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MAJOR ARTICLE (FAST TRACK – FIDSA MEMBERS ONLY)

Clostridioides difficile binary toxin binding component (cdtb) increases virulence in a hamster model

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***Background*:** *Clostridioides difficile* is the leading cause of hospital-acquired gastrointestinal infection, in part due to the existence of binary toxin (CDT)-expressing hypervirulent strains. While the effects of the CDT holotoxin on disease pathogenesis have been previously studied, we sought to investigate the role of the individual components of CDT during *in vivo* infection.

***Methods*:** To determine the contribution of the separate components of CDT during infection, we developed strains of *C. difficile* expressing either CDTa or CDTb individually. We then infected

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both mice and hamsters with these novel mutant strains and monitored them for development of severe illness.

Results: While expression of CDTb without CDTa did not induce significant disease in a mouse model of *C. difficile* infection, we found that complementation of a CDT-deficient *C. difficile* strain with CDTb alone restored virulence in a hamster model of *C. difficile* infection.

Conclusions: Overall, this study demonstrates that the binding component of *C. difficile* binary toxin, CDTb, contributes to virulence in a hamster model of infection.

Keywords *Clostridioides difficile*, binary toxin, CDT, hamster model

INTRODUCTION

Clostridioides difficile, a gram-positive, spore-forming anaerobe, is the causative agent of *Clostridioides difficile* infection (CDI), a gastrointestinal infection typically characterized by high levels of inflammation and diarrhea. This bacterium is considered an urgent health threat by the CDC [1], and was shown in a 2015 study to be responsible for approximately 500,000 infections and 29,000 deaths [2]. *C. difficile* typically infects those with dysbiosis, a state of disruption in the healthy intestinal microbiota leading to reduced and/or skewed microbial diversity, commonly induced through use of broad-spectrum antibiotics [3]. This dysbiosis allows *C. difficile* to establish a niche and begin toxin production, which lead to disruption of the host intestinal epithelial barrier, production of pro-inflammatory cytokines, and recruitment of inflammatory immune cells to the site of infection [4]. The host immune response to CDI is critical in determining patient outcome, as immune biomarkers have been shown to be more predictive of time to disease resolution than bacterial burden [5]. While effective antibiotic treatment is available, one in five patients will experience recurrent infection [2], highlighting the need for further understanding of the host response to aid in the development of improved or novel therapeutics.

The past few decades have seen an overall increase in both the frequency and severity of CDI, a phenomenon that has been primarily associated with the emergence of hypervirulent strains of *C. difficile* [6]. In addition to the primary virulence factors Toxin A and Toxin B, these strains express a third toxin called *C. difficile* transferase, or CDT [7]. CDT is a binary toxin with ADP-ribosyltransferase activity and consists of an enzymatic component, CDTa, and a binding component, CDTb. Following the binding of CDTb to its host cell receptor, lipolysis-stimulated lipoprotein receptor (LSR) [8], CDTa binds to CDTb and induces endocytosis into the host cell. Upon acidification of the endosome, CDTb inserts itself into the endosomal membrane and forms a pore through which CDTa escapes into the cytosol. CDTa then ADP-ribosylates actin, thereby preventing its elongation and leading to cytoskeletal disruption [7]. CDT also induces the formation of microtubule protrusions at the apical surface which are thought to aid in bacterial

adherence to host epithelial cells [9]. Previous work from our group demonstrated that CDT expression is associated with increased mortality in patients [10] and can enhance virulence in a mouse model of CDI through suppression of protective eosinophilic responses [11]. Additionally, it has been shown that CDTb alone is sufficient to induce cytotoxicity *in vitro* [12,13], though it remains unknown whether this contributes to disease pathogenesis *in vivo*.

In this study, we sought to investigate the role of the individual components of CDT during *in vivo* infection. To do this, we used allelic exchange to delete *cdtA* and *cdtB* expression and then genetically complemented with either CDTa or CDTb, thus generating strains of *C. difficile* producing CDTa or CDTb alone. We then infected both mice and hamsters with these strains to determine how differences in individual CDT component expression would affect disease severity.

MATERIALS AND METHODS

Generation of CDT Mutant Strains

Strains and plasmids used in this study are listed in Table 1, while primers are listed in Table 2. The genes encoding *cdtA* and *cdtB* were deleted from *C. difficile* R20291Δ*pyrE* using allelic-exchange (AE) technology [14]. To achieve this, left and right homology arms, corresponding to the regions annealing immediately upstream and downstream of *cdtA/B*, were amplified by PCR using *cdtAB* LAF/RAR and *cdtAB* RAF/RAR primer sets respectively. The homology arms were then spliced together by splicing by overlap-extension (SOEing) PCR by means of their overlapping 20bp homologous regions before cloning the ensuing product into pMTL-YN4 using flanking *Sbf*I-*Asc*I restriction sites, thus generating the knockout cassette (KOC) pMTL-YN4-*cdtAB* KOC. The plasmid was then conjugated into *C. difficile* R20291Δ*pyrE* exactly as described previously and transconjugants were selected on the basis of thiamphenicol resistance [15]. Thereafter, single cross-over integrants (SCOs) were identified by two parallel PCR screens using *cdtAB* diag F/ YN4 primers for left arm recombinants and YN4 F/ *cdtAB* diag R primers for right arm recombinants respectively (data not shown). To select for double cross-over recombinants, SCO integrants were harvested, diluted $1x10^{-3}$ and cultured onto *Clostridium difficile* minimal medium (CDMM) [16] containing 500µg/ml 5-fluoroorotic acid (FOA) and 1µg/ml uracil, to force plasmid loss through the counter-selection marker *pyrE*, and to select for double cross-over mutants before confirming plasmid loss on the basis of thiamphenicol sensitivity. Deletion mutants were confirmed to be as intended by PCR analysis using *cdtAB* diag F/R primers, where the deletion mutant generated a circa 4kbp product compared to the 4.6kbp product of its wild-type counterpart (Figure 1A). Finally, the *pyrE* allele was restored to wild-type using pMTL-YN2 exactly as described previously [15].

Strains differentially producing CDTa or CDTb, were generated by the integration of either *cdtA* or *cdtB* at the *pyrE* locus, under the control of *cdtA* promoter P_{cdtA}. Firstly, *cdtA* coupled with its

native promoter, was amplified by PCR using P_{cdtA} F and *cdtA* R primers. The product of which was cloned into pMTL-YN2C by means of flanking *Not*I-*Bam*HI restriction sites thus generating the complementation cassette (CS) pMTL-YN2C- P_{cdtA}-*cdtA.* In a similar fashion, pMTL-YN2C-P_{cdtA}-*cdtB,* was generating by amplifying P_{cdtA} using P_{cdtA} F/P_{cdtA} LAR primers and *cdtB* using *cdtB* RAF/*cdtB* RAR primers, before SOEing the products together and cloning them into pMTL-YN2C by means of flanking *Not*I-*Sal*I restriction sites. The CDTb-encoding construct could only be generated with an SNP in the promoter region of P_{cdtA} ensuing an A-G substitution at position -124 relative to the start codon. The resultant plasmids were applied in parallel, to individually integrate the respective CDT constructs at the *pyrE* locus of R20291Δ*pyrE*Δ*cdtAB* concomitant with the repair of *pyrE,* following successful conjugation and selection for uracil prototrophs on CDMM lacking uracil. PCR analysis using primer *pyrE* WT F, coupled with either *cdtA* R or *cdtB* RAR, demonstrated effective knock-in at the *pyrE* locus (Figure 1B), thus generating strains R20291Δ*cdtAB**P_{cdtA}-*cdtA* and R20291Δ*cdtAB**P_{cdtA}-*cdtB.*

Analysis of CDT production by Western blot

Secreted CDTa/b was assessed by Western blot analysis of 48h culture-free supernatants exactly as described previously [15], using an HRP-Chicken anti-*Clostridium difficile* Binary Toxin Subunit A or B antibody (Gallus-Immunotech, USA).

***C.* Difficile spore preparation and bacterial culture**

C. difficile spore stocks were generated as described previously [17]. Briefly, *C. difficile* strains were grown in 2 mL of Columbia broth overnight at 37 °C anaerobically. The 2 mL inoculum was then added to 40 mL of Clospore media. The culture was incubated anaerobically at 37 °C for 5-7 days. Following the incubation, spores were harvested by centrifuging the culture at 3200 rpm for 20 min at 4 °C, then resuspending in cold sterile water. After washing the spores at least three times, the spore stocks were stored at 4 °C in sterile water. The stocks were heat treated at 65 °C for 20 min to eliminate any remaining vegetative cells. The concentration of spores in each stock was determined by serially diluting the stocks in anaerobic PBS and plating on BHI agar supplemented with 1% sodium taurocholate. Once the CFU/mL of each stock was determined, the infection inoculum was prepared by diluting the appropriate *C. difficile* strain spore stock to the appropriate concentration. Animals received 100 µL of inoculum each via oral gavage.

To determine *C. difficile* colonization in infected animals, cecal contents were resuspended and serially diluted in reduced PBS. Serial dilutions were plated on BHI agar supplemented with 1% sodium taurocholate, 1 mg/mL cycloserine, and 0.032 mg/mL cefoxitin (Sigma), then incubated at 37 °C overnight in an anaerobic chamber. Bacterial burden was normalized to cecal content sample weight.

Mice and *C. Difficile* Infection

Experiments were carried out using 8 to 12-week-old male C57BL/6J mice from the Jackson Laboratory. All animals were housed under specific-pathogen free conditions at the University of Virginia's animal facility, and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. Mice were infected using a previously established murine model for CDI [11]. Six days prior to infection, mice were given an antibiotic cocktail within drinking water consisting of 45 mg/L vancomycin (Mylan), 35 mg/L colistin (Sigma), 35 mg/L gentamicin (Sigma), and 215 mg/L metronidazole (Hospira). Three days later, mice were switched to regular drinking water for 2 days and the day prior to infection, given a single intraperitoneal injection of 0.016 mg/g clindamycin (Pfizer). The day of infection, mice were orally gavaged with vegetative (1×10^8 CFUs) or spores (1×10^3) of *C. difficile* strains as indicated (R20291 wildtype, R20291 Δ*cdtAB*, R20291 CDTb+). Mice were monitored daily during the course of infection and twice daily during the acute phase (days 2 and 3). Mice were immediately euthanized following the development of severe illness as measured by clinical scoring parameters. These parameters included weight loss, coat condition, eye condition, activity level, posture, and diarrhea, which were evaluated to give a clinical score between 0 and 20. Severe disease was indicated by a clinical score of 14 or higher and any mouse scoring at or above that cutoff was immediately euthanized.

Hamsters and *C. Difficile* Infection

Experiments were carried out using 90-100 g adult male Syrian Golden hamsters from Charles River Laboratory. All animals were housed under specific-pathogen free conditions at the University of Virginia's animal facility, and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. Hamsters were infected using a previously established hamster model for CDI [18], with minor modifications. Hamsters were orally gavaged with 0.03 mg/g clindamycin (Pfizer) five days prior to infection. On the day of infection, hamsters were orally gavaged with 10^2 spores of *C. difficile* mutant strains as indicated (R20291 Δ*cdtAB*, R20291 CDTb+). Hamsters were monitored twice daily over the course of infection, and were euthanized immediately upon development of severe illness as assessed via clinical scoring. Parameters used included weight loss, coat condition, eye condition, activity level, posture, and diarrhea, which were evaluated to give a clinical score between 0 and 20. Severe disease was indicated by a clinical score of 14 or higher. Any hamster receiving a clinical score at or above a 14 was immediately euthanized.

Statistical analysis

For animal work, survival curves were generated using the Kaplan-Meier estimator. Significance between groups was determined using ordinary one-way ANOVA, while Tukey's test was used for multiple comparisons. Comparisons between two groups were done using a two-tailed *t* test. All statistical analyses were performed using GraphPad Prism software.

RESULTS

Generation of CDT mutant strains

To determine the contribution of the separate components of CDT to disease pathology *in vivo*, CDTa(+)CDTb(-) and CDTa(-)CDTb(+) strains of R20291 were generated as outlined in the Methods section. Mutants were generated in the SBRC Nottingham lineage of R20291 (CRG 0825) [19]. *cdtA* and *cdtB* were knocked out to generate a CDT-deficient strain of *C. difficile*, which was subsequently complemented with either *cdtA* (R20291Δ*cdtAB**P*cdtA*-*cdtA*) or *cdtB* alone (R20291Δ*cdtAB**P*cdtA*-*cdtB*). These two strains are hereafter referred to at CDTa+ and CDTb+. Deletion of *cdtAB* and complementation with *cdtA* or *cdtB* was confirmed using PCR analysis (Figure 1A-B). Following successful strain development, we validated their phenotype regarding CDT production. To do this, we cultured R20291, R20291Δ*cdtAB*, CDTa+, and CDTb+ in TY broth and at the 48h time point, assessed each strain for CDTa/b production by Western blot analysis of culture-free supernatants using antibodies developed against CDTa or CDTb (Figure 1C). Analysis of the Western blots demonstrated that the *cdtAB* deletion mutant was devoid of detectable CDTa or CDTb production. As expected, individual complementation of *cdtA* restored CDTa production whilst complementation of *cdtB* restored CDTb production, thus generating strains differentially expressing CDTa or CDTb. The strains will hereafter be referred to as CDTa+ and CDTb+.

The binding component cdtb does not increase virulence in a mouse model of *C. Difficile* infection

Because CDTb has been shown to induce cytotoxicity independently of CDTa [12,13], we asked whether expression of CDTb alone could enhance virulence *in vivo*. To test this, we utilized a previously published [11] mouse model of *C. difficile* infection (CDI) (Figure 2A). We infected adult C57BL/6J mice (n=10) with 1 x 10^3 spores of either the R20291 wildtype strain, the Δ*cdtAB* strain (lacking CDTa and CDTb), or the CDTb+ strain (expresses CDTb but not CDTa). Two days post-infection, the mice were sacrificed and cecal tissue and contents were harvested for analysis. At this time point, mice infected with the wildtype strain began to experience moderate weight loss (Figure 2B) and significantly more severe disease as measured by clinical scoring (Figure 2C). However, mice infected with either the Δ*cdtAB* or the CDTb+ strain did not show any signs of weight loss or significant disease. There was no qualitative difference in Toxin A and Toxin B production *in vivo* (Figure 2D), indicating that the difference in virulence seen in the mutant strains was not due to a deficiency in production of either of these primary clostridial toxins. We examined whether the differences in disease severity between the wildtype strain and the CDT mutant strains were due to differences in bacterial colonization, but no significant difference in bacterial burden was measured (Figure 2E). We also infected mice with vegetative cells of each strain (Supplementary Figure 1A-D) and found that similarly to the spore infection, the wildtype strain induced significant mortality, weight loss, and clinical scores

while both the Δ*cdtAB* and CDTb+ strains were avirulent. Overall, this indicates that expression of CDTb without CDTa is not sufficient to increase virulence in a mouse model of CDI.

The binding component cdtb enhances virulence in a hamster model of *C. Difficile* infection

We hypothesized that the contribution of CDTb to disease pathology could be subtle, and any differences between the Δ*cdtAB* strain and the CDTb+ strain may be overshadowed in the relatively resistant mouse model of CDI. Therefore, we asked whether a more sensitive animal model would reveal more minute differences in disease phenotype between infections with the mutant strains. To investigate this, we utilized a hamster model of CDI [18] (Figure 3A). We infected adult Syrian Golden hamsters (n=10) with 1 x 10^2 spores of the Δ*cdtAB* strain or the CDTb+ strain, then monitored the animals twice daily for mortality (Figure 3B), weight loss, and clinical scores (Figure 3C). Because we wanted to determine how the presence of CDTb alone would affect disease severity, only the Δ*cdtAB* and the CDTb+ strains were used in the hamster model. Infection with the CDTb+ strain induced significantly higher mortality as compared to the Δ*cdtAB* strain, and while the CDTb+-infected hamsters did experience more severe disease, the swiftness and severity of the infection prevented any statistically significant comparison between clinical scores. Indeed, the hamsters experienced a much more severe and much faster course of disease as compared to the mice, with the time between symptom onset and mortality being much shorter in the hamsters than in the mice. Overall, we concluded that the binding component of CDT was sufficient to increase virulence in a hamster model of CDI.

DISCUSSION

We have found that the binding component of the CDT binary toxin has *in vivo* toxin activity as assessed in a hamster model of CDI. That CDTb might function independently of the holotoxin as a pore-forming toxin has been suggested given its pore-forming activity in cultured cells [12,13], however, its contribution to pathogenesis independent of CDTa had not been assessed in an *in vivo* infection model. In this study we developed and utilized a strain of *C. difficile* expressing CDTb but not CDTa to further investigate the role of CDTb during infection and found that expression of CDTb alone is sufficient to enhance virulence. Previous work has shown that CDT holotoxin expression has been associated with increased mortality in both humans [10] and mice [11]. Additionally, while not common, clinical strains of *C. difficile* that are negative for the primary clostridial toxins TcdA and TcdB but positive for CDT have been isolated from symptomatic patients [20], further supporting that the binary toxin CDT plays a significant role in the pathogenesis of CDI. However, studies of CDT have primarily centered on its role as a binary AB toxin and have not focused on the independent contributions of its two components. Our finding that CDTb enhanced virulence in the absence of CDTa suggests a new model for AB toxins in which the B subunit can have independent and/or synergistic effects on disruption of the gut epithelial barrier.

One unexpected finding in this study is that the strain lacking CDTa/b was avirulent in both mice and hamsters. This strain expresses the primary toxins Toxin A and B, which have been shown to be capable of causing disease on their own [21]. Additionally, there are strains of *C. difficile* that only express Toxin A and Toxin B and not CDT which are capable of causing symptomatic disease in patients [22]. Because of this, one would expect that the CDT-deficient strain would still be capable of causing disease in both animal models due to the maintained expression of Toxin A and Toxin B. However, our findings show that the strain lacking CDTa/b causes no disease in either animal model. Some potential differences could be in the production or effectiveness of Toxin A and Toxin B in the mutant strain as compared to the wild type. Consequently, further study into the role of Toxin A/B in this strain is needed.

In this study, we saw that the CDTb+ mutant strain did not cause disease in a mouse model, but did induce severe disease and mortality in a hamster model of infection. This striking difference suggests that there may be entirely different mechanisms responsible for driving disease severity and mortality between these models. Further supporting this idea are the marked differences in disease progression we observed. In the mouse model, the animals experienced a more prolonged course of disease following the initial onset of symptoms as compared to the hamsters and were capable of potentially recovering back to their baseline weight and appearance. In the hamster model, however, the animals underwent a very rapid and severe course of symptomatic disease, in some cases progressing from no outward signs of disease to moribund in less than six hours. In addition, no recovery took place for any animal once they developed symptomatic disease. It is unclear what is responsible for such a high degree of variation in disease progression following symptom onset. One possible difference may be in the kinetics of bacterial colonization, as more rapid or more gradual colonization could be responsible for the contrasting disease progression. Another difference that was not explored here but may be investigated in the future is how differences in the mice and hamster microbiota may influence disease severity. The extent of epithelial barrier disruption may also play a role, as differences in toxin receptor expression could affect the degree of damage induced by the toxins. Similarly, innate immune receptor expression may influence the scale of the inflammatory response induced in response to infection. While not performed as part of this work, measuring inflammatory biomarkers in future experiments may help to clarify potential reasons behind the differing responses between these animal models.

While we were able to determine that the CDTb+ strain was more virulent in the hamster model, it is not yet known how exactly CDTb is contributing to worsened disease. It has been shown that CDTb possesses an LSR-dependent ability to form pores in the plasma membrane, thus inducing cytotoxicity independently of its role in delivering CDTa inside the cell [12,13]. Therefore, it may be that compared to mice, hamsters are more sensitive to epithelial damage caused by this CDTb-induced cytotoxicity. Similarly, it is possible that the epithelial damage induced by CDTb promotes the translocation of microbes across the gut barrier, leading to bloodstream infections such as candidemia [23, 24]. This may be another potential reason behind

the increased clinical severity seen in hypervirulent strains [10], further emphasizing the need to study the effects of CDTb during infection.

Overall, we have found that the binding component of the *C. difficile* binary toxin contributes significantly to disease in a hamster model. To our knowledge, this is the first work demonstrating the impact of CDTb alone *in vivo* and helps to further explain the heightened virulence displayed by the epidemic strains of *C. difficile*. Understanding the significance and impact of CDT and its individual components during infection can help in developing therapeutic strategies against these more severe hypervirulent strains.

Potential Conflicts of Interest: WAP Jr. is a consultant for TechLab, Inc.

Patient Consent Statement: This study does not include factors necessitating patient consent

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Author Contributions: MS designed, performed, and analyzed the data from the animal experiments. JL, AD, JU, and WP helped with tissue processing and provided invaluable advice. NM constructed the initial *cdtAB* deletion mutant. TB repaired the *pyrE* allele, constructed the *cdtA/cdtB* complements and analyzed CDT production. SK and NPM supervised NM and TB. WAP Jr. supervised MS and supported all aspects of the work.

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FIGURES

Figure 1

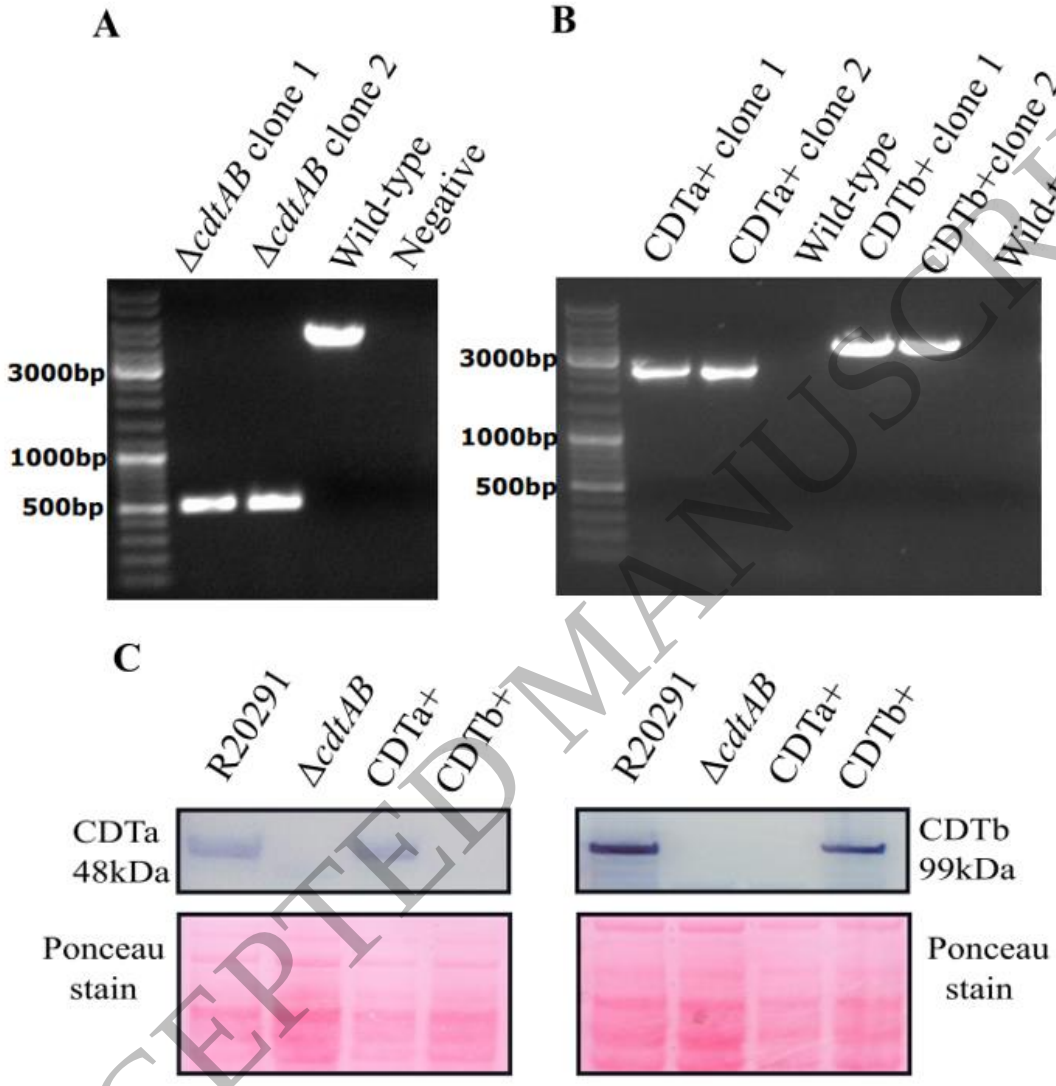

Figure 1: Authentication of *C. difficile* CDT mutant strains. Gel image following PCR analysis for A) deletion of *cdtAB* using *cdtAB* diag F/R primers and B) individual complementation of *cdtA* or cdtB at *pyrE* using *pyrE* WT F with *cdtA* R or *cdtB* R respectively. Gels ran alongside a GeneRuler DNA Ladder Mix (Thermo, USA). C) Western blot analysis of secreted CDT for 48 h culture-free supernatants detected with an HRP-Chicken anti-*Clostridium difficile* Binary Toxin Subunit A or B antibody. Ponceau staining was performed immediately following protein transfer and before blocking to ensure equal loading/transfer.

Figure 2

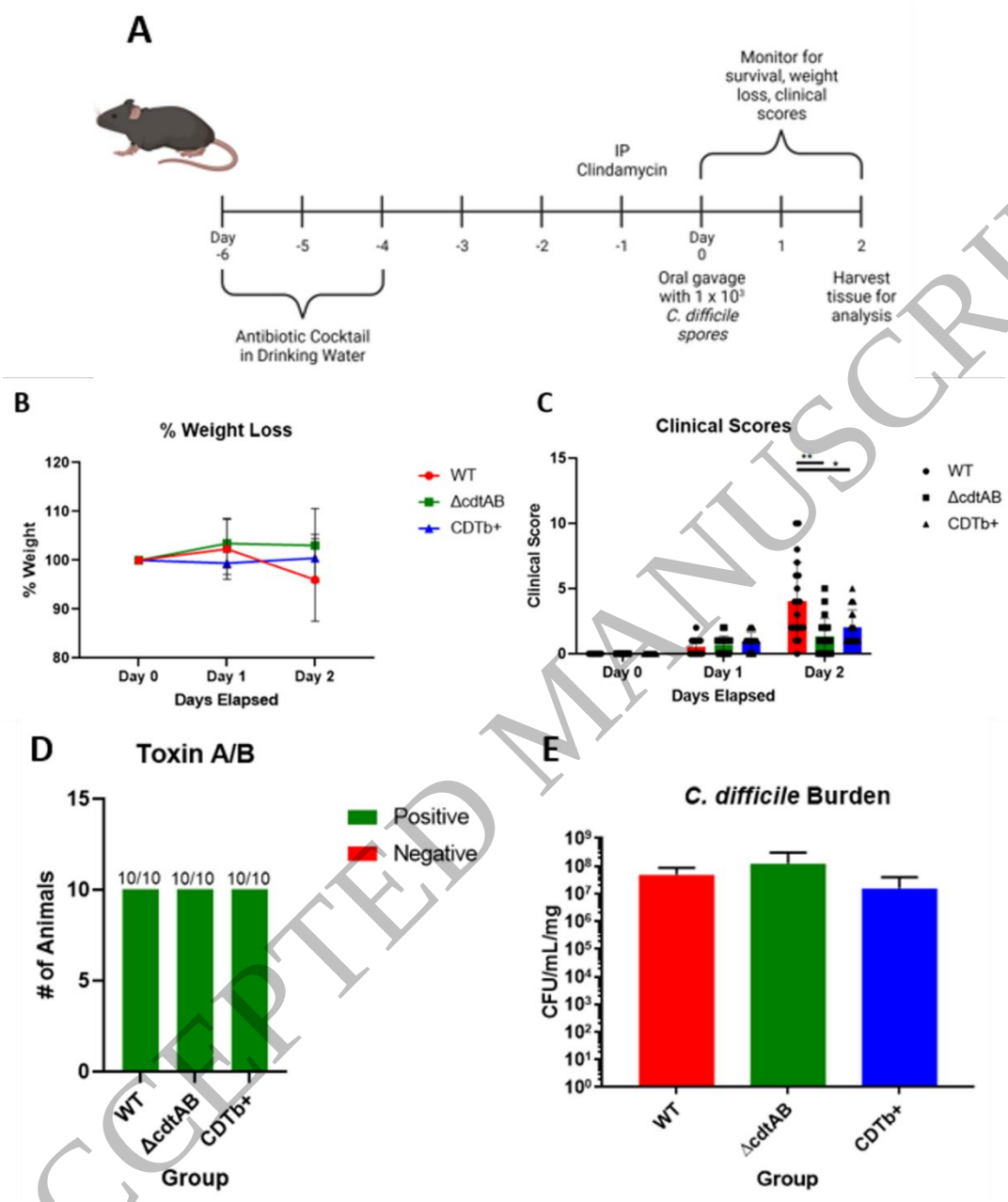

Figure 2: The binding component CDTb does not increase virulence in a mouse model of *C. difficile* infection

A) C57BL/6J mice were treated with antibiotics then infected with 1 x 10^3 spores of R20291 wild-type (WT), R20291 Δ*cdtAB*, or R20291 CDTb+ strain of *C. difficile*. Following infection, mice were monitored for B) weight loss and C) clinical signs of disease (**p=0.023, *p=0.0265). Data are combined from two independent experiments, n=20. D) Presence of ToxinA/B in cecal contents was assessed via ELISA provided by TechLab. E) Cecal contents were suspended and

serially diluted in anaerobic PBS, then plated on BHI agar with taurocholate and *C. difficile* supplement. Following overnight anaerobic incubation at 37 °C, colony growth was assessed and normalized to cecal content weight (n=10).

Figure 3

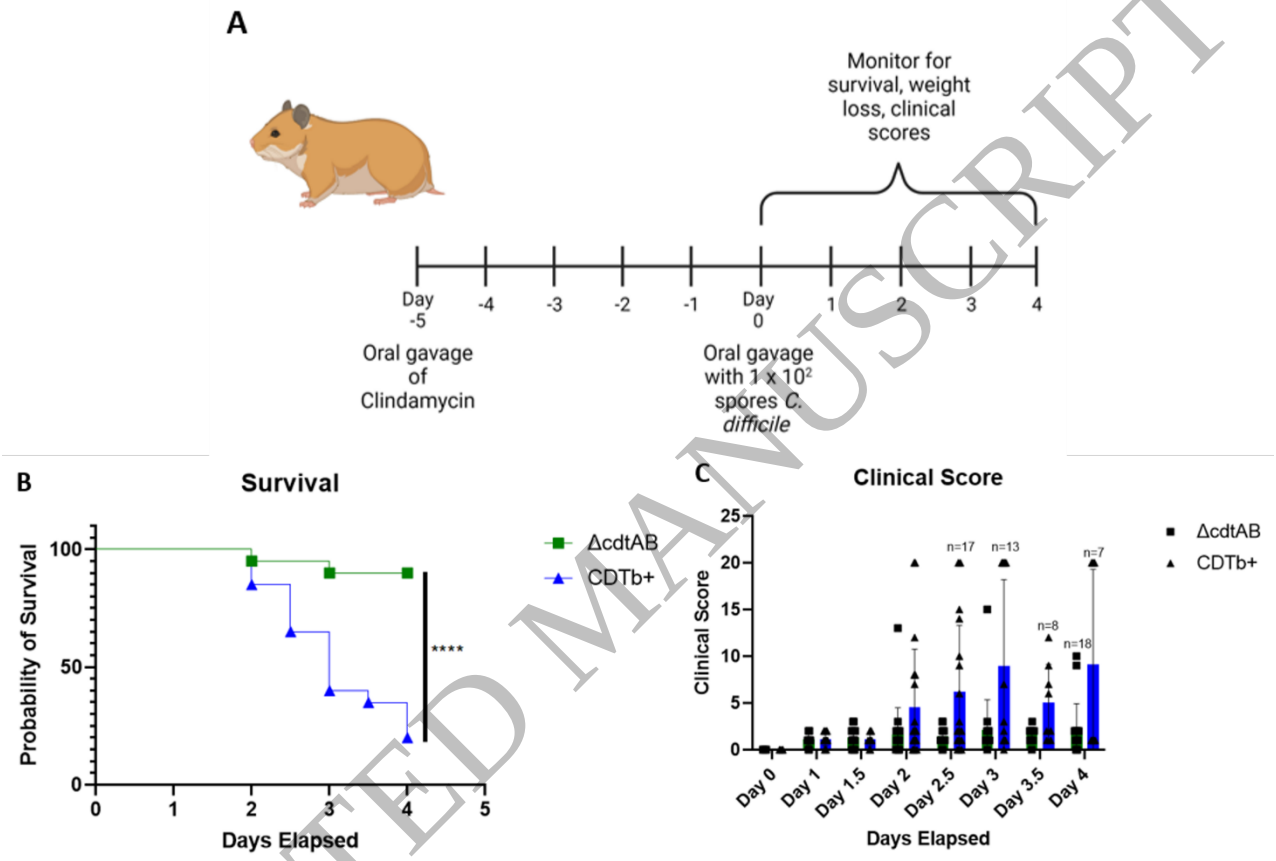

Figure 3: The binding component CDTb enhances virulence in a hamster model of *C. difficile* infection

A) Adult Golden Syrian hamsters were given oral clindamycin then infected with 1 x 10^2 spores of *C. difficile* R20291 Δ*cdtAB* or R20291 CDTb+. Following infection, hamsters were monitored for B) survival (****p<0.0001) and C) clinical signs of disease. Data were combined from two separate experiments, n=20.

Table 1: Strain and plasmids used in this study

Strain/Plasmid	Description	Reference
Strains	Cloning host.	Stratagene, USA
E. coli		
XL-1 blue		
CA434	Conjugal donor.	[25]
C. difficile		
R20291 (CRG 0825)	Clinical RT 027 isolate, SBRC	[19]
R20291Δ*pyrE*	Nottingham lineage	[14]
R20291Δ*pyrE*Δ*cdtAB*	*pyrE* mutant for AE	This study
R20291Δ*cdtAB*	initial *cdtAB* mutant	This study
R20291Δ*cdtAB**P_{cdtA}-*cdtA*	*pyrE*-restored *cdtAB* mutant	This study
R20291ΔcdtAB*P_{cdtA}-*cdtB*	*cdtA* complemented strain	This study
	cdtB complemented strain	

Plasmids		
pMTL-YN4	Knock-out vector for R20291	[14]
pMTL-YN4-*cdtAB* KOC	KOC for *cdtAB*	This study
pMTL-YN2	Complementation vector for R20291	[14]
pMTL-YN2C- P_{cdtA}-*cdtA*	Complementation cassette for *cdtA*	This study
pMTL-YN2C- P_{cdtA}-*cdtB*	Complementation cassette for *cdtB*	This study

Table 2: Oligonucleotide primers used in this study

Primer	**Sequence 5'-3'**
***cdtA* deletion**	
cdtAB LAF	TTTTTTcctgcaggTTTTTACTATCTACTCAGATTCCTCACTATGGAA
cdtAB LAR	TCATTTGATATTATTCTCCCTCCCAATATTAGTT
cdtAB RAF	GAGGGAGAATAATATCAAATGATTTAAATTTGTCC
cdtAB RAR	AAAAAAggcgcgccTCTTAGAAAAGTTTATAAAAAAGTTGGATTTATA
cdtAB diag F	GAGATGTCTCAAGATAAGAATTTG
cdtAB diag R	GATAATTATCTTTTTAATACAATATAGTTC

***cdtA* complementation**	
P_{cdtA} F	TTTTTgcggccgcGTTCCTAAGAATCCTCTATATAATAATCG
cdtA R	TTTTTggatccTTAAGGTATCAATGTTGCATCAAC
***cdtB* complementation**	GTATTTTCATTTATTCTCCCTCCCAATATTAG
P_{cdtA} LAR	GGGAGAATAAATGAAAATACAAATGAGGAATAAAAAGG
cdtB RAF	TTTTTgtcgacTTACTAATCAACACTAAGAACTAATAAC
cdtB RAR	
Single cross-over (SCO)	CTCCATCAAGAAGAGCGAC
determination	CTTATCCAGGGTGCTATC
YN4 F	CTCCATCAAGAAGAGCGAC
YN4	CTTTCTATTCAGCACTGTTATGCC
***pyrE* restoration**	
pyrE F	
pyrE R	