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REVIEW

Large Clostridial Toxins: Mechanisms and Roles in Disease

Kathleen E. Orrell,[a,b] Roman A. Melnyk[a,b]

[a]Molecular Medicine Program, The Hospital for Sick Children Research Institute, Toronto, Ontario, Canada
[b]Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

SUMMARY Large clostridial toxins (LCTs) are a family of bacterial exotoxins that infiltrate and destroy target cells. Members of the LCT family include *Clostridioides difficile* toxins TcdA and TcdB, *Paeniclostridium sordellii* toxins TcsL and TcsH, *Clostridium novyi* toxin TcnA, and *Clostridium perfringens* toxin TpeL. Since the 19th century, LCT-secreting bacteria have been isolated from the blood, organs, and wounds of diseased individuals, and LCTs have been implicated as the primary virulence factors in a variety of infections, including *C. difficile* infection and some cases of wound-associated gas gangrene. Clostridia express and secrete LCTs in response to various physiological signals. LCTs invade host cells by binding specific cell surface receptors, ultimately leading to internalization into acidified vesicles. Acidic pH promotes conformational changes within LCTs, which culminates in translocation of the N-terminal glycosyltransferase and cysteine protease domain across the endosomal membrane and into the cytosol, leading first to cytopathic effects and later to cytotoxic effects. The focus of this review is on the role of LCTs in infection and disease, the mechanism of LCT intoxication, with emphasis on recent structural work and toxin subtyping analysis, and the genomic discovery and characterization of LCT homologues. We provide a comprehensive review of these topics and offer our perspective on emerging questions and future research directions for this enigmatic family of toxins.

KEYWORDS *Clostridium difficile*, large clostridial toxin, toxin, toxin-mediated diseases, toxin-receptor interaction

INTRODUCTION

Clostridia are a polyphyletic class of anaerobes that are prolific producers of toxins. Well-known clostridial toxins include pore-forming toxins, such as *Clostridium perfringens* epsilon toxin (Etx) (1), binary toxins, such as *Clostridioides difficile* binary toxin

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Address correspondence to Roman A. Melnyk, roman.melnyk@sickkids.ca.

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TABLE 1 Sequence identity and similarity of LCT holotoxins

	% sequence identity (% similarity)[a]				
LCT	TcdA	TcdB	TcsH	TcsL	TcnA
TcdB	46 (66)				
TcsH	77 (87)	48 (68)			
TcsL	46 (66)	76 (88)	49 (70)		
TcnA	31 (51)	30 (50)	32 (52)	31 (50)	
TpeL	41 (61)	39 (60)	42 (61)	40 (61)	31 (51)

[a]Sequence identity and similarity were calculated using Water (EMBOSS) local alignment. LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* VPI 9048, *C. novyi* 19402, and *C. perfringens* JGS1495. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138).

(CDT) (2), and large clostridial toxins (LCTs) (3). LCTs are a family of six bacterial exotoxins secreted by Gram-positive, spore-forming clostridial species. Members of the LCT family include *Clostridioides difficile* toxins TcdA and TcdB, *Paeniclostridium sordellii* toxins TcsL and TcsH, *Clostridium novyi* toxin TcnA, and *Clostridium perfringens* toxin TpeL. LCTs were first grouped together as a family of related toxins on basis of their large size (>200 kDa), similarities in primary structure (Table 1), and unusual ability to induce profound changes in cell morphology (3). Members of the LCT family have been implicated as the primary virulence factors in a variety of human and animal infections, including *C. difficile* infection (CDI), some cases of wound-associated gas gangrene, toxic shock syndrome, and severe soft tissue infections in injection drug users (4–7).

LCTs are single-chain multidomain polypeptides with similar gene organization, regulation, and overall domain architecture. Clostridia express LCTs in response to various environmental and physiological signals, enabling LCTs to infiltrate and ultimately destroy eukaryotic cells to promote bacterial infection. Symptoms of infection are thought to arise owing to the cytosolic delivery of the N-terminal glycosyltransferase domain (GTD) through concerted actions of the cysteine protease domain (CPD), the central translocation and receptor-binding domain (T domain) and combined repeating oligopeptide (CROP) (8) (Fig. 1). In brief, LCTs first bind target receptors on host cells, and become internalized into vesicles. The internalized LCT-containing vesicles subsequently become acidified. In response to acidic pH, conformational changes occur within LCTs, triggering translocation of the glycosyltransferase and cysteine protease domain across the endosomal membrane and into the cytosol. The cysteine protease domain autocatalytically cleaves and releases the glycosyltransferase from the rest of the polypeptide, freeing the glycosyltransferase to access membrane-tethered Rho and Ras guanine triphosphatases (GTPases), leading first to cytopathic effects (cell rounding) and later to cytotoxic effects (cell death).

Here, we provide a comprehensive review of the LCT family. We detail seminal contributions spanning the 19th, 20th, and 21st centuries that have established LCTs as potent poisons, highlight recent structural work and toxin subtyping analysis to provide a thorough understanding of LCT structure, function, diagnostic, and therapeutic development, and discuss the burgeoning field of LCT homologue identification and characterization. We end this review by providing our perspective on pressing questions and pertinent future research directions.

ROLE OF LCTs IN CLOSTRIDIAL INFECTION AND DISEASE

C. difficile, TcdA, and TcdB

C. difficile was first described by I. C. Hall and E. O'Toole in 1935 (9). During their investigations of the intestinal microbiota of newborns, Hall and O'Toole isolated *C. difficile* from the stools of healthy infants. The researchers found that cell-free supernatants of *C. difficile* cultures were lethal to a variety of animals (10), suggesting the presence of secreted factors that were toxic toward animals. Hall and O'Toole named the bacterium *Bacillus difficilis*: *Bacillus* for the rod-like morphology and *difficilis* for the difficulty in cultivating the anaerobic bacterium. Since then, the bacterium formally known as *B. difficilis* has been renamed

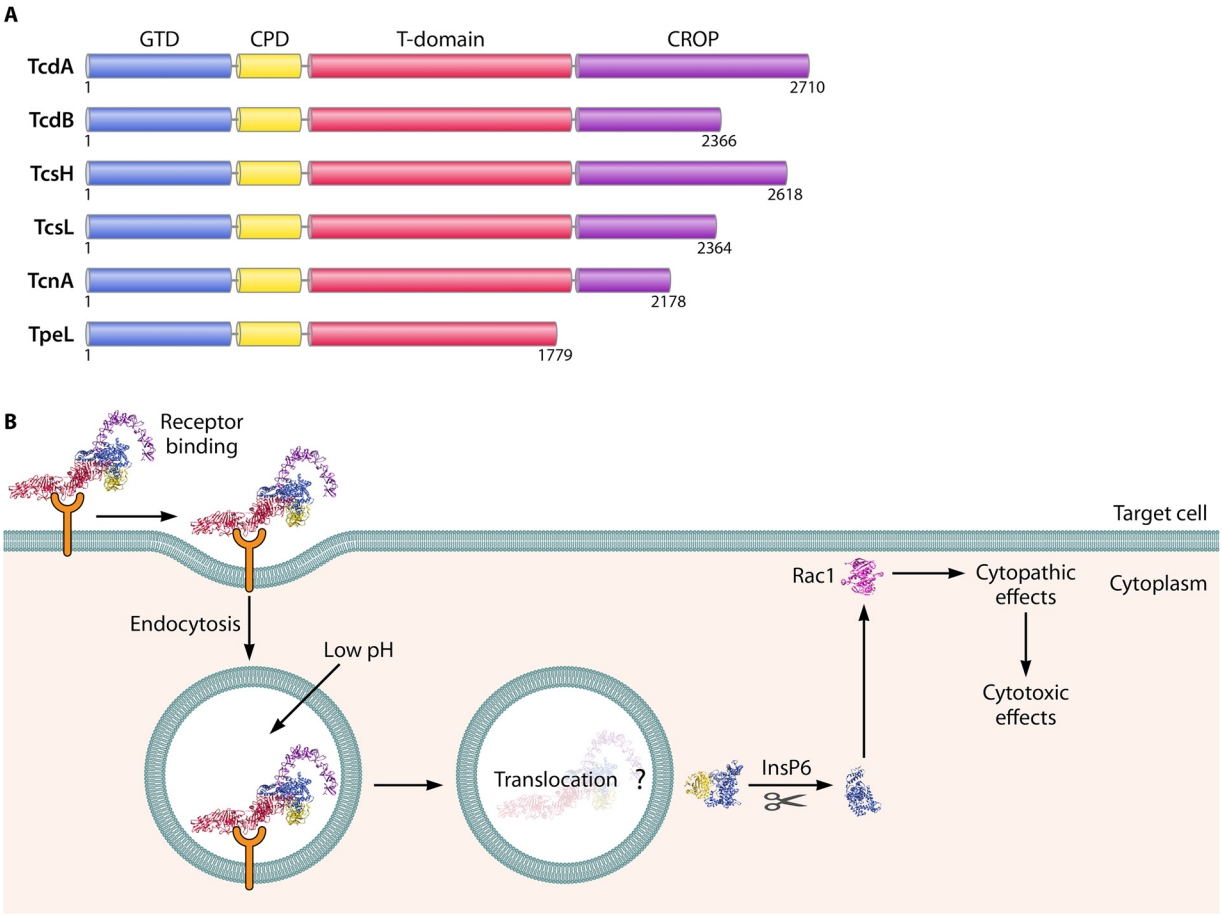

FIG 1 LCT domain architecture and mechanism of action. (A) Individual domains are colored as follows: glycosyltransferase domain (GTD) in blue, cysteine protease domain (CPD) in yellow, translocation and receptor-binding domain (T domain) in red, and combined repeating oligopeptide (CROP) in purple. (B) Schematic illustrating the major steps of LCT intoxication of host cells, using TcdB (PDB 6OQ5).

several times. First, to *Clostridium difficile*, then to *Peptoclostridium difficile* (11), and finally to *Clostridioides difficile* in 2016 by Lawson et al. (12).

Because *C. difficile* was originally isolated from healthy infants, the bacterium was considered a normal component of the intestinal flora in humans. In the 1970, J. G. Bartlett was the first to determine that *C. difficile* was the causative agent of *C. difficile* infection (CDI) (13–15). CDI is characterized by damage and injury of the colonic epithelium, with clinical symptoms ranging from diarrhea to pseudomembranous colitis and toxic megacolon (16). Bartlett's work connected years of puzzling findings on antibiotic-associated colitis in guinea pigs and hamsters, first noted during animal studies of penicillin during World War II, and the rapid increase of antibiotic-associated colitis in hospitals during the 1950s, 60s, and 70s. In animal and human studies, Bartlett and colleagues identified undescribed toxins in the stools of infected patients (14, 15). The toxic components were later identified as TcdA, toxin *C. difficile* A, or simply toxin A/toxA, and TcdB, toxin *C. difficile* B, or simply toxin B/toxB (17–22).

CDI is the leading cause of antibiotic-associated diarrhea in the developing world (23), and in the United States alone, there are approximately a quarter of a million cases of CDI annually, resulting in 12,800 deaths and attributable health care costs of $1 billion (23). CDI is most prevalent in hospitalized elderly patients (>65 years) who have recently taken antibiotics (24). In animals, antibiotic usage has also been identified a major risk factor for CDI (25). It has been hypothesized that antibiotic usage wipes out the normal gut microflora, enabling *C. difficile* to colonize the gastrointestinal tract, eventually leading to toxin production (26). Although *C. difficile* is considered

TABLE 2 Large clostridial toxins: overview

Organism	Toxin	Mol wt (kDa)	Biological activity	Role in infection
C. difficile	TcdA	308	Enterotoxic[a] (269)	Primary virulence factor in TcdB$^-$ TcdA$^+$ strains, less central role in TcdB$^+$ TcdA$^+$ strains
	TcdB	270	Enterotoxic[a] (270)	Primary virulence factor in TcdB$^+$ TcdA$^{(+/-)}$ strains
P. sordellii	TcsL	270	Necrotizing, edematizing (45)	Primary virulence factor in TcsL$^+$ TcsH$^{(+/-)}$ strains
	TcsH	299	Hemorrhaging (45)	Unknown
C. novyi	TcnA	250	Necrotizing, edematizing (271)	Primary virulence factor in *C. novyi* type A and B infections
C. perfringens	TpeL	206	Unknown	May enhance virulence of *C. perfringens* type G strains

[a]In some animal models, TcdA and TcdB also cause extraintestinal damage (272). CDI-related extraintestinal effects are extremely rare and poorly understood (273, 274).

a hospital-acquired infection, the epidemiology of CDI is changing, with increasing reports of community-acquired CDI in populations without established risk factors (27). Multiple reports have also identified *C. difficile* in livestock and in the food chain and have suggested that the presence of *C. difficile* in the agricultural industry may provide a reservoir for common community-acquired CDI (25, 28).

In animal models, TcdA and TcdB recapitulate the symptoms associated with CDI, including disruption of tight junctions, epithelial cell death, and mucosal inflammation (16), and are thus believed to be the major virulence factors in infection (16) (Table 2). Furthermore, *C. difficile* strains lacking TcdA and TcdB are avirulent and nonpathogenic (29, 30), and TcdA and TcdB levels correlate with the severity of *C. difficile* infection in epidemic *C. difficile* strains (31, 32). It remains contentious whether TcdA or TcdB is the major virulence factor in infection. In support of TcdB as the major virulence factor, TcdA$^-$ TcdB$^+$ clinical isolates cause CDI in in humans (33, 34). Additionally, laboratory TcdA$^-$ TcdB$^+$ strains cause CDI in animal models (29, 30, 35), and TcdB alone is directly responsible for severe intestinal damage (36). In contrast, laboratory TcdA$^+$ TcdB$^-$ strains are attenuated in virulence compared to TcdA$^+$ TcdB$^+$ strains, and TcdA alone is capable of only causing minimal intestinal damage in animal models (36). Recently, the first ever TcdA$^+$ TcdB$^-$ clinical isolate was isolated from an individual with antibiotic-associated diarrhea (37). The existence of a TcdA$^+$ TcdB$^-$ clinical strain suggests that TcdA may be capable of causing CDI, although it is not yet clear if TcdA can cause more severe disease symptoms. Notably, hamsters infected with the TcdA$^+$ TcdB$^-$ strain did not recapitulate the clinical symptoms associated with human infection, suggesting potential limitations of animal models in understanding and defining the role of *C. difficile* toxins in human infection.

P. sordellii, TcsL, and TcsH

P. sordellii was first isolated by A. Sordelli in 1922 (5). Sordelli isolated the bacterium from acute edematous human wound infections and named it *Bacillus oedematic sporogenes*. The name of the bacterium was derived on the basis of shared features to *Bacillus oedematiens* (presently known as *C. novyi*), which causes edema (fluid retention and swelling), and *Bacillus sporogenes*, which has a similar rod-like morphology. To avoid confusion with *B. oedematiens* and *B. sporogenes*, the bacterium was renamed *Bacillus sordellii* in 1927, in honor of Sordelli (38), and later *Clostridium sordellii* (39, 40). Recently, the bacterium was reclassified as a species of the genus *Paeniclostridium*, a new closely related genus to *Clostridium* (41).

TcsL, toxin *C. sordellii* lethal, also known as lethal toxin (LT), and TcsH, toxin *C. sordellii* hemorrhagic, also known as hemorrhagic toxin (HT), were first described in 1969 by Arseculeratne and colleagues as two independent toxins secreted by *P. sordellii* with edematizing and hemorrhagic activities (42). In 1987, Popoff purified TcsL and described TcsL as an ~250-kDa cytotoxin that was immunologically related to TcdB (43). One year later, Martinez and Wilkins purified and characterized TcsH, describing it as an ~300-kDa protein that was immunologically similar to TcdA (44).

P. sordellii has been implicated in a myriad of sporadic infections in both humans and animals which are characterized by a mild or completely absent inflammatory response (45). *P. sordellii* has been associated in wound-associated gas gangrene in

humans and animals (46, 47). Gas gangrene is typified by large amounts of gas, which can form bubbles and blisters in tissue that can lead to necrotizing infection and tissue death. More recently, *P. sordellii* infections have been identified in deep tissue infections (most often in injection drug users), and during childbirth, abortion, and surgery (5, 48–51). *P. sordellii* soft tissue infections in injection drug users are characterized by a rapid onset of illness, massive edema, and, in some cases, necrotizing fasciitis, also known as flesh-eating disease (48, 49). In toxic shock syndrome, *P. sordellii* colonizes the genital tract and causes a rapidly fatal onset of infection. Toxic shock syndrome is characterized by several clinical features, including leukocytosis, edema, and refractory hypotension (5, 50, 51).

Most pathogenic strains of *P. sordellii* produce TcsL, while few produce TcsH, suggesting that TcsL has a more central role in virulence than TcsH (52–54) (Table 2). In support of TcsL as the major virulence factor in *P. sordellii* infections, animal studies have demonstrated that TcsL alone causes extensive tissue edema and death, particularly in the lung vascular endothelium (55, 56), and inactivation of the *tcsL* gene prevents mice from developing tissue edema or dying (57). Amimoto et al. have, however, shown that vaccination with toxoids of both TcsH and TcsL was required to protect guinea pigs against a *P. sordellii* spore challenge (58), suggesting a possible role of TcsH in virulence. In *P. sordellii* culture supernatants, however, TcsH has been shown to account for a marginal amount of toxicity, with ~98% of supernatant toxicity attributable to TcsL, suggesting that TcsH may have a minimal, if any, role in infection (52).

C. novyi and TcnA

C. novyi was first isolated in the late 19th century by F. G. Novy (4). An excellent historical review of *C. novyi* is provided by Aronoff and Kazanjian (4). In brief, to determine the bacteriological components of food substances, Novy injected nuclein isolated from milk into rabbits. Unexpectedly, the rabbits developed septicemia with malignant edema and rapidly died. From the deceased rabbits, Novy was able to, albeit with great difficulty due to its extreme oxygen sensitivity, cultivate and isolate the bacterium responsible for infection and death, naming it *Bacillus novyi* in 1897. The bacterium was again isolated in 1915 from a combat wound in a soldier who later developed gas gangrene and named *Bacillus oedematiens* (59). In 1923, the bacterium was formally reclassified as *Clostridium novyi* (60).

C. novyi alpha toxin, also known as TcnA for toxin *C. novyi* alpha, or simply alpha toxin, was first isolated from *C. novyi* by Izumi et al. in 1983 and was shown to have lethal and edematizing activity in mice (61, 62). TcnA was later shown to have similar cytopathic effects and sequence homology to other members of the LCT family (3, 63, 64).

C. novyi is a rare pathogen of both animals and humans (46). Owing to its extreme oxygen sensitivity, *C. novyi* is very difficult to cultivate, which may contribute to the rarity in which *C. novyi* is implicated in infection. Like *P. sordellii*, *C. novyi* has been implicated in some cases of wound-associated gas gangrene that resulted in lethal infections of deep soft tissue (46, 47). *C. novyi* has also been implicated in hepatic damage and infectious necrotic hepatitis in animals, also known as black disease, due to the dark discoloration of subcutaneous tissue caused by severe congestion of blood vessels (65, 66). Infectious necrotic hepatitis is usually accompanied by subcutaneous edema, hemorrhaging, and necrotic lesions in the liver (65). More recently, *C. novyi* has been identified as the causative agent in severe soft tissue infections in injection drug users (67–69). *C. novyi* infection in injection drug users has similar clinical features as infection with *P. sordellii*, including leukocytosis, edema, and refractory hypotension, which can lead to necrotizing infection (67–69).

TcnA is produced by *C. novyi* type A and B (Table 2). *C. novyi* type A is associated with gas gangrene infections in humans and animals and infections in injection drug users (67–69). *C. novyi* type B is associated with necrotic enteritis in animals (66, 70, 71). TcnA is believed to be the major virulence factor in *C. novyi* type A and B infections, largely owing to its lethal and edematizing activity *in vitro* and *in vivo* in animal models (4) (Table 2). For livestock infected with necrotic hepatitis, detection of the *tcnA* gene has

been used to confirm *C. novyi* type B infection (66). Interestingly, *C. novyi* infection in injection drug users has very similar clinical manifestations as those in patients with *P. sordellii*-induced toxic shock syndrome, suggesting that both diseases may be mediated by toxins with similar biochemical and biological activities (4).

C. perfringens and TpeL

C. perfringens was first isolated near the end of the 19th century by two independent research groups. For an excellent review on the history and isolation of *C. perfringens*, please consult Rood et al. (72). In brief, in 1891, W. H. Welch isolated *C. perfringens* from the blood and organs of a deceased male who had died of an aortic aneurism (73). Concurrently, M. P. Achalme isolated *C. perfringens* from a patient with acute articular rheumatism (74). Welch and colleagues named the bacterium *Bacillus aerogenes capsulatus* (*aerogenes* for air/gas producing and *capsulatus* for capsule), and Achalme named the bacterium *Bacillus phlegmonis emphysematosae* (*phlegmonis* for phlegmon, an area of acute inflammation in soft tissue, and *emphysematosae* for emphysema, a condition of abnormal enlargement of tissues). Since then, *C. perfringens* has been renamed multiple times before the formal adoption of *Clostridium perfringens* (*perfringens* for per, meaning through, and frango for burst) in the 1930 (75, 76).

C. perfringens large toxin, also known as TpeL for toxin *perfringens* large, is the most recently discovered LCT. TpeL was isolated from *C. perfringens* culture filtrate in 2007 by Amimoto et al. (77). The researchers noted that TpeL had sequence homology to other LCTs and was toxic when injected into mice.

C. perfringens has been implicated in numerous diseases in humans and animals (78). Like *P. sordellii* and *C. novyi*, *C. perfringens* is associated with some cases of wound-associated gas gangrene, with reports suggesting that *C. perfringens* is responsible for up to 90% of all clostridium-mediated gas gangrene (46, 47). In humans, *C. perfringens* is one of the leading causes of bacterium-mediated food poisoning (79) and is associated with antibiotic-associated diarrhea (80) and necrotizing enterocolitis, a lethal infection characterized by profound inflammation of the intestine that occurs mostly in neonates (81). In animals, particularly poultry, *C. perfringens* is implicated in severe and often fatal disease, including necrotic enteritis, which costs the agricultural industry in excess of two billion dollars per year in the United States alone (82). Necrotic enteritis, also known as pulpy kidney or overeating disease, is an acute enterotoxemia that has clinical manifestations that differ between animals. Clinical manifestations include enterocolitis, a soft consistency of the kidney, and encephalomalacia within the brain (83, 84).

TpeL is present in isolates of *C. perfringens* types B, C, and G (85–87) (Table 2). The majority of *C. perfringens* type B and C strains are associated with hemorrhagic and necrotic enteritis in animals and enteritis necroticans in humans, respectively (88). Chen and McClane have shown that natural production levels of TpeL in *C. perfringens* type C supernatants contribute to cytotoxic activity, suggesting a potential role of TpeL in type C infections (87). However, there is no direct evidence that TpeL contributes to *C. perfringens* type B and C infections. Strong evidence instead supports a central role for other *C. perfringens* toxins, including β-toxin and ε-toxin in type B infections and β-toxin and enterotoxin (CPE) in type C infections (88). *C. perfringens* type G strains are associated with necrotic enteritis in poultry, and there is some evidence to suggest that TpeL may contribute to virulence in type G strains (89). TpeL-positive strains are associated with a more rapid course of infection and a higher fatality rate than TpeL-negative strains (89). *C. perfringens* toxin NetB is, however, believed to be the major virulence factor in type G infections (90). It is yet unclear if TpeL contributes to virulence, by acting either alone or synergistically with NetB.

LCT GENE ORGANIZATION AND REGULATION

In 1996, a 19.6-kb chromosomal region termed the pathogenicity locus (PaLoc) was uncovered in *C. difficile* strain VPI 10463 (91). The *C. difficile* PaLoc contains genes for both TcdA and TcdB as well as three accessory genes: *tcdR*, *tcdC*, and *tcdE* (Fig. 2). The

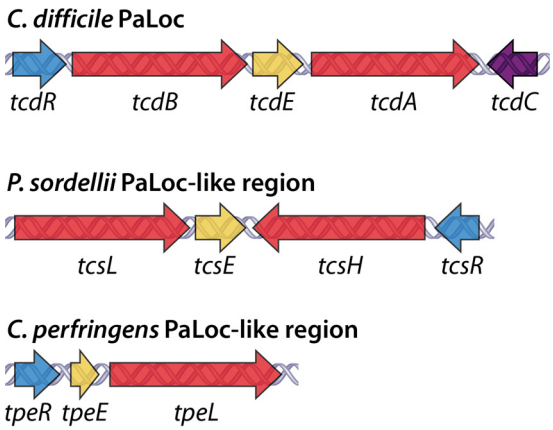

FIG 2 LCT gene organization. The PaLoc of *C. difficile* and PaLoc-like regions of *P. sordellii* and *C. perfringens*, first identified in *C. difficile* strain VPI 10463, *P. sordellii* strain VPI 9048, and *C. perfringens* strain ATCC 3626. LCT genes are colored red. Accessory genes are colored as follows: alternative σ factor (*tcdR/tcsR/tpeR)* in blue, anti-σ factor (*tcdC*) in purple, and holin-like protein (*tcdE/tcsE/tpeE*) in yellow.

accessory gene *tcdR* encodes an alternative RNA polymerase sigma (σ) factor, TcdR. TcdR belongs to the σ^{70} family of alternative σ factors, which includes alternative σ factors of other pathogenic clostridia, such as BotR of *C. botulinum* and UviA of *C. perfringens* (92). TcdR is a positive regulator of LCT expression and is critical for the initiation of TcdA and TcdB gene expression (92). The accessory gene *tcdC* encodes an anti-σ factor, TcdC. TcdC negatively regulates LCT expression by directly interacting with TcdR or the TcdR-RNA polymerase holoenzyme (93, 94). TcdC may have a role in controlling TcdA and TcdB levels, as *C. difficile* strains with frameshift mutations or deletions of the *tcdC* gene have increased toxin production (31, 95). The extent to which TcdC contributes to LCT regulation remains controversial, as several studies have demonstrated that TcdC only moderately contributes to LCT expression (96, 97). The accessory gene *tcdE* encodes a bacteriophage holin-like protein, TcdE. Holins are bacteriophage-encoded membrane proteins that oligomerize and form holes in the host cell membrane to release progeny phage (98). Multiple reports have suggested that holin-like proteins may be responsible for release of proteins from bacteria (99–101). Based on sequence homology to holins and the lack of obvious export signatures on TcdA and TcdB, TcdE was proposed to regulate TcdA and TcdB release from *C. difficile* (102). In 2012, Govind and Dupuy demonstrated that TcdE facilitates the release of TcdA and TcdB without inducing cell lysis (103), providing the first experimental evidence of holin-like proteins mediating the secretion of proteins from bacteria. In addition to holin-dependent secretion, there is evidence that *C. difficile* can also release TcdA and TcdB by bacteriolysis mediated by the cell surface peptidoglycan hydrolase Cwp19 (104, 105).

The *C. difficile* PaLoc is located at the same chromosomal site in the majority of *C. difficile* strains (91, 106, 107). *C. difficile* strains lacking the PaLoc harbor a 75/115-bp noncoding region, are nontoxigenic, and do not cause disease (91, 108). The *C. difficile* PaLoc can be transferred from toxigenic to nontoxigenic strains and thus has characteristics of a mobile genetic element (109). There is considerable genetic variation in PaLocs of different *C. difficile* strains, including truncated and monotoxin PaLoc variants (110, 111). Genetic variations of the *C. difficile* PaLoc have been assessed by toxinotyping, a PCR restriction fragment length polymorphism method that distinguishes strains into 34 toxinotypes based on the PaLoc (107). Based on genetic studies of the PaLoc, it has been suggested that the bitoxin PaLoc (i.e., containing TcdA and TcdB) may have evolved from the merging of two monotoxin PaLocs (i.e., containing either TcdA or TcdB) (111).

A PaLoc-like region has been identified in strains of *P. sordellii* (112) and *C. perfringens* (113) (Fig. 2). In contrast to the chromosomally localized *C. difficile* PaLoc, the *P. sordellii* and

C. perfringens PaLoc-like regions are located on conjugative plasmids (54, 85, 114, 115). Both the *P. sordellii* and *C. perfringens* PaLoc-like regions contain accessory genes that are homologous to the *C. difficile* genes *tcdR* and *tcdE* and encode TcdR- and TcdE-like proteins, respectively. In *P. sordellii*, the genes *tcsR* and *tcsE* encode TcsR and TcsE (112), and in *C. perfringens*, the genes *tpeR* and *tpeE* encode TpeR and TpeE (113) (Fig. 2). TcdR, TcsR, and TpeR are all members of the σ^{70} family of alternative σ factors (113). TcsR and TpeR have been demonstrated to regulate the expression of TcsL and TpeL, respectively (112, 113). Notably, TcdR and TcsR are functionally interchangeable, while neither TcdR or TcsR can be functionally exchanged with TpeR (113). TcdE and TcsE belong to superfamily 4 of bacteriophage holins, and TpeE belongs to the DUF2762 superfamily of bacteriophage holins (116). Recently, TpeE was demonstrated to facilitate the secretion of TpeL without cell lysis, supporting a model of holin-dependent toxin secretion in *C. perfringens* (117). Due to the similarities in structure and function of TcdR and TcsR and of TcdE and TcsE, it has been suggested that the *C. difficile* PaLoc and *P. sordellii* PaLoc-like region may share a recent common ancestor, while the PaLoc-like region of *C. perfringens* may be more divergent (113). This is consistent with *C. difficile* and *P. sordellii* both belonging to the *Peptostreptococcaceae* family, while *C. perfringens* belongs to the *Clostridiaceae* family.

There are few studies on *tcnA* gene organization. In *C. novyi*, the *tcnA* gene is phage localized (118), and toxigenic strains of *C. novyi* can transduce nontoxigenic strains of *C. novyi* to produce TcnA, indicating mobile transfer of the *tcnA* gene (119). It is not currently known whether the *tcnA* gene resides within a PaLoc-like region or how *tcnA* expression is regulated.

The *C. difficile* PaLoc and the *P. sordellii* and *C. perfringens* PaLoc-like regions are influenced by environmental, physiological, and nutrient signals. Regulation of the PaLoc has been extensively studied in *C. difficile* (120) and has been studied in less detail in *P. sordellii* and *C. perfringens* (113). In *C. difficile*, *P. sordellii*, and *C. perfringens*, LCT production follows a similar pattern of temporal expression, with increases in toxin production as bacterial cells approach the stationary phase of growth (113, 121, 122). The regulatory pathways that coordinate LCT production and bacterial growth are not well understood. Quorum sensing has been proposed to coordinate LCT production and bacterial growth, enabling the clostridia to modulate LCT production in response to bacterial cell densities (113). In *C. difficile*, *P. sordellii*, and *C. perfringens*, LCT production is repressed by glucose (112, 113). In *C. difficile*, the catabolite control protein A (CcpA) mediates the bacterium's response to glucose, by directly and indirectly inhibiting transcription of numerous regulators (123, 124). Both *P. sordellii* and *C. perfringens* harbor CcpA homologues (113), which may mediate the glucose response in these organisms, although this has not been experimentally demonstrated. It has been proposed that TcdA and TcdB production is triggered by *C. difficile* in response to particular states of nutrient availability during infection and that toxin production improves nutrient availability for the bacterium, thus enabling *C. difficile* to persist and cause damage to the host (120). The integration of regulation, toxin production, and infection in *P. sordellii* and *C. perfringens* is not well understood.

In addition to growth conditions and glucose, the *C. difficile* PaLoc is regulated by a complex array of environmental and physiological factors through several global regulators, including CodY, SigD, PrdR, Rex, RstA, and Spo0A (120, 125–128). These environmental and physiological factors include temperature (129), amino acids such as proline and cysteine (130), short-chain fatty acids such as butyric acid that are present in the gut (131), subinhibitory concentrations of antibiotics (132, 133), stress responses (134), and sporulation (135, 136). Interestingly, several studies have reported that regulation of the *C. difficile* PaLoc is strain specific, with the PaLoc across different strains responding differently to antibiotics (120) and sporulation cues (126, 128). Furthermore, epidemic *C. difficile* RT027 strains encode a binary toxin locus, CdtLoc, which has been suggested to regulate PaLoc expression (137).

LCT Classification and Subtyping Analysis

With the increasing number of sequenced bacteria, LCTs are being detected in an expanding number of clostridial genomes. A key question that has emerged is whether

LCT genes vary across clostridial strains, and if so, if sequence variation contributes to biological and functional differences, which may manifest in different clinical presentations of infection and disease. Recently, Mansfield et al. (138) and Shen et al. (139) and proposed a method of sequence-based subtyping of TcdA and TcdB to enable more accurate predictions of variations in toxin activity. The subtyping analysis on a larger set of *C. difficile* genomes by Mansfield et al. partitions TcdA and TcdB into 7 and 12 distinct subgroups, referred to as A1-7 and B1-12, respectively (138). TcdA and TcdB from *C. difficile* VPI 10463, which has been the reference strain since the 1980s, belong to the A1/B1 subtype, and TcdA and TcdB from epidemic strains, such as RT027, tend to cluster outside the A1/B1 subtype (138). Interestingly, TcdA variants differ mainly in the number of repeats in the C-terminal repetitive region, while TcdB has diversified through extensive homologous recombination throughout its entire sequences (138). Variations in TcdB sequence have been correlated with distinct antigenic, receptor-binding, and phenotypic properties, which will be reviewed in the upcoming sections on LCT structure and function. To the best of our knowledge, sequence variations in TcsL, TcsH, TcnA, and TpeL across different strains have not been investigated.

STRUCTURE AND FUNCTION OF LCTs

Holotoxin Structure

Over the years, numerous structures have been solved for individual LCT domains as well as multidomain and full-length fragments of TcdA and TcdB, providing enormous insights into LCT structure and function. In 2010, the structure of full-length TcdA was determined by negative stain electron microscopy (EM) and small-angle X-ray scattering (SAXS) (140, 141). TcdA was shown to have a bilobed organization, with a globular "head" region consisting of the glycosyltransferase and cysteine protease domain, from which the T domain extends to the opposite end of the molecule. The structure of the CROP-less TcdA later confirmed the organization of the glycosyltransferase domain, the cysteine protease domain, and the T domain and enabled refinement of structural changes at neutral and acidic pH by fitting the high-resolution TcdA structure to EM maps of the TcdA holotoxin (142) (Fig. 3A and B). At neutral pH, the TcdA CROP makes structural contacts with the T domain, and at acidic pH, the CROP extends away from the T domain (Fig. 3A and B). The X-ray structure of full-length TcdB later revealed the precise positioning of the TcdB CROP at acidic pH and the dynamism of the CROP at neutral pH (143) (Fig. 3C). At acidic pH, the TcdB CROP extends ~130 Å from the base of the cysteine protease domain and T domain, curving around the glycosyltransferase domain like a hook in a "open" configuration (143). Although the full-length TcdB structure was solved at acidic pH, no major structural changes were observed for the glycosyltransferase, cysteine protease, and T domain compared to that at neutral pH (143). Notably, the glycosyltransferase domain and T domain were bound to nanobodies, which may have prevented the full suite of pH-mediated conformational changes.

Combined Repeating Oligopeptide

The combined repeating oligopeptide (CROP) is the C-terminal domain of LCTs, except the naturally CROP-less TpeL. As the name suggests, the CROP is composed of multiple repeating units: 19- to 24-amino-acid short repeats (SRs) and 29- to 31-amino-acid long repeats (LRs). The LCT CROP is variable in sequence, sharing between 27% and 75% sequence identity among LCTs (Table 3). The LCT CROP ranges in size, from 40 kDa to 100 kDa, and correspondingly, in number of SRs and LRs (Table 4). The CROPs of TcdA and TcsH are the largest, with 30 and 33 SRs and 6 and 7 LRs, respectively, while the CROP of TcnA is the shortest, with 13 SRs and 3 LRs.

At present, there is structural information for the TcdA and TcdB CROP (Fig. 3C and D). The SRs and LRs of the CROP consist of one and three β-hairpins, respectively, with a variable loop region of 7 to 10 amino acids for SRs and ~18 amino acids for LRs (144, 145) (Fig. 3D). SRs pack together, forming a solenoid-like fold, and LRs introduce kinks and curvature into the CROP structure. Using the structure of the TcdA CROP fragment

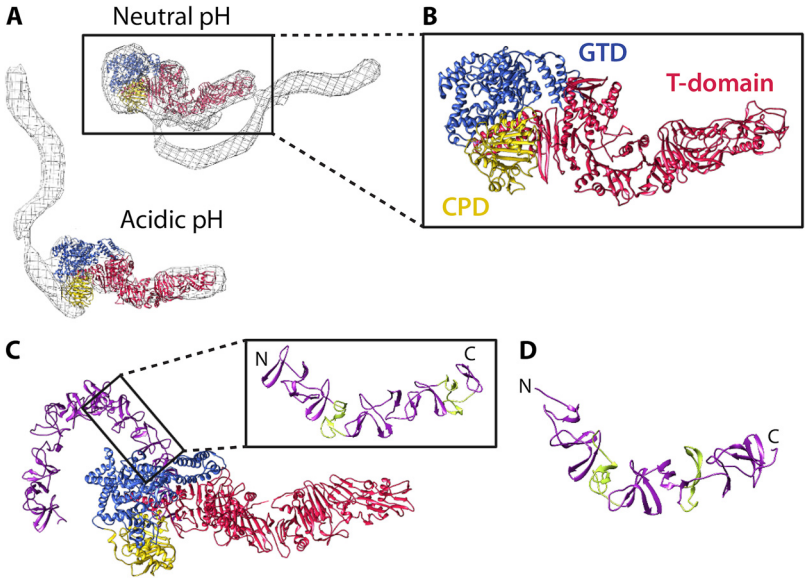

FIG 3 Structure and organization of the *C. difficile* holotoxin. (A) $TcdA_{1–1832}$ (PDB 4R04) fit into the EM map of the TcdA holotoxin at neutral and acidic pH (140) using Chimera (267). The TcdA EM maps were kindly provided by Borden Lacy and are reproduced with permission. (B) Focused view of $TcdA_{1–1832}$. (C) Structure of $TcdB_{1–2366}$ at acidic pH (PDB 6OQ5). In the focused image, short repeats (SRs) are colored purple and long repeats (LRs) are colored green. (D) Structure of a fragment of the TcdA CROP (PDB 2G7C), with SRs colored purple and LRs colored green.

as a framework, Ho et al. built models of the full-length TcdA and TcdB CROPs (144). Ho et al. were the first to propose the S-shape and hook-shape structures of the TcdA and TcdB CROPs, respectively; these predictions were later supported by EM (140) and X-ray crystallography (143) (Fig. 3A, B, and C). Furthermore, work by Chen et al. has shown that the TcdB CROP has a C-terminal SR hinge region that confers conformational mobility to the CROP, enabling the CROP to adopt "open" and "closed" conformations at acidic and neutral pHs, respectively (143).

The CROP has long been assumed to mediate cellular binding and entry into host cells. Antibodies against the CROP prevent cell-surface binding and toxicity (146, 147), and recently, the TcdA CROP was shown to mediate binding to the colonic epithelium (148). Due to homology with streptococcal glycosyltransferases, the CROP was first postulated to mediate LCT attachment to cell surfaces by binding to carbohydrate moieties (20), a common strategy of bacterial and viral pathogens (149). In support of the CROP as a carbohydrate binding region, the TcdA and TcdB CROPs have been demonstrated to bind cell surface carbohydrates with low affinity (150–153). The physiological relevance of TcdA and TcdB binding to carbohydrates in the context of cellular intoxication has not been clarified. Recent work has demonstrated that the protein receptor chondroitin sulfate proteoglycan 4 (CSPG4) binds in part to the TcdB CROP (amino acids 1831 to 1850) and to the T domain (154), providing evidence that regions of the

TABLE 3 LCT CROP sequence identity and similarity

	% sequence identity (% similarity)[a]			
LCT	**TcdA**	**TcdB**	**TcsH**	**TcsL**
TcdB	37 (51)			
TcsH	68 (77)	43 (57)		
TcsL	36 (50)	75 (87)	43 (59)	
TcnA	34 (45)	27 (40)	39 (57)	31 (48)

[a]Sequence identity and similarity were calculated using Water (EMBOSS) local alignment. LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* VPI 9048, and *C. novyi* 19402. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138).

TABLE 4 LCT CROP repeating units

LCT[a]	CROP bounds	No. of short repeats	No. of long repeats	Mol wt (kDa)
TcdA	1812–2710	33	7	102
TcdB	1814–2366	21	4	64
TcsH	1812–2618	30	6	92
TcsL	1815–2364	21	4	63
TcnA	1822–2178	13	3	41

[a]LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* VPI 9048, and *C. novyi* 19402. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138).

CROP are involved in receptor binding. Presently, no other protein receptors have been identified that bind to the CROP of other LCTs.

It is clear that the CROP is not the sole LCT domain that mediates cell surface binding and entry, as was originally hypothesized. Multiple receptors have been identified that bind in the LCT T domain (148, 155–159) (reviewed in the upcoming section), including a receptor for the CROP-less TpeL (160). Functional studies with TcdA and TcdB have also demonstrated that toxin entry is attenuated or not affected by truncations or complete removal of the CROP (77, 161, 162). Interestingly, several studies have demonstrated that the LCT CROP has functions apart from binding to cell surface carbohydrates and protein receptors. These functions include aiding in holotoxin folding, stabilization, and prevention of premature autoprocessing (163–165). Recently, it was shown that the TcdB CROP directly and reversibly binds intestinal bile acids, inhibiting toxin uptake and thereby intoxication (166). Bile acids prevent TcdB from binding to cell surface receptors and induce conformational changes that enable TcdB to become more resistant to proteolytic digestion (166). Notably, intestinal bile acids do not bind to the TcdA CROP or to the CROP-less TpeL. Although the role of bile acid binding to TcdB requires further study, this work suggests that bile acids may impact the timing of TcdB intoxication of cells, modulating virulence with respect to bile acid concentration in the gastrointestinal tract. It is not yet known if bile acids bind to other LCTs to modulate cellular intoxication.

Bezlotoxumab is a neutralizing antibody against TcdB that is used in the treatment of recurrent CDI (167). To neutralize TcdB, bezlotoxumab binds two epitopes in the N terminus of the TcdB CROP (146). Notably, bezlotoxumab was generated using TcdB1 antigens (from strain VPI 10463) and has exhibited reduction in neutralization efficacy against TcdB B2/4/5 subtypes from RT027, 8864, and RT078 strains (138, 168, 169). Alignment of key residues in the epitope region across all TcdB subtypes by Mansfield et al. revealed residue changes in B2/4/5 TcdB subtypes (138), providing strong evidence that the reduced efficacy of bezlotoxumab is related to sequence variations in the epitope binding region of subtypes. TcdB subtyping is clearly essential to direct the clinical use of bezlotoxumab and other emerging toxin-targeted therapies.

Translocation and Receptor-Binding Domain

The translocation and receptor-binding domain (T domain) is an ~1,000-amino-acid domain that shares between 28% and 79% sequence identity among LCTs (Table 5; Fig. 4A). At present, there are high-resolution structures of the full-length T domains of TcdA (142) and TcdB (143, 170) and of a fragment of TcsL (158). The LCT T domain is structurally unique and, at neutral pH, is composed largely of β-sheets, with a short helical region (amino acids 956 to 1135) that extends from one end of the T domain to the other, wrapping around β-sheet structures (142) (Fig. 4A). Interestingly, although no major structural rearrangements were observed for the T domain at acidic pH, there is a loss of electron density within the helical stretch (944 to 949 and 1032 to 1047), suggesting structural flexibility of this region (143).

Several cell surface receptors have been identified that bind to the LCT T domain. Receptors for TcdA (148), TcdB (155–157), TcsL (158, 159), and TpeL (160) have been identified (Table 6), while the receptors for TcsH and TcnA remain more elusive.

TABLE 5 Sequence identity and similarity of LCT translocation and receptor-binding domains

	% sequence identity (% similarity)[a]				
LCT	TcdA	TcdB	TcsH	TcsL	TcnA
TcdB	47 (69)				
TcsH	79 (92)	48 (70)			
TcsL	47 (69)	77 (88)	48 (70)		
TcnA	28 (50)	28 (51)	28 (51)	28 (50)	
TpeL	36 (56)	33 (56)	37 (56)	34 (56)	28 (47)

[a]Sequence identity and similarity were calculated using Water (EMBOSS) local alignment. The following boundaries of the LCT T domains were used for sequence comparison: TcdA (802 to 1812), TcdB (800 to 1814), TcsH (802 to 1812), TcsL (800 to 1815), and TcnA (800 to 1822). LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* VPI 9048, *C. novyi* 19402, and *C. perfringens* JGS1495. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138).

Notably, LCTs do not bind to the same receptors, and several LCTs have been demonstrated to bind multiple receptors (Table 6).

Sulfated glycosaminoglycans (sGAGs) and low-density lipoprotein receptor (LDLR) have been identified as host cell factors that mediate cell surface binding and entry of TcdA (148) (Table 6). Both factors were identified using genome-wide CRISPR-Cas9 screens using a truncated TcdA lacking the majority of the CROP. Biolayer interferometry confirmed that sGAGs bound directly to TcdA independent of the CROP. Binding could not be directly detected between LDLR and TcdA, suggesting that TcdA and LDLR bind weakly or require other cellular factors (148). While both sGAGs and LDLR mediate cellular binding and entry, the former were suggested to be the major attachment factors in the colonic epithelium (148). The exact role of LDLR binding in the context of TcdA intoxication remains to be clarified. Due to the major function of LDLR family receptors in mediating endocytosis (171), LDLR may facilitate endocytosis of TcdA bound to sGAGs (148). It is possible that TcdA binds to structurally similar LDLR family receptors, many of which are cell surface receptors for other pathogens (160, 172, 173). In support of TcdA binding structurally similar LDLR family members, Schöttelndreier et al. have recently provided evidence that TcdA binds to low-density lipoprotein receptor-related protein-1 (LRP1) (174).

At present, three receptors for TcdB have been identified: Wnt receptor frizzled family (FZD) FZD1/FZD2/FZD7 (156), chondroitin sulfate proteoglycan 4 (CSPG4) (155), and poliovirus receptor-like protein 3 (PVRL3/nectin 3) (157) (Table 6). Competition studies have demonstrated that FZD and CSPG4 bind independently of each other, indicating distinct nonoverlapped binding sites for these two receptors (156). It is not known whether PVRL3 binds at distinct sites or competes with binding to TcdB with FZD and CSPG4. FZD binds TcdB in the central region of the T domain, with palmitoleic acid (PAM) mediating binding of TcdB and FZD by making extensive contacts with both TcdB and FZD (170) (Table 6; Fig. 4A and B). Several reports have indicated that FZD binding varies between TcdB subtypes, with B2 and B4 subtypes having reduced affinity for FZD compared to that of B1 and B3 subtypes (175–178). Mansfield et al. have shown that the FZD binding motif is not conserved in B2/4/7/10/11 subtypes, suggesting that these subtypes may all have reduced affinity for FZD (138). The exact binding sites of CSPG4 and PVRL3 on TcdB are unknown. Functional studies have indicated that CSPG4 binds to both the T domain and the CROP (154) (Table 6) and that B3 and B4 subtypes have reduced affinity for CSPG4 binding compared to that of B1 and B2 subtypes (178). Recently, truncation and mutational analysis of TcdB revealed that the GTD and CPD contribute to CSPG4 binding, suggesting that the CSPG4 binding interface may be composed of multiple TcdB domains that converge in the holotoxin (178). PVRL3 is believed to bind to the central region of the TcdB T domain (162) (Table 6). Chung et al. have demonstrated that the B2 subtype has lower affinity for PVRL3 than B1 and suggested that the CROP may modulate PVRL3 binding, although it is not yet clear how this modulation occurs (176).

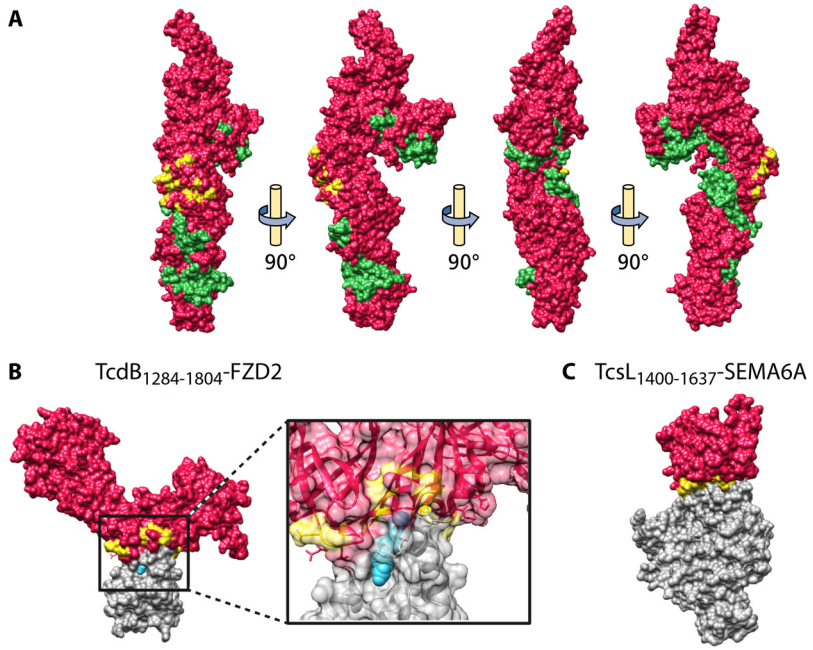

FIG 4 Structure of the LCT T domain and LCT-receptor complexes. (A) T domain of TcdB (PDB 6OQ5), with the hydrophobic helical stretch (residues 956 to 1135) colored green and the interface residues for FZD2 binding colored yellow. (B) $TcdB_{1284–1804}$-FZD2 (PDB 6C0B), with focus on the receptor-binding interface. TcdB is colored red, FZD2 interface residues on TcdB are colored yellow, FZD2 is colored gray, and palmitoleic acid (PAM) is colored blue. (C) $TcsL_{1400–1637}$-SEMA6A (PDB 6WTS). TcsL is colored red, SEMA6A interface residues on TcsL are colored yellow, and SEMA6A is colored gray.

TcdB may utilize multiple receptors with different binding sites to broaden the selection of mammalian cells it can target. Both PVRL3 and FZDs are highly expressed on the surface epithelium of the human colon (156, 157), while CSPG4 is predominantly expressed in the multinucleated intestinal subepithelial myofibroblasts (ISEMFs) (179). The expression of PVRL3 and FZDs on the colonic epithelium suggests that PVRL3 and FZD might be the first receptors TcdB encounters when released into the lumen of the colon, and CSPG4 might serve as an important target to cause further tissue damage by exposing subepithelial myofibroblast cells. Notably, FZDs are receptors in the Wnt signaling pathway, an essential pathway for maintaining colonic stem cells (180). Healthy colonic stem cells constantly supply new colonic epithelial cells, which is central to colonic epithelial cell renewal and repair. TcdB competes with Wnt for binding to FZDs and, subsequently, inhibits Wnt signaling (156), suggesting that colonic stem cells are a potential target in *C. difficile* pathogenesis (181). Interestingly, recent work by Mileto et al. has shown that the B2 subtype can induce stem cell damage in an FZD-independent manner, suggesting the involvement of other TcdB receptors in mediating colonic epithelial damage (182). More recently, Pan et al. have also demonstrated that TcdB subtypes induce different pathological effects in mouse colonic tissue, suggesting that receptor preference can mediate colonic pathology (178).

Semaphorins 6A and 6B (SEMA6A/6B) have been identified as cellular receptors for TcsL by two independent genome-wide CRISPR-Cas9 screens (158, 159) (Table 6). SEMA6A binds to the central region of the T domain, forming a discontinuous binding interface along the T domain (158) (Table 6; Fig. 4C). Interestingly, the interface on TcsL that binds SEMA6A corresponds to the same interface on TcdB that binds FZD2, indicating that LCTs bind structurally unrelated receptors using the same receptor-binding interface (158). Furthermore, mutation of multiple residues in TcsL changes binding specificity to FZD2, suggesting that LCT receptor binding can be fine-tuned by changing key residues in the interaction surfaces (158, 159).

Intraperitoneal injection of TcsL causes major damage to lung endothelial cells, resulting in increased vascular permeability and edema in the lungs (55). Therefore,

TABLE 6 Host cell factors that mediate cell surface binding and internalization of LCTs

Host cell factor[a]	Binding region[b]	Expression in human tissue[c]	PDB[d]
TcdA			
Sulfated glycosaminoglycans (sGAGs) (148)	1–1832 (148)	Colonic epithelium (148)	NA
Low-density lipoprotein receptor (LDLR) (148)	1–1832 (148)	ND	NA
Low-density lipoprotein receptor-related protein-1 (LRP1) (174)	ND	ND	NA
TcdB			
Chondroitin sulfate proteoglycan 4 (CSPG4) (155)	1500–1850 (154)	Multinucleated intestinal subepithelial myofibroblasts (179)	NA
Frizzled family receptors (FZD)1/2/7 (156)	Discontinuous surface, 1433–1599 (170)	Colonic epithelium (156)	6C0B
Poliovirus receptor-like protein 3 (PVRL3) (157)	1372–1493 (162)	Colonic epithelium (157)	NA
TcsL			
Semaphorins (SEMA)6A/6B (158, 159)	Discontinuous surface, 1433–1601 (158)	Vascular endothelium (158, 159)	6WTS
TpeL			
LRP1 (160)	1335–1779 (160)	ND	NA

[a]LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* JGS6382 and 6018, and *C. perfringens* JGS1495. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138). Sucrase isomaltase (275) and glycoprotein 96 (Gp96) (276) have also been identified as TcdA receptors, but their lack of expression on the intestinal epithelial cells makes their physiological relevance to *C. difficile* pathogenesis unclear.

[b]Binding region indicates the minimal region demonstrated to bind to the receptor or the stretch of LCT interface residues that bind to the receptor, as determined from the LCT-receptor complex. ND, not determined if the full-length LCT was used to detect binding.

[c]Includes human tissues that are physiologically relevant for infection. ND, not determined.

[d]NA, not available.

the vascular endothelium is believed to be the primary target of TcsL *in vivo* (55). Mice coinjected with TcsL and SEMA6A fused to an Fc fragment are protected from developing fluid edema in lung tissues, indicating that blocking SEMA6A-mediated TcsL entry into cells prevents toxin-induced symptoms (158). The physiological consequences of TcsL binding SEMA are not entirely clear. TcsL binds to SEMA6A at a position partially overlapping the functional site used by the plexin A2 cognate ligand; thus, TcsL binding to SEMA6A/6B has been suggested to interfere with semaphorin-plexin signaling in the vascular endothelium (158, 159). It is not yet known whether TcsL binding inhibits or disrupts semaphorin-plexin mediated downstream signaling pathways, many of which have key roles in controlling cell shape and movement (183).

LRP1 was identified as a receptor for TpeL using a haploid genetic screen (160) (Table 6). Functional studies have indicated that LRP1 binds the C-terminal region of TpeL, although the exact binding site of TpeL on LRP1 has not been determined (160) (Table 6). In a similar manner to that of LDLR family receptors and TcdA, LRP1 has been hypothesized as an endocytic receptor for TpeL (160).

For LCTs to gain entry into cells, LCTs must be internalized. TcdB, TcsL, and TcnA are endocytosed in a dynamin- and clathrin-dependent manner (184), while TcdA endocytosis is clathrin independent but dependent on dynamin and on the host factor protein kinase C and casein kinase substrate in neurons 2 (PACSIN2) (185). Investigation into the mechanism of LCT-mediated endocytosis and the role of cell surface receptors is ongoing. Schöttelndreier et al. have provided evidence that LRP1 contributes to TcdA internalization (174) and that FZD2/7, CSPG4, and PVRL3 do not contribute to TcdB internalization, suggesting the presence of a yet-unidentified receptor that facilitates TcdB endocytosis (186). The latter finding is in direct contrast to previous reports that CSPG4 and PVRL3 facilitate receptor-mediated endocytosis of TcdB (155, 157). To account for binding to both nonendocytic and endocytic receptors, Schöttelndreier et al. (174, 186) have proposed a model for TcdA/TcdB uptake and entry. In this model, TcdA/TcdB first bind nonendocytic receptors, enriching the cell surface. Nonendocytic receptors then associate with endocytic receptors, facilitating TcdA/TcdB binding to the endocytic receptor and internalization (174, 186). This model for TcdA/TcdB internalization requires experimental testing. It is not yet known if other LCTs exploit a similar mechanism of cellular internalization.

For LCTs to modify cytosolic factors and exert their toxic effects, LCTs must escape from internalized vesicles. Early on, it was noted that blocking endosomal acidification with small molecule inhibitors of v-ATPases such as bafilomycin prevented LCT toxicity (187–189). Furthermore, acidic pH was shown to trigger conformational changes within LCTs, leading to exposure of hydrophobic surfaces and changes to protease susceptibility (187–189). The optimal pH for the hydrophobic transition of LCTs is between pH 4.0 and 5.0 (187–189), although differences in the optimal pH have been reported for a TcdB subtype (190). The requirement of endosomal acidification for toxicity and acidic pH-induced conformational changes suggest that LCTs translocate out of endosomes in a similar manner to that of other bacterial toxins, including diphtheria toxin (191) and botulinum neurotoxin (192). These bacterial toxins have long been thought to form membrane-inserted pores in the endosomal membrane using their central translocation domains that act as conduits for translocation of toxin enzymatic domains into the cytosol. Several functional studies have shown that LCTs insert into the membrane at acidic pH, leading to the release of rubidium from preloaded cells and formation of ion-conductive pores (193, 194). TcdA has been demonstrated to require cholesterol-enriched membranes for insertion, which may indicate that LCTs preferentially insert into membranes, or regions of membranes, with a distinct lipid composition. Notably, unlike diphtheria toxin (195) and botulinum neurotoxin (196), LCTs do not form stable ion-conductive pores, instead exhibiting characteristic "flickering" electrophysiological behavior, with large conductances of up to ~1 to 2 nS and lifetimes of several milliseconds (193, 194, 197).

Since the primary sequences of LCTs were determined, it was postulated that the ~172-residue marginally hydrophobic stretch near the N terminus of the T domain

(TcdB amino acids 956 to 1128) was involved in membrane insertion, pore formation, and translocation (20, 22, 140, 193, 194). In 2014, a site-directed loss-of-function mutagenesis screen was performed on conserved LCT residues in the hydrophobic region of TcdB (197). Highly sensitive residues were identified that were >100-fold defective in both pore formation and cellular toxicity when mutated (197). Several years later, regions of the LCT hydrophobic region were directly shown to insert into the membrane, demonstrating the membrane insertion propensity of the hydrophobic region (198). Additionally, an aspartate residue in the hydrophobic region, D1037 in TcdB, was identified as a part of a yet-incompletely described "pH sensor" for membrane insertion (198). A model for how the hydrophobic region may facilitate translocation was proposed, based largely on similarities in hydropathy to the translocation domain of diphtheria toxin and membrane insertion data of the LCT hydrophobic region (197, 198). This model posits that the LCT hydrophobic region forms a membrane-inserted pore, inserting as a "double dagger" of two α-helical hairpins in the membranes, with a nonhydrophobic inserting element located at the N-terminal edge of the hydrophobic region. Importantly, this model has not been experimentally tested and many details of translocation are not known, including the toxin oligomeric state and whether the glycosyltransferase and cysteine protease domain must unfold in order to translocate.

Recently, hundreds of LCT T domain homologues were identified, providing an unprecedented opportunity to gain insights into the elusive mechanism of translocation (199). LCT homologues share on average 18.6% amino acid identity with the TcdB T domain (199) and retain important LCT translocation features (199). Unlike the canonical LCT family, T domain homologues are found elsewhere in addition to clostridia and have variable domain architectures. Nearly 150 (~20% of all homologues) have an upstream glycosyltransferase and cysteine protease domain, and >300 (~40% of all homologues) have either an upstream glycosyltransferase or cysteine protease (199). The remaining 40% of homologues have an upstream sequence with no known domain annotation (199). The diversity of the upstream protein region suggests that LCT-like translocases are permissible in the types of proteins they can translocate. Several LCT T domain homologues have been functionally characterized as protein translocases, including an LCT homologue from *Serratia marcescens* (199) and two LCT homologues from *Yersinia mollaretii* (200). The *S. marcescens* LCT homologue has been demonstrated to translocate its upstream cysteine protease and a domain of unknown function into cells, while the LCT homologues from *Yersinia mollaretii* translocate their upstream enzymatic domains (a cysteine protease domain and either an ADP-ribosyltransferase or glycosyltransferase domain) into cells, which inactivate Rab proteins through ADP ribosylation and glycosylation, respectively (200).

Strikingly, distant T domain homologues have the highest degree of conservation across the N terminus of the LCT T domain; this region was termed the "evolutionarily conserved translocase" (199) (Fig. 5). In TcdB, the evolutionarily conserved translocase was demonstrated to be a functional domain intertwined within receptor-binding sites of the T domain that could independently facilitate translocation of the glycosyltransferase and cysteine protease domain (199). The evolutionarily conserved translocase spans a region of the T domain that extends beyond the hydrophobic region, indicating that the hydrophobic region is not necessary and sufficient for translocation. It is not clear if the evolutionarily conserved translocase is an independently folded domain in other LCTs or LCT homologues or whether this region requires other parts of the protein for stability/solubility.

In a recent analysis of >8,000 *tcdB* genes, the majority of conserved surfaces across the entire toxin were located in the evolutionarily conserved translocase (138). Thus, the evolutionarily conserved translocase is also an attractive target for broad-spectrum therapeutics, which could target multiple TcdB subtypes, other LCTs, and LCT T domain homologues.

Cysteine Protease Domain

The LCT cysteine protease domain (CPD) autocatalytically cleaves the N-terminal glycosyltransferase from the polypeptide, resulting in the release of the glycosyltransferase into the

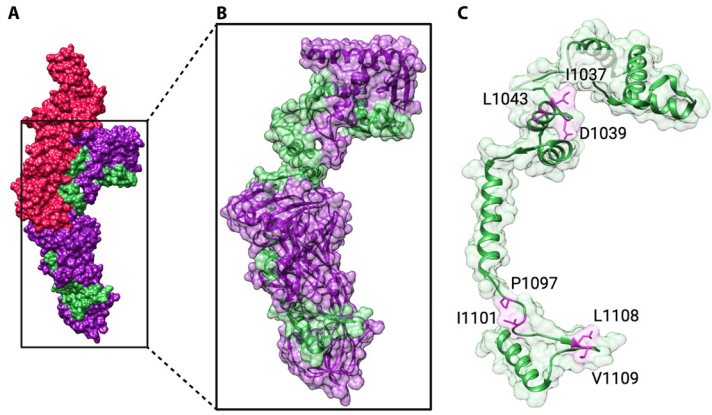

FIG 5 Translocation features of the TcdA T domain. (A) Structure of the TcdA T domain (PDB 4R04), with the evolutionarily conserved translocase colored purple (residues 853 to 1475) and the hydrophobic region (residues 958 to 1137) colored green. (B) Focus on the evolutionarily conserved region and (C) the hydrophobic region without the rest of the T domain. Residues with important functions in translocation identified by Zhang et al. (197) are shown as pink sticks.

cytosol (201, 202). The LCT cysteine protease domain shares between 33% and 86% sequence identity among LCTs (Table 7) and belongs to the C80 family of proteases (203). C80 cysteine proteases are found in bacterial pathogens, such as the multifunctional autoprocessing repeats-in-toxin (MARTX) from *Vibrio cholerae* (204). All LCTs have a conserved histidine, cysteine, and aspartate, which form a catalytic triad that is essential for autoprocessing (201, 205). Autoprocessing is not essential for cytotoxicity, but mutation of the catalytic triad render LCTs less toxic (201, 205–207). It has been hypothesized that autoprocessing is required for optimal activity of the LCT glycosyltransferase, most likely by improving the access of the glycosyltransferase to cellular substrates (208). Enhanced autoprocessing activity has been observed for a TcdB subtype from the epidemic *C. difficile* RT027, suggesting that more efficient autoprocessing may be responsible for increased toxicity of toxin subtypes (209).

To induce autoprocessing, LCTs must bind to the cellular host factor inositol hexakisphosphate (InsP6), which is found exclusively in the cytosol of eukaryotic cells (210). The equilibrium dissociation constant (K_D) values for InsP6 binding to TcdA, TcdB, TcsL, TcnA, and TpeL range from ~2.0 μM to 9.0 μM (189, 205). The structures of the TcdA and TcdB cysteine protease domains bound to the cellular factor InsP6 were solved in 2009 and 2010, respectively (211, 212) (Fig. 6). The TcdA and TcdB cysteine protease domains have a central β-sheet flanked by a number of α-helices. Structurally, the cysteine protease domains of TcdA and TcdB are very similar to, albeit larger than, the cysteine protease domains of MARTX (213, 214), containing additional helical regions and an additional β-strand. The catalytic triad and InsP6-binding site are on opposite faces of the domain and are separated by a three-stranded β-sheet, termed a β-flap. Binding of InsP6, which is negatively charged, occurs at a basic, lysine-rich positively charged pocket.

TABLE 7 Sequence identity and similarity of LCT cysteine protease domains

	% sequence identity (% similarity)[a]				
LCT	**TcdA**	**TcdB**	**TcsH**	**TcsL**	**TcnA**
TcdB	57 (77)				
TcsH	86 (95)	58 (77)			
TcsL	57 (77)	79 (90)	59 (79)		
TcnA	33 (57)	35 (57)	36 (59)	36 (60)	
TpeL	53 (71)	51 (70)	54 (72)	51 (72)	38 (60)

[a]Sequence identity and similarity were calculated using Water (EMBOSS) local alignment. LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* VPI 9048, *C. novyi* 19402, and *C. perfringens* JGS1495. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138).

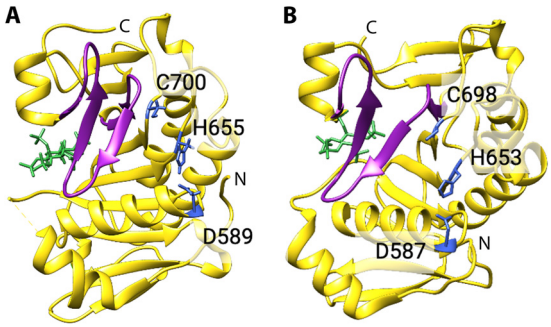

FIG 6 Structural features of the *C. difficile* toxin cysteine protease domain. (A) TcdA cysteine protease domain (PDB 3HO6) and (B) TcdB cysteine protease domain (PDB 3PEE) bound to InsP6 (268). The β-flap is colored purple, residues of the catalytic triad are depicted as blue sticks, and InsP6 is colored green.

In TcdA and TcdB, InsP6 binding to the cysteine protease domain induces conformational changes to the β-flap, which in turn transduces conformational changes to the active site region (211, 215). Cleavage occurs after a conserved leucine residue and at neutral pH (205, 216), resulting in release of the glycosyltransferase domain into the cytosol, while the remainder of the toxin is localized in endosomes (217).

The mechanism of autoprocessing is highly similar among LCTs, although a few key differences have emerged (189, 205). The TcsL holotoxin requires acidic pH to bind InsP6 with high affinity, while all other LCTs preferentially bind InsP6 at neutral pH (205). Binding of TcsL to InsP6 at acidic pH is unexpected, as InsP6 is present solely in the cytosol of eukaryotic cells (210). Interestingly, the TcsL cysteine protease domain binds to InsP6 with ~10-fold higher affinity at neutral pH than at acidic pH, suggesting that the TcsL holotoxin must undergo major conformational changes at acidic pH before binding to InsP6 at neutral pH (205). Studies have also revealed differences in sensitivity to InsP6-induced cleavage between the TcdA and TcdB holotoxins (165). TcdA is less sensitive to InsP6-induced cleavage, probably due to extensive domain interactions between the TcdA CROP and N terminus at neutral pH (164, 206). It is possible that holotoxins with similar-sized CROP domains to that of TcdA, such as TcsH, may be less sensitive to InsP6-induced cleavage, although to the best of our knowledge, this has not been investigated. Several features relating specifically to TcdA and TcdB autoprocessing activity and regulation have been reported. The histidine and cysteine of the TcdA and TcdB catalytic triad coordinate a zinc ion that is essential for autoprocessing activity (142), and the cysteine of the TcdA and TcdB catalytic triad is regulated through endogenous *S*-nitrosylation (218). Additionally, autoprocessing has been implicated in regulating the proinflammatory activities of TcdA and TcdB (219). It has not been demonstrated if zinc and *S*-nitrosylation are involved in autoprocessing activity and regulation or if autoprocessing mediates inflammatory activity of other LCTs.

Glycosyltransferase Domain

Once autocatalytically cleaved from the polypeptide and released into the cytosol, the LCT glycosyltransferase modifies Rho and Ras GTPases by glycosylation, using UDP-glucose or UDP-*N*-acetylglucosamine (GlcNAc) as a cosubstrate (189, 202, 220–223) (Table 8). Related glycosyltransferases have been identified in pathogenic bacteria, including *Escherichia coli* (NleB) (224), and species of *Legionella* (Lgt1, 2, 3) (225) and *Photorhabdus* (PaTox) (226). Numerous *in vitro* studies have provided evidence that LCT-mediated glycosylation of Rho and Ras GTPases is essential for cellular toxicity (189, 222, 227–229). Recently, Bilverstone et al. demonstrated that animals infected with strains of *C. difficile* with glycosyltransferase-defective mutations were unable to induce CDI, providing strong support that glycosyltransferase activity is essential in *C. difficile* disease pathogenesis (230). At high nanomolar (>1 nM)

TABLE 8 Substrate specificity of LCT glycosyltransferase domains[a]

LCT	Sugar donor(s)	Transfer(s)	Strain	Target(s)
TcdA	UDP-glucose (220)	Glucose	VPI 10463 (A1)	RhoA/B/C, Rac1, RhoG, Cdc42, Rap1/2, H/K/N-Ras (277, 278)
			C34	Rho, Rac, Cdc42, Rap (279)
TcdB	UDP-glucose (220)	Glucose	VPI 10463 (B1)	RhoA/B/C, Rac1/2/3, Cdc42, RhoG (177, 277, 278, 280)
			RT027 (B2)	RhoA/B, Rac1/2/3, Cdc42, Rap1A/2A, R-Ras (177, 277, 281)
			1470 (B3)	Rac, Cdc42, Rap, Ral, R-Ras (282)
			8864 (B4)	Rac, Cdc42, Rap, Ral, R-Ras (279, 283)
			C34 (B7)[b]	Rho, Rac, Cdc42, Rap, Ral, R-Ras (279)
			RT019 (B7)	Rac1/2/3, Cdc42, Rap2A/1B, R-Ras1/2, (H/K)-Ras (177, 281)
			HMX-152 (B7)	RhoA (110)
			HSJD-312 (B7)	RhoA (110)
			HMX-149 (B11)	RhoA (110)
TcsL	UDP-glucose (221)	Glucose	VPI 9048	Rac, Cdc42, Rap, Ras (221, 284)
			IP 82	Rac, Rap, Ral, Ras (221, 285)
			6018	Rac1, RhoG, Rap1/2, Ral, (H/K/N)-Ras (277, 284)
TcsH	UDP-glucose[c] (221)	Glucose	VPI 9048	Rho, Rac, Cdc42, Ras (221, 286)
TcnA	UDP-GlcNAc (222)	*N*-Acetylglucosamine	590, 19402	Rho, Rac, Cdc42 (221, 287)
TpeL	UDP-glucose, UDP-GlcNAc[d] (189, 223)	Glucose, *N*-acetylglucosamine	MC18	Rac, Rap, Ral, Ras (223)
			JGS1495	(H/K/N)-Ras (288)

[a]This table is based in part on data from Chandrasekaran and Lacy (239). If the sequence of the *C. difficile* subtype was available, the TcdA or TcdB subtype is indicated in parentheses. Subtyping based on that reported by Mansfield et al. (138).
[b]Based on partial sequencing of the toxin gene.
[c]Based on conservation of key UDP-glucose binding features.
[d]Preferentially modifies GTPases by GlcNAcylation using UDP-GlcNAc (189).

concentrations of TcdB (163), glycosylation-independent effects, including necrosis (231) and pyknosis (232), have also been reported. The physiological relevance of glycosylation-independent effects of TcdB remains unclear and requires further investigation.

LCTs glycosylate Rho and Ras GTPases by cleaving the UDP-glucose or UDP-GlcNAc cosubstrate and transferring glucose or *N*-acetylglucosamine onto the conserved Thr35/37 of the target GTPase (233). LCT glycosyltransferases have conserved residues that are essential for catalysis, most notably the aspartate-X-aspartate (DXD) motif (TcdB D286/D288) and tryptophan (TcdB W102, W520) (52, 234, 235) that are important for binding and coordination of the cosubstrate and a manganese ion (189, 227, 228, 234, 236, 237) (Fig. 7A). The glycotransferase domain shares between 34% and 85% sequence identity among LCTs (Table 9), and differences in sequence have been ascribed to differences in cosubstrate and GTPase specificity. In LCTs, specificity for UDP-glucose or UDP-GlcNAc is dictated by two residues (I383 and Q385 in TcdB) (223, 238, 239). The molecular basis for GTPase specificity in LCTs has not been completely defined. Preferential targeting of Rho and Ras GTPases in TcdB and TcsL has been attributed to specific residues in the glycosyltransferase domain, such as those in α-helix 17 (residues 444 to 455) in TcdB (45).

The structures of the TcdA (240, 241), TcdB (237), TcsL (242), and TcnA (242) glycosyltransferase domains have been solved by X-ray crystallography (Fig. 7). LCT glycosyltransferases belong to the GT-A family of glycosyltransferases (243), which are defined by a core $\alpha/\beta/\alpha$ sandwich that resembles a Rossmann fold, a tertiary fold found in many nucleotide-binding proteins (244). In addition to the Rossmann-like fold, LCTs have multiple α-helical subdomains. The N-terminal subdomain ($\sim$1 to 90 amino acids) is a membrane localization domain that targets the glycosyltransferase to the cytosolic leaflets of the cell membrane, where it can access membrane-bound Rho and Ras GTPases. The nucleotide sugar-binding pocket is formed by the edge of the β-sheet and several α-helices and is overlaid with a flexible loop that is involved in binding of the phosphate of the nucleotide sugar (245, 246). The flexible loop undergoes conformational changes upon substrate binding, defining open and closed conformations of the LCT glycosyltransferase. The TcdA and TcdB glycosyltransferase domains have been shown to interact with the TRiC/CCT chaperonin system (247), which may aid in glycosyltransferase refolding in the cytoplasm after unfolding in the acidic environment of the endosome. It is not yet known if the TcsL, TcsH, TpeL, or TcnA glycosyltransferase interacts with cytosolic chaperones.

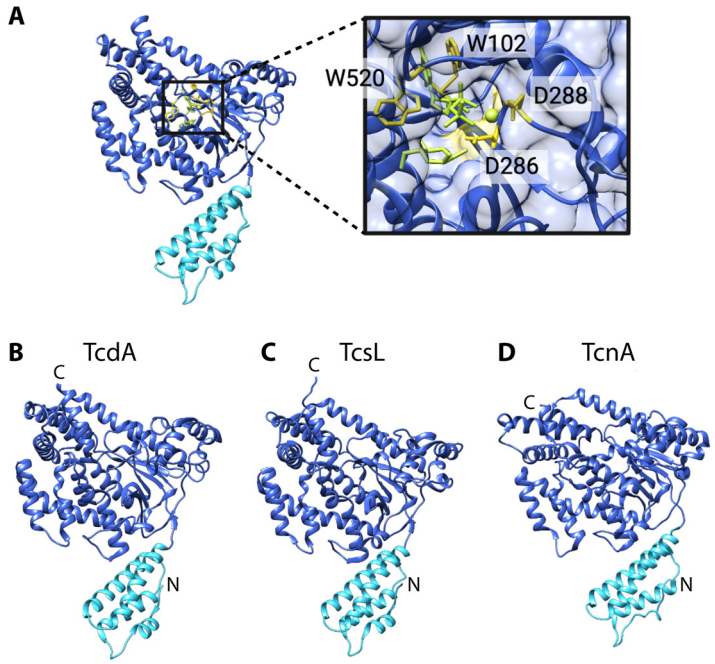

FIG 7 Structural features of the LCT glycosyltransferase domain. (A) TcdB glycosyltransferase domain (PDB 2BVM) in complex with the cosubstrate and a manganese ion, depicted as green sticks and a sphere, respectively. Residues important for catalytic function are depicted as yellow sticks. Structures of the (B) TcdA (PDB 3SRZ), (C) TcsL (PDB 2VKD), and (D) TcnA (PDB 2VK9) glycosyltransferase domains. The membrane localization domain (MLD) is colored aqua.

GTPases are molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. LCTs preferentially target GTPases in their inactive GDP-bound state (202, 233). Rho GTPases are master regulators of the actin cytoskeleton, control motile cellular processes, and are involved in cell cycle control and polarity (248). Ras GTPases are essential for assembly and function of cell-cell junctions, cell differentiation, and proliferation (249).

LCT-mediated glycosylation of Rho and Ras GTPases has been correlated with both cytopathic effects (i.e., loss of cytoskeletal structure, resulting in the characteristic cell rounding phenotype) and cytotoxic effects (i.e., apoptosis, resulting in cell death) (Tables 9 and 10). In addition to the major cytopathic and cytotoxic effects, glycosylation of Rho and Ras proteins has also been correlated with cell cycle arrest and defects in cell proliferation in TcdB and TcsL (250–252). Glycosylation-dependent cytopathic and cytotoxic effects are believed to be primarily responsible for LCT-mediated tissue damage, although the precise mechanisms underlying tissue damage and cell death are not well defined. Broadly, two types of cytopathic effects have been described for LCTs: cell rounding with protrusions radiating around rounded cells, also referred to as

TABLE 9 Sequence identity and similarity of LCT glycosyltransferase domains

	% sequence identity (% similarity)[a]				
LCT	**TcdA**	**TcdB**	**TcsH**	**TcsL**	**TcnA**
TcdB	51 (72)				
TcsH	85 (92)	51 (73)			
TcsL	53 (73)	76 (88)	55 (75)		
TcnA	34 (53)	34 (54)	34 (52)	36 (54)	
TpeL	46 (66)	44 (65)	46 (67)	48 (66)	34 (57)

[a]Sequence identity and similarity were calculated using Water (EMBOSS) local alignment. LCTs are from the following strains: *C. difficile* VPI 10463, *P. sordellii* VPI9048, *C. novyi* 19402, and *C. perfringens* JGS1495. Under new subtyping analysis, TcdA and TcdB from *C. difficile* VPI 10463 are referred to as TcdA1 and TcdB1 (138).

TABLE 10 LCT-mediated glycosylation of Rho and Ras GTPases and correlated cellular effects[a]

LCT	Cytopathic effect	Cytotoxic effect	Pyroptosis
TcdA	Rho GTPases (Rac) (289)	Rho GTPases (290)	RhoA (260)
TcdB	Rho GTPases (Rac) (291)	Rho GTPases (RhoA) (292)	RhoA (258)
TcsL	Rho GTPases (Rac) (250, 293)	Ras GTPases ([H/K/N]-Ras) (250)	ND
TcsH	Rho GTPases (221, 286)	Rho GTPases (221, 286)	ND
TcnA	Rho GTPases (221, 287)	Rho GTPases (221, 287)	ND
TpeL	Rho GTPases (Rac) (223)	Ras GTPases (189)	ND

[a]The following strains are used for each LCT: VPI10463 (TcdA, TcdB); VPI 9048 (TcsL); VPI 9048 (TcsH); 590 (TcnA); MC18 (TpeL). ND, not determined.

the "arborizing" cytopathic effect, and cell rounding without protrusions (253). TcdA, TcnA, and TcdB subtypes B1 and B2 have been reported to induce the arborizing cytopathic effect, while TcsL and TcdB subtypes B3, B4, and B7 have been reported to induce cell rounding without protrusions (110, 138, 253, 254). It is not yet clear why some LCTs induce the arborizing phenotype, although altered specificity for GTPases has been proposed (138, 253, 254).

It is well established that TcdA and TcdB induce an inflammatory response in intestinal epithelial cells (239). In the context of CDI, inflammation may be both beneficial as a host defense mechanism for pathogen eradication and harmful if the inflammatory response is prolonged and uncontrolled (255, 256). There are conflicting reports on the requirement for glycosyltransferase activity in the TcdA- and TcdB-induced inflammatory response (229, 239, 257–259). In myeloid cells, glycosylation of RhoA by TcdA and TcdB has been correlated with the activation of the pyrin inflammasome, a multimeric protein complex that activates procascapse-1, which then activates proinflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18 (258, 260, 261). Activation of the pyrin inflammasome can lead to pyroptosis, a caspase-1-dependent cell death that is highly inflammatory and characterized by cell swelling and lysis (258, 262, 263). A recent report by Saavedra et al. provided evidence that the pyrin inflammasome does not have a role in TcdA/TcdB-induced killing of mouse intestinal epithelial cells, due to the absence of pyrin expression (264). The role of TcdA/TcdB-induced activation of the pyrin inflammasome in human intestinal epithelial cells has yet to be investigated. Interestingly, TcsL has been reported to induce an anti-inflammatory response to inactivation of Ras proteins (45). To the best of our knowledge, it is not known whether TpeL, TcnA, and TcsH induce an inflammatory response.

NEWLY IDENTIFIED LCT HOMOLOGUES

The recent identification of hundreds of LCT homologues (199) has opened up a new avenue of LCT research and has provided a novel lens for understanding toxin structure and function. As detailed in this review, LCTs were first identified in context of disease and defined by their toxicity to humans at low doses. The genomics-driven identification of hundreds of LCT homologues has reversed the paradigm of LCT discovery, as bioinformatically identified homologues are identified on the basis of sequence similarity alone. It is not known if LCT homologues are involved in disease processes or what the ecological function of homologues may be.

On the basis of their frequent co-occurrence with virulence and mobile genes and presence in pathogenic bacteria, recently identified LCT homologues have been hypothesized to be putative toxins (199). Notably, the majority of recently identified LCT homologues are present in nonclostridial species that are directly pathogenic to insects (i.e., *Pseudomonas*, *Photorhabdus*, and *Yersinia*) or in species that, due to plant growth-promoting properties, may be pathogenic to insects or other plant pathogens, such as nematodes (199). Interestingly, as reported by Mansfield and Doxey (265) and Contreras et al. (266), numerous links to insects have also been made for botulinum neurotoxin homologues. The significance and evolutionary implications of LCT

homologues—and more broadly, bacterial toxins—targeting insects and other plant pathogens is unclear. It may be suggested that human-targeted LCTs evolved from insect-targeting homologues and/or that LCTs pose a selective advantage to clostridia in the environment.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Through decades of work spanning the fields of clinical and veterinary medicine, microbiology, biochemistry, and structural biology, LCTs have emerged as some of the deadliest poisons of humans and animals. While members of the LCT family share many similar features, including sequence homology and general mechanism of action, LCTs also have distinct clinical and molecular features. Recent work has demonstrated that sequence variation in TcdA and TcdB across different *C. difficile* strains results in toxin subtypes with different functional and immunological activities, warranting the creation of a toxin subtyping system. *C. difficile* toxin subtyping is directly relevant for diagnostic and therapeutic development and should be used to guide the use of toxin-mediated treatments, such as bezlotoxumab, in the clinic. It remains to be determined whether sequence variation exists in other LCTs across clostridial strains and, if so, whether sequence variations result in toxins with different biological and functional activities.

A clearer picture of LCT-mediated infection is emerging, particularly for TcdA, TcdB, and TcsL; the roles in virulence of TcnA, TpeL, and TcsH remain less well defined. Although great strides have been made in LCT structure and function, a detailed mechanistic understanding of LCT intoxication is still elusive. Central to understanding LCT intoxication is identifying the receptors LCTs bind to mediate cell surface attachment and internalization and the GTPases LCTs target. The additional layer of complexity that also must be addressed is how the difference in receptor engagement and substrate specificity between LCTs and toxin subtypes contributes to differences in toxin-mediated pathology and disease progression. Perhaps the greatest challenge is determining how LCTs translocate their glycosyltransferase and cysteine protease domain across the endosomal membrane and into the cytosol. Based on conserved sequence features in membrane-inserting regions, it seems likely that the mechanism of translocation is highly similar among LCTs and subtypes. Additional work is required to gain a deeper and more precise understanding of the enigmatic mechanism of translocation.

Genomics has expanded the LCT family from six proteins to several hundred, thus establishing a new frontier in LCT research. Characterization and determination of the receptors that homologues engage and the intracellular substrates they modify—and by extension, the organisms they target—remain difficult. Elucidating the function of LCT homologues, particularly in their native environment, will clarify their ecological roles and potential adaptive value in bacteria. Importantly, homologues also hold clues to how LCTs evolved to become deadly human poisons and may lead us closer to identifying the ancestral LCT toxin.

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Kathleen E. Orrell, Ph.D., is a postdoctoral fellow in the Molecular Medicine Program at The Hospital for Sick Children in Toronto, Canada. She received her doctoral degree in biochemistry from the University of Toronto in 2021. Her doctoral work focused on large clostridial toxin translocation across host cell membranes. Dr. Orrell's current work investigates structural and functional features of novel bacterial toxin effectors.

Roman A. Melnyk, Ph.D., is an Associate Professor of Biochemistry at the University of Toronto and Senior Scientist in Molecular Medicine at The Hospital for Sick Children. He received his Ph.D. in biochemistry from the University of Toronto in 2004 and then was a postdoctoral fellow at Harvard Medical School from 2004 to 2006. Following this, he was a Sr. Scientist at Merck Frosst Centre for Therapeutic Research between 2006 and 2011. His research focuses on understanding the structure and function of bacterial toxins and using this information both to develop novel small molecule antitoxins and for engineering intracellular delivery platforms.