence of the causative strains, antibiotic usage patterns, an aging population that includes more immunocompromised patients, and lack of proper infection control strategies (143, 160, 177). The *C. difficile* toxins A and B are directly responsible for disease and are essential in pathogenesis (73, 123, 138, 185, 219). Strains that do not produce either of these toxins are not associated with disease. Treatment of CDI has been hampered by recurrence, multi-drug resistance, and a lack of drugs with reasonable bioavailability and activity in the colon. Moreover, a large number of *C. difficile* isolates are resistant to the majority of antibiotics currently used in hospitals and outpatient settings (6, 16, 164, 193). Bacterial infections have long been treated with antibiotics, but for CDI the repertoire of available antibiotics to effectively clear the infection and prevent recurrence is dwindling. As a result, an alternative non-antibiotic treatment approach to CDI is necessary.

The myriad of disease symptoms in CDI is variable and the severity of disease depends on the amount of toxins produced by the infecting strain (3). This indicates

that toxin synthesis is a critical and significant element in CDI. Therefore, an important approach to combat CDI is inhibition of toxin synthesis and /or toxin activity. Targeting the regulatory mechanisms that control the synthesis of these toxins or inhibition of toxin activity are unlikely to pose a selective pressure on the bacterium, thus minimizes the risk of resistance development compared to antibiotics. In order to find an appropriate and specific target to effectively disrupt *C. difficile* toxin synthesis, it is necessary to understand the mechanism and the key players that are involved. Furthermore, finding an effective inhibitor of toxin activity requires a quantitative toxin-specific assay that directly detects the activities of the toxins. In addition, early detection of the bacterium during infection is critical to prevent further damage by these toxins. Thus, an assay that combines detection of the *C. difficile* bacterium with toxin activity would shorten the diagnosis of CDI for early treatment intervention.

In Chapters 2 and 3, I described two related *C. difficile* toxin assays (the Cdifftox activity and Cdifftox agar plate assays) developed as part of this project. The Cdifftox activity assay enables the detection of active toxins in culture supernatant fluid, whereas the Cdifftox agar plate assay (CDPA) detects active toxin-producing *C. difficile* colonies on an agar plate. This agar plate assay allows colonies of the infecting bacteria to be selected for further testing such as antibiotic susceptibility testing, which is routinely done in clinical microbiology laboratories. Recently, there has been a major shift away from the use of traditional *C. difficile* diagnostic methods such as culture, ELISA, and tissue culture cytotoxicity assay to real-time PCR-based detection of the toxin genes from stool. PCR-based methods are quick

and sensitive, but they are based on the assumption that detection of the DNA that encode the toxins is indicative of the presence of actively growing toxin-producing *C. difficile* cells capable of causing disease. I have provided data in Chapter 3 showing that it is not unusual to detect a non-toxin-producing strain bearing the toxin genes. There are a number of possible reasons for lack of toxin synthesis; the strain may contain a mutation in the regulatory region or a region that encodes a critical feature of the toxins, thereby either blocking toxin synthesis or causing the production of inactive toxins, both of which would render a toxin gene-positive strain clinically irrelevant. Hence, detection of the *C. difficile* toxin genes in stools does not automatically translate to active *C. difficile* infection capable of causing disease. An unproven concern but likely limitation of PCR-based diagnostics is that patients with a non-CDI cause of diarrhea may be colonized by *C. difficile* and cause a positive test for the toxin genes leading to an improper diagnosis. The use of PCR in CDI diagnosis may lead to treatment for a perceived infection that is clinically irrelevant. Overuse of antibiotics also drives resistance, which is a major problem with *C. difficile*. Therefore, a diagnostic method that combines the detection of the bacterium with active toxin production such as the CDPA, offers a better approach to CDI diagnosis. A major limitation of CDPA is that it depends on production of the toxins to differentiate toxin-producing strains from non-toxin producing strains. These toxins are regulated and so a growth condition that is not conducive for toxin synthesis may result in a false negative diagnosis. Identification of the inducing activity discovered in this study that activates *C. difficile* toxin synthesis may help eliminate this problem. I plan to modify the published CDPA assay by incorporating

this inducing factor into the CDPA medium to activate toxin synthesis when colonies begin to grow on the plate. This should improve the sensitivity and eliminate the potential problem of false negative results. It is also anticipated that it will allow the assay to detect toxin-producing strains within 24 hrs, which would be useful for diagnosis.

The Cdifftox activity assay was used to screen for a number of compounds that could inhibit toxin activity. Taurocholate (a common component of human bile) was found to inhibit both toxins A and B activity. In Chapter 4, I provided data demonstrating that taurocholate protected human colonic intestinal epithelial cells (Caco-2 cells) from the damaging effects of the toxins. One of the downstream effects of the toxins when internalized into the host cell cytoplasm is apoptosis. This has been demonstrated using only toxin A (23, 29, 71). Caspase-3 is a pro-apoptotic protease, which commits cells to apoptosis. Using a caspase-3 assay, I have shown for the first time that toxin B induces apoptosis in Caco-2 cells and that taurocholate decreased the extent of toxin B-mediated apoptosis. All these experiments were performed either *in vitro* or *ex vivo*. I plan to repeat these experiments in a *C. difficile* animal model to investigate whether taurocholate could also protect a *C. difficile*-infected animal model. The therapeutic benefits of bile salts are well documented; they prevent hepatocyte injury and cholestasis (93, 94, 172), drug-induced cholestasis (174), and endotoxin absorption (9, 80). Therefore, developing a method to deliver high or sustained concentrations of bile salts and/or their derivatives, perhaps in conjunction with antibiotics into the colon of individuals suffering from recurrent CDI may help protect the colon from the damaging effects

of the *C. difficile* toxins and facilitate clearance of the pathogen. I also plan to take advantage of the Cdifftox activity assay to conduct a high throughput screening of a number of small molecule drug libraries in search of other potent inhibitors of toxin activity, toxin synthesis, or *C. difficile* growth. This may lead to the discovery of more potent drugs that could be effectively harnessed to treat CDI.

The development of the toxin assays enabled me to conduct the experiments necessary to prove the hypothesis of this dissertation. I have demonstrated for the first time that a small (< 1 kDa), heat-stable factor is released by *C. difficile* into the extracellular *milieu* during growth, which peaks at high cell density. When incubated with low-density log-phase *C. difficile* cells, this factor activated early and elevated toxin synthesis. These results demonstrated for the first time that toxin synthesis in *C. difficile* is regulated by quorum signaling. The predicted signals based on the two known quorum signaling systems present in the genome of *C. difficile* were tested, but did not appear to be involved in toxin synthesis. However, mutants deficient in these systems are necessary to completely eliminate the involvement of these systems in *C. difficile* toxin synthesis.

A purification method was developed that enabled precipitation and concentration of the toxin-inducing factor from stationary phase culture supernatant fluid. This partially purified toxin-inducing factor was very potent at inducing toxin synthesis. Analysis of *tcdA* and *tcdC* mRNA transcripts in the presence of this partially purified toxin-inducing factor revealed an early induction of transcription of the *tcdA* gene. It is widely accepted that the transcription of *tcdC* is inversely proportional to the transcription of the toxin genes, suggesting a negative regulatory

role of TcdC protein. However, this inverse relationship was not observed in the presence of the toxin-inducing factor, suggesting that the quorum signaling mechanism is independent of TcdC-mediated regulation. This finding was confirmed when the toxin-inducing factor was determined to be able to induce toxin activity in different non-epidemic and epidemic strains of *C. difficile* with non-functional TcdC due to a deletion or mutation in the *tcdC* gene. Elevated levels of the toxins were produced in the presence of the toxin-inducing factor by all the strains, independent of a functional or defective TcdC. Without the toxin-inducing factor, the differences in the amount of toxins produced by these strains were significant between the normal strains and the *tcdC* mutant strains. However, no significant differences were observed in the toxin amounts produced when the tested strains were incubated with the toxin-inducing factor, supporting the idea that the toxin-inducing activity is acting independent of TcdC. This finding is interesting because it is the first evidence that other factors are involved in the regulation of toxin synthesis in *C. difficile* and that TcdR and TcdC are not the only key players in toxin regulation.

With the goal of identifying the molecule responsible for the toxin-inducing activity, I further purified the activity using HPLC. I was able to isolate the inducing activity in a single peak. However, mass spectrometry analysis of this peak showed that there are other molecules co-eluting with the activity. The molecular weight of the major compound in the active HPLC fraction was a 655.8 Da, which was consistent with a cyclic compound shown in Figure 5.16. I plan to repeat the purification to confirm the presence of the cyclic compound as the major component of the fraction. This will be followed by testing the activity of the synthetic compound

to confirm its role in toxin induction. The following are some of the many questions that would be addressed once the toxin-inducing factor is identified:

1. Identification of the key genes and proteins involved in the generation of the toxin-inducing activity (signal).
2. Identification of the key genes and proteins that sense and transduce the signal.
3. The mechanism that governs the release, sensing, and transduction of the signal.
4. The connection between the signal and the pathogenicity locus, which ultimately lead to the transcription of the toxin genes.
5. Evaluation of the amount of the signal produced by different strains of *C. difficile*.
6. The role of the signal in the pathogenesis of *C. difficile*.
7. Identify key therapeutic targets to inhibit toxin synthesis.
8. Other target genes regulated by the signal.

The role of the toxins in the natural history of *C. difficile* and why they are produced are not known. However, the syntheses of these toxins are triggered by an event that occurs at high cell density during the stationary phase. Thus, the benefit of these toxins to *C. difficile* may be related to stationary phase-associated events such as digestion of complex biomolecules to generate readily available nutrients to sustain the high cell density during stationary phase, detoxification of harmful metabolites, and competition with other gut microflora for survival

advantage. Nutrients may become limited at high cell density. These toxins are hydrolytic with enterotoxic and/or cytopathic properties. In the mammalian host, it is possible that these toxins are released to proteolyze tissues, thereby releasing readily metabolisable nutrients to sustain the burgeoning cell density. CodY, is a highly conserved pleiotropic transcription factor in low G+C bacteria that control genes involved in adjustment to poor growth conditions (194). Dineen, et al. reported that CodY repressed the toxin genes expression in *C. difficile* (51), suggesting a link between nutrient limitation and the expression of the *C. difficile* toxin genes. However, in this study nutrient limitation was not a condition that preceded and precipitated the activation of toxin synthesis. This is because the experiments in this study were conducted under conditions of abundant nutrients in the medium. Thus, nutrient limitation was neither a factor nor a co-factor in the activation of toxin synthesis. It is possible that these toxins may be produced to release nutrients in anticipation of an impending nutrient limitation and starvation associated with high cell density. Alternatively, it can be speculated that these toxins are produced to release nutrients to ensure adequate nutrient supply at high cell density. The toxins may also be produced to detoxify harmful metabolic wastes that may build up during anaerobic growth or harmful molecules produced by other gut flora. The effect of the toxins on other gut microflora has not been studied and it is possible that these toxins may have a deleterious effect on other gut flora. In the highly competitive environment of the colon, release of a molecule that could potentially harm or impede the successful colonization of the competitor may be

advantageous. I plan to pursue this line of research to provide more insight into the natural history of *C. difficile* and how it affects the dynamics of the gut microflora.

Based on all the possible reasons presented above regarding why *C. difficile* produces the toxins, it is plausible to think that the syntheses of these toxins must be coordinated by the bacterial population producing it. This ensures that the beneficial effects of producing the toxins are maximized. For instance, if the toxins are produced to harm other competing bacteria, a coordinated process that enables a large population of *C. difficile* cells producing the toxins at the same time would be more effective. This is because a large amount of the toxins will be made available to successfully eliminate the competing bacteria. This concept is supported by the data from this study demonstrating that the *C. difficile* toxin synthesis is mediated by quorum signaling. Furthermore, it appears obvious from the natural history of *C. difficile* that when antibiotic therapy wipes out the normal gut flora, the overgrowth of *C. difficile* may lead to rapid accumulation of the quorum signal in the colon and induce toxin synthesis. In the presence of the normal gut flora, the cell density of *C. difficile* is reduced and so the quorum signal produced does not reach the critical concentration necessary to activate toxin synthesis. This may explain why *C. difficile* do not cause disease as a commensal.

In conclusion, I have provided evidence demonstrating for the first time that toxin synthesis in *C. difficile* is regulated by quorum signaling. This regulation is independent of TcdC and mediated by the release of a small heat-stable factor that accumulates during growth, and ultimately leads to activation of toxin synthesis. The toxin-inducing factor was precipitated from the stationary phase culture supernatant

fluid and partially purified by HPLC. Efforts are being made to identify this toxin-inducing factor, which would lead to understanding of the mechanism involved in regulation of toxin synthesis in *C. difficile*. Such knowledge may lead to the discovery of novel therapeutic targets that could be harnessed for the treatment of both initial and recurrent *C. difficile* infections.

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VITA

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