**Clostridioides difficile** Binary Toxin Binding Component 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. Although 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 both mice and hamsters with these novel mutant strains and monitored them for development of severe illness.

**Results.** Although 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.** binary toxin; CDT; *Clostridioides difficile*; hamster model.

*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 Centers for Disease Control and Prevention [1], and it was shown in a 2015 study to be responsible for approximately 500,000 infections and 29,000 deaths [2]. *Clostridioides 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 proinflammatory 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, because immune biomarkers have been shown to be more predictive of time to disease resolution than bacterial burden [5]. Although effective antibiotic treatment is available, 1 in 5 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.

In the past few decades, researchers 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]. *Clostridioides difficile* transferase is a binary toxin with ADP-ribosyltransferase activity and consists of an enzymatic component, CDTa, and a binding component, CDTb. After 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 adds an ADP-ribose moiety onto actin, preventing actin elongation and subsequently inducing...
cytoskeletal disruption [7]. Clostridioides difficile transferase 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]. In addition, it has been shown that CDTb alone is sufficient to induce cytotoxicity in vitro [12, 13], although 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.

**METHODS**

**Generation of Clostridioides difficile Binary Toxin Mutant Strains**

Strains and plasmids used in this study are listed in Table 1, whereas primers are listed in Table 2. The genes encoding cdtA and cdtB were deleted from C difficile R20291ΔpyrE using allelic-exchange technology [16]. To achieve this, left and right homology arms corresponding to the regions annealing immediately upstream and downstream of cdtA/B were amplified by polymerase chain reaction (PCR) using cdtAB LAI/RAR and cdtAB RAF/RAR primer sets, respectively. The homology arms were then spliced together by overlap-extension (SOEing) PCR by means of their overlapping 20-base pair (bp) homologous regions before cloning the ensuing product into pMTL-YN4 using flanking SflI-AseI restriction sites, thus generating the knockout cassette (KOC) pMTL-YN4-cdtAB KOC. The plasmid was then conjugated into C difficile R20291ΔpyrE exactly as previously described, and transconjugants were selected on the basis of thiamphenicol resistance [17]. Thereafter, single-crossover integrants (SCOs) were identified by 2 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-crossover recombinants, SCO integrants were harvested, diluted 1 × 10⁻³, and cultured onto C difficile minimal medium (CDMM) [18] containing 500 µg/mL 5-fluoroorotic acid and 1 µg/mL uracil, to force plasmid loss through the counter-selection marker pyrE and to select for double crossover mutants before confirming plasmid loss on the basis of thiamphenicol sensitivity. The intended deletions were confirmed by PCR analysis using cdtAB diag F/R primers. The deletion mutant generated an approximately 4-kbp product, while its wild-type (WT) counterpart generated a 4.6-kbp product (Figure 1A). Finally, the pyrE allele was restored to WT using pMTL-YN2 exactly as described previously [17].

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 PcdtA. First, cdtA coupled with its native promoter was amplified by PCR using PcdtA F and cdtA R primers, the product of which was cloned into pMTL-YN2C-ΔcdtA using the complementation cassette pMTL-YN2C-PcdtA-ΔcdtA. In a similar fashion, pMTL-YN2C-PcdtB-ΔcdtB was generated by amplifying PcdtB using PcdtB F/PcdtB LAR primers, and cdtB was generated using cdtB RAF/ΔcdtB RAR primers, before SOEing the products together and cloning them into pMTL-YN2C by means of flanking NotI-Sall restriction sites. The CDTb-encoding construct could only be generated with a single-nucleotide polymorphism in the promoter region of PcdtA using 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 R20291ΔpyrEΔcdtAB concomitant with the repair of pyrE, after successful conjugation and selection for uracil prototrophs on CDMM lacking uracil. The 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 R20291ΔcdtAB△PcdtA-ΔcdtA and R20291ΔcdtAB△PcdtA-ΔcdtB.

***Table 1. Strain and Plasmids Used in This Study***

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td>Cloning host</td>
<td>Stratagene, USA</td>
</tr>
<tr>
<td>XL-1 blue</td>
<td>Conjugal donor</td>
<td>[14]</td>
</tr>
<tr>
<td>Clostridioides difficile</td>
<td>Clinical RT 027 isolate, SBRC Nottingham lineage</td>
<td>[15]</td>
</tr>
<tr>
<td>R20291 (CRG 0825)</td>
<td>pyrE mutant for AE</td>
<td>[16]</td>
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<td>R20291ΔpyrE</td>
<td>initial cdtA mutant</td>
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<td>pMTL-YN4</td>
<td>Knockout vector for R20291</td>
<td>[16]</td>
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Abbreviations: AE, allelic exchange; KOC, knockout cassette; SBRC, Synthetic Biology Research Centre.
Clostridioides difficile Analysis of Clostridioides difficile Binary Toxin Production by Western Blot

Secreted CDTa/b was assessed by Western blot analysis of 48-hour culture-free supernatants exactly as described previously [17], using an HRP-Chicken anti-Clostridium difficile Binary Toxin Subunit A or B antibody (Gallus-Immunotech, Shirley, MA).

### Clostridioides difficile Spore Preparation and Bacterial Culture

Clostridioides difficile spore stocks were generated as described previously [19]. Briefly, C difficile strains were grown in 2 mL Columbia broth overnight at 37°C anaerobically. The 2-mL inoculum was then added to 40 mL Clospore media. The culture was incubated anaerobically at 37°C for 5–7 days. After the incubation, spores were harvested by centrifuging the culture at 3200 rpm for 20 minutes at 4°C, then resuspending in cold sterile water. After washing the spores at least 3 times, the spore stocks were stored at 4°C in sterile water. The stocks were heat treated at 65°C for 20 minutes to eliminate any remaining vegetative cells. The concentration of spores in each stock was determined by serially diluting the stocks in anaerobic phosphate-buffered saline (PBS) and plating on brain-heart infusion (BHI) agar supplemented with 1% sodium taurocholate. Once the colony-forming units (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, St. Louis, MO), then incubated at 37°C overnight in an anaerobic chamber. Bacterial burden was normalized to cecal content sample weight.

### Table 2. Oligonucleotide Primers Used in This Study

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<tr>
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<tr>
<td>cdtA R</td>
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<tr>
<td>cdtB complementation</td>
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<tr>
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<td>Single crossover determination</td>
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<tr>
<td>pyrE F</td>
<td>CTTTCTATTACGCAGTGTATTGAC</td>
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</table>

### Figure 1. Authentication of Clostridium difficile transferase (CDT) mutant strains.

Gel image after polymerase chain reaction analysis for (A) deletion of cdtAB using cdtAB diag F/R primers and (B) individual complementation of cdtA or cdtB at pyrE using pyrE wild-type 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-hour culture-free supernatants detected with an HRP-Chicken anti-Clostridium difficile Binary Toxin Subunit A or B antibody. Ponceau staining was performed immediately after protein transfer and before blocking to ensure equal loading/transfer.

### Analysis of Clostridioides difficile Binary Toxin Production by Western Blot

Secreted CDTa/b was assessed by Western blot analysis of 48-hour culture-free supernatants exactly as described previously [17],
Mice and *Clostridioides 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 before infection, mice were given an antibiotic cocktail in drinking water consisting of 45 mg/L vancomycin (Mylan, Canonsburg, PA), 35 mg/L colistin (Sigma, St. Louis, MO), and 215 mg/L metronidazole (Hospira, Lake Forest, IL). Three days later, mice were switched to regular drinking water for 2 days, and the day before infection, they were given a single intraperitoneal injection of 0.016 mg/g clindamycin (Pfizer, New York, NY). The day of infection, mice were orally gavaged with vegetative (1 × 10⁸ CFUs) or spores (1 × 10⁵) of *C. difficile* strains as indicated (R20291 wild-type, R20291 ΔcdtAB, 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 after 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 and assigned a numerical score and then added together to give a final 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 *Clostridioides difficile* Infection

Experiments were carried out using 90- to 100-gram 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 [20], with minor modifications. Hamsters were orally gavaged with 0.03 mg/g clindamycin 5 days before infection. On the day of infection, hamsters were orally gavaged with 10⁵ spores of *C. difficile* mutant strains as indicated (R20291 ΔcdtAB, 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 and assigned a numerical score and then added together to give a final 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 analysis of variance, whereas Tukey’s test was used for multiple comparisons. Comparisons between 2 groups were done using a 2-tailed *t* test. All statistical analyses were performed using GraphPad Prism software.

RESULTS

Generation of *Clostridioides difficile* Binary Toxin 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 under Methods. Mutants were generated in the Synthetic Biology Research Centre Nottingham lineage of R20291 (CRG 0825) [15]. *cdtA* and *cdtB* were knocked out to generate a CDT-deficient strain of *C. difficile*, which was subsequently complemented with either *cdtA* (R20291ΔcdtAB+PcdtA-cdtA) or *cdtB* alone (R20291ΔcdtAB+PcdtA-cdtB). These 2 strains are hereafter referred to as CDTa⁺ and CDTb⁺. Deletion of *cdtAB* and complementation with *cdtA* or *cdtB* was confirmed using PCR analysis (Figure 1A and B). After successful strain development, we validated their phenotype regarding CDT production. To do this, we cultured R20291, R20291ΔcdtAB, CDTa⁺, and CDTb⁺ in TY broth, and at the 48-hour time point, we assessed each strain for CDTa/b production by utilizing 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, whereas 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 *Clostridioides 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 used a previously published [11] mouse model of CDI (Figure 2A). We infected adult C57BL/6J mice (n = 10) with 1 × 10⁵ spores of either the R20291 wild-type strain, the ΔcdtAB strain (lacking CDTa and CDTb), or the CDTb⁺ strain (expresses CDTb but not CDTa). Two days postinfection, the mice were killed and cecal tissue and contents were harvested for analysis. At this time point, mice infected with the wild-type 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 ΔcdtAB 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 wild-type 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 wild-type strain induced significant mortality, weight loss, and clinical scores, whereas both the ΔcdtAB and CDTb+ strains were avirulent. Overall, this

Figure 2. The binding component CDTb does not increase virulence in a mouse model of Clostridium difficile infection. (A) C57BL/6J mice were treated with antibiotics then infected with 1 × 10^3 spores of R20291 wild-type (WT), R20291 ΔcdtAB, or R20291 CDTb+ strain of C. difficile. After infection, mice were monitored for (B) weight loss and (C) clinical signs of disease (**P = .023, *P = .0265). Data are combined from 2 independent experiments, n = 20. (D) Presence of ToxinA/B in cecal contents was assessed via enzyme-linked immunosorbent assay provided by TechLab. (E) Cecal contents were suspended and serially diluted in anaerobic phosphate-buffered saline, then plated on BHI agar with taurocholate and C. difficile supplement. After overnight anaerobic incubation at 37°C, colony growth was assessed and normalized to cecal content weight (n = 10).
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 Clostridioides difficile Infection**

We hypothesized that the contribution of CDTb to disease pathology could be subtle, and any differences between the ΔcdtAB 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 used a hamster model of CDI [20] (Figure 3A). We infected adult Syrian Golden hamsters (n = 10) with 1 × 10⁵ spores of the ΔcdtAB strain or the CDTb⁺ strain, then we 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 ΔcdtAB and the CDTb⁺ strains were used in the hamster model. Infection with the CDTb⁺ strain induced significantly higher mortality compared with the ΔcdtAB strain, and although 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 compared with 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 used a strain of *C difficile* expressing CDTb but not CDTa to further investigate the role of CDTb during infection, and we 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]. In addition, although 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 [21], 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 2 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 [22]. In addition, there are strains of C. difficile that only express Toxin A and Toxin B, which are capable of causing symptomatic disease in patients [23]. 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 compared with 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 it 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 after the initial onset of symptoms compared with 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 6 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 after symptom onset. One possible difference may be in the kinetics of bacterial colonization, because 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, because differences in toxin receptor expression could affect the degree of damage induced by the toxins. Likewise, innate immune receptor expression may influence the scale of the inflammatory response induced in response to infection. Although 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.

Although 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 with 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 [24, 25]. 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.

CONCLUSIONS

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.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Clostridioides difficile Toxin A/B ELISA kits were provided by TechLab. Images from BioRender were used in Figures 2, 3, and Supplementary Figure 1.

Author contributions. MS designed, performed, and analyzed the data from the animal experiments. JL, AD, JU, and WAP 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 C. difficile transferase production. SK and NPM supervised NM and TB. WAP Jr. supervised MS and supported all aspects of the work.

Disclaimer. The views expressed are those of the authors and not necessarily those of the National Health Service, the National Institute for Health and Care Research (NIHR), or the Department of Health.

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Potential conflicts of interest. WAP Jr. is a consultant for TechLab, Inc. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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