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Toxin B is essential for virulence of *Clostridium difficile*

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Abstract

Clostridium difficile is the leading cause of infectious diarrhea in hospitals worldwide, because of its virulence, spore-forming ability and persistence^{1,2}. *C. difficile*-associated diseases (CDAD) are induced by antibiotic treatment or disruption of the normal gastrointestinal flora^{3,4}. Recently, morbidity and mortality resulting from CDAD have increased significantly due to changes in the virulence of the causative strains and antibiotic usage patterns^{1,2,5,6}. Since 2002, epidemic toxinotype III NAP1/027 strains^{1,2}, which produce high levels of the major virulence factors, toxin A and toxin B, have emerged. These toxins have 63% amino acid sequence similarity⁷ and are members of the large clostridial glucosylating toxin family, which are monoglucosyltransferases that are proinflammatory, cytotoxic and enterotoxic in the human colon^{8–10}. Inside host cells, both toxins catalyze the transfer of glucose onto the Rho family of GTPases, leading to cell death^{8, 11}. However, the role of these toxins in the context of a *C. difficile* infection is unknown. Here we describe the construction of isogenic *tcdA* and *tcdB* mutants of a virulent *C. difficile* strain and their use in the hamster disease model to show that toxin B is a key virulence determinant. Previous studies showed that purified toxin A alone can induce most of the pathology observed following infection of hamsters with *C. difficile*^{8,9, 12} and that toxin B is not toxic in animals unless it is co-administered with toxin A, suggesting that the toxins act synergistically¹². Our work provides evidence that toxin B, not toxin A, is essential for virulence, which represents a major paradigm shift. Furthermore, it is clear that the importance of these toxins in the context of infection cannot be predicted exclusively from studies using purified toxins, reinforcing the importance of using the natural infection process to dissect the role of toxins in disease.

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Author Contributions D.L., J.O'C., G.C., V.A., and J.R. designed the genetic and molecular aspects of the research, which were carried out by D.L., P.H. and G.C. S.J., S.S., J.O'C., and D.G. planned and developed the animal experiments, which were carried out by S.S. and J.O'C. Tissue culture assays were designed, planned and carried out by D.L., G.C., R.P., T.P. and G.V. All authors were involved in data analysis and interpretation. The manuscript was primarily written by D.L. and J.R. but all authors had very significant input into its content and reviewed and edited the manuscript.

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C. difficile is difficult to genetically manipulate; reproducible methods for the construction of chromosomal mutants have only recently become available^{13,14}. We have developed a method that involves a plasmid, designated the recombination vector, which is unstable in *C. difficile*; this plasmid carries DNA homologous to part of the genome and becomes inserted at that genomic location, resulting in a specific gene disruption. Using this process, we have successfully mutated two regulatory genes¹³ and others have generated mutations in additional genes¹⁵. This methodology has now made possible studies aimed at dissecting the role of toxin A and toxin B in CDAD by constructing and analyzing isogenic strains of this bacterium.

Toxin A and toxin B are encoded by the *tcdA* and *tcdB* genes, respectively, located within a 19.6kb pathogenicity locus, the PaLoc9 (Fig. 1a), which encodes three other genes involved in toxin production^{16,17}. To construct the plasmids required for the targeted inactivation of *tcdA* and *tcdB*, 566 bp *tcdA*- or *tcdB*-specific PCR products were cloned into pJIR1456. These PCR products corresponded to an essential region of the N-terminal domain between the DXD catalytic motif and amino acid 546 (Fig. 1b)¹⁸. Insertion of these plasmids into the corresponding chromosomal gene region by homologous recombination should lead to disruption of the gene, resulting in a truncation of the toxins (at leucine residues 492 and 493 in toxins A and B, respectively), thereby attenuating toxin function.

The *tcdA*- and *tcdB*-specific recombination vectors were transferred to JIR809413, a derivative of *C. difficile* strain 630, by RP4-mediated conjugative transfer from *Escherichia coli*. Two putative independently derived toxin A (*tcdA*Δ1 and *tcdA*Δ2) and five putative independently derived toxin B (including *tcdB*Δ1 and *tcdB*Δ2) mutants were isolated. Note that since each of these mutants was derived from a separate conjugation experiment, the mutants are truly independent. Their genotypes were confirmed by PCR analysis (data not shown) and Southern blotting (Fig. 1c). The insertion of the recombination vectors was shown to be specific and to have disrupted the respective *tcdA* and *tcdB* genes, with the wild-type bands replaced by bands corresponding to insertion of the recombination vectors. For each specific mutant the other toxin gene had a profile identical to wild-type. Even though the insertions were generated by single crossovers, and were therefore reversible¹³, the resultant chromosomal mutants were very stable; no evidence of deletion of the inserted plasmids was detected in the Southern blots (Fig. 1c). Autonomously replicating plasmids could not be extracted from these strains, suggesting that once chromosomal insertion had occurred, the plasmid was maintained in an inserted state, as previously found¹³.

Western blots using toxin A-specific antibodies showed that the *tcdA* mutants no longer produced toxin A and had a similar profile to an avirulent strain that does not carry the PaLoc (Fig. 2a). By comparison, the *tcdB* mutants produced toxin A, although they reproducibly appeared to make more toxin A than the wild-type. This finding was confirmed by quantitative real time reverse transcriptase PCR (QRT-PCR), which showed that the *tcdB* mutants expressed *tcdA* at 2.2–3.3-fold higher levels than the wild-type. The basis for this increased expression of toxin A is not known. QRT-PCR showed that no differences in expression of the other PaLoc genes (*tcdR*, *tcdE*, and *tcdC*) were observed in any of the mutants (data not shown).

Cytotoxicity assays using HT29 cells, which are more sensitive to toxin A than other cell lines^{19,20} showed that supernatants from the toxin A mutants were significantly less toxic ($p=0.021$) than supernatants from the wild-type (Fig. 2b). These assays also showed that toxin A produced by the wild-type strain and by the *tcdB* mutants was cytotoxic and therefore functional (Fig. 2b). The latter activity was neutralised by toxin A-specific antibodies (Fig. 2c), verifying that the observed toxicity resulted from the action of toxin A. Vero cell cytotoxicity assays were also carried out on these supernatants, the cytotoxic activity observed is predominantly toxin B-mediated^{8,19}. Consequently, the *tcdA* mutants exhibited the same cytotoxic phenotype as the wild-type strain (Fig. 2d). These cytopathic effects were neutralized by toxin B-specific antibodies, confirming that toxin B was responsible for the observed Vero cell toxicity (Fig. 2e). By contrast, supernatants from the *tcdB* mutants were significantly less cytotoxic ($p=0.0058$) for Vero cells than wild-type supernatants. These mutants exhibited a similar phenotype to the avirulent strain although they did have low levels of toxicity (Fig. 2d), consistent with the relatively lower cytopathic effects of toxin A on Vero cells. This low level of cytotoxicity was not neutralized by preincubation with toxin B antibodies (Fig. 2e).

All of the genetic and phenotypic assays used to analyze the mutant strains therefore showed that each pair of mutants, although isolated independently, behaved identically. Attempts were made to complement these mutations with the wild-type genes, but these experiments were not successful. The intact genes are very difficult to clone into shuttle vectors because they are very large (8.1- and 7.1-kb for *tcdA* and *tcdB*, respectively) and contain repeat regions^{21,22}. For this reason, in subsequent virulence experiments we used two independently isolated mutants for each gene, to rule out the possibility that any observed phenotypic effects were the result of other mutations.

The hamster disease model parallels most of the recognized features of human CDAD, especially with respect to the susceptibility of the animal to *C. difficile* infection following the administration of antimicrobial agents. Since it is currently the best animal model available²³ it was used to compare the virulence of the *C. difficile* mutants.

Upon challenge with the wild-type, all ten infected hamsters were colonized approximately two days after inoculation and 9/10 animals died approximately five days after colonization (Fig. 3). The two independent toxin A mutants, which produced wild-type levels of toxin B *in vitro*, yielded similar results, with 9/10 and 8/10 hamsters being colonized following challenge with the *tcdA* Δ 1 and *tcdA* Δ 2 mutants, respectively. The mean number of days (\pm SD) to colonization was similar following inoculation with wild-type (2.1 ± 1.2), *tcdA* Δ 1 (1.9 ± 0.3) and *tcdA* Δ 2 (1.9 ± 1.1) strains. These results matched *in vitro* studies that investigated the adherence of these strains to polarized Caco-2 human intestinal epithelial cells under anaerobic conditions, where no significant differences in adherence were observed (data not shown).

The mean time from colonization to death was similar for hamsters colonized with the wild-type (9/10) and the (*tcdA* Δ 1 (9/9) and *tcdA* Δ 2 (7/8) mutants (Fig.3). Fecal pellets from hamsters infected with the *tcdA* Δ 2 mutant were analyzed for plasmid stability, toxin analysis was also performed on these specimens. All of the colonies (n=800) recovered from the

eight *tcdA*Δ2 colonized hamsters were thiamphenicol resistant, confirming that the inserted recombination vector was retained in these isolates. Seven of the eight colonized hamsters died (Fig. 3c), the one surviving animal lost colonization after only two days. Confirmatory PCRs, with primers specific for the gene disruption, were performed on a minimum of four post-colonization isolates (from different hamsters) for each group of animals; the results showed that they all carried an insertionally inactivated *tcdA* gene.

All of the fecal pellets isolated from *tcdA*Δ2-infected hamsters were strongly cytotoxic (using a cytotoxin assay that primarily measures toxin B), indicating the presence of active toxin B, and were negative for toxin A in an enzyme immunoassay, as expected. Overall, 16/17 (94%) of the hamsters colonized with the toxin A mutants died, providing clear evidence that the *tcdA* mutants were still virulent even though they no longer produced toxin A, either *in vitro* or *in vivo*. These data provide clear evidence that toxin A is not essential for virulence in the hamster model, at least for a derivative of strain 630.

By contrast, the virulence trials involving the toxin B mutants demonstrated that toxin B was required for virulence (Fig. 3). For each of the *tcdB* mutants tested, 9/10 hamsters were colonized after challenge. The time from challenge to colonization (*tcdB*Δ1 (2.6±2.9); *tcdB*Δ2 (1.9±0.3)) was similar to that observed for the wild-type and the toxin A mutants. However, only 4/19 of the hamsters (21%) colonized with the toxin B mutants died (Fig. 3c), which was statistically different ($P=0.001$) from the wild-type strain (10/10 deaths). The hamster that died following challenge with the *tcdB*Δ1 mutant died 12 days after colonization although the interval from colonization to death in the three hamsters that died following challenge with the *tcdB*Δ2 mutant was similar to that of the wild-type. Plasmid stability analysis and toxin testing of fecal pellets from the *tcdB*Δ2 trial explained these results. All of the colonies (n=600) recovered from the six surviving hamsters were thiamphenicol resistant, indicating that the integrated recombination vector was carried by these strains. Fecal pellets from 5/6 surviving hamsters were EIA positive for toxin A and were either cytotoxin negative (n=2) or weakly cytotoxic (n=4), consistent with the absence of toxin B and the relatively lower cytotoxicity of toxin A. These results provided evidence that the isolates recovered from the surviving hamsters were no longer virulent, even though they continued to produce toxin A *in vivo*. Of the three hamsters that died, colonies from two animals (n=200) were all thiamphenicol resistant. However, only 74% of the isolates from the third hamster were thiamphenicol resistant. Restriction endonuclease analysis (REA) typing of two of the thiamphenicol sensitive isolates confirmed that they were derived from the wild-type strain, suggesting that they represented revertants that had lost the integrated plasmid and therefore displayed a wild-type (toxin A⁺B⁺) phenotype. In addition, fecal pellets from all three hamsters that died were strongly cytotoxic (toxin B positive) and toxin A positive by EIA. We conclude that the thiamphenicol sensitive isolates were revertants and were able to produce toxin B, reinforcing the requirement for toxin B in the virulence of *C. difficile*. We suggest that the two hamsters that carried thiamphenicol resistant strains, but whose fecal isolates were toxin A and B positive, had mixed infections of the mutant strain and a revertant strain capable of producing wild-type toxin B, at a level that was not detected by our analysis.

In summary, hamsters challenged with the toxin A mutants were as likely to die as those infected with the wild-type strain, even though these strains produce more toxin A than the wild-type (Fig. 2a), and animals challenged with the toxin B mutants were much more likely to survive (Fig. 3c). Among the few hamsters that died following challenge with the toxin B mutants, our analysis suggested that a proportion of the *C. difficile* population infecting those animals produced toxin B. These results support the conclusion that the production of toxin B, not toxin A, correlates with a virulent *C. difficile* phenotype.

In conclusion, in this study, four independently derived toxin A or toxin B mutants of *C. difficile* were constructed and characterized *in vitro* and *in vivo* using the hamster CDAD model. The results demonstrated that toxin B is an essential virulence factor since disruption of the *tcdB* gene led to a significantly attenuated virulence phenotype. Isolates that produced toxin B but not toxin A retained a wild-type virulence phenotype, which provides further confirmation of the paramount role of toxin B in disease pathogenesis. Unexpectedly, the presence of toxin A in the absence of toxin B, even at levels higher than in the wild-type strain, was not lethal in the hamster model. Although this finding ostensibly contradicts a study which showed that antibodies to toxin A protect toxoid A-immunised hamsters from disease²⁴, vaccination studies do not provide direct evidence of the role of a bacterial component in disease. Therefore, it is concluded that toxin A is not an essential virulence determinant in a derivative of the virulent *C. difficile* strain 630, although it may still play a non-essential role in the disease process.

Some previous studies have suggested that toxin A may not be the major virulence factor of *C. difficile* and that toxin B might not be dependent on toxin A for function^{23,25}, however, a lack of genetic tools did not allow these hypotheses to be tested. Other studies suggested that toxin B was unable to initiate disease unless toxin A was present^{9,12}. The data generated from our study do not support the hypothesis that both toxin A and toxin B are required to cause disease. Clinical variants expressing toxin B but not toxin A are being isolated with increasing frequency²⁶ and disease resulting from these strains is similar to that following infection with strains producing both toxins²⁷. The observation that clinical isolates that lack toxin A are still capable of causing severe disease reinforces the proposition that toxin A is not essential for virulence and that toxin B does not require the presence of toxin A for its *in vivo* activity^{9,23,26,27}. Note that naturally occurring toxin A-positive, toxin B-negative isolates of *C. difficile* have not been reported. Earlier studies on toxin A and toxin B function and their putative cooperativity involved purified toxins or culture supernatants^{9,12}. The disparity between these studies and those described here accentuates the importance of using the natural infection process to dissect the role of toxins in disease pathogenesis and reinforces the critical need for the use of isogenic strains in such studies.

The clinical diagnosis of *C. difficile* predominantly involved immunoassays for the detection of toxin A until the discovery of clinical disease caused by toxin A⁻B⁺ isolates that were not detected by these assays. It is now evident from our data that in the absence of culturing for the organism, the diagnostic emphasis should focus on the detection of both toxins by EIA and/or a standard cytotoxin assay that measures primarily toxin B²⁶, especially with toxin A-deficient strains being isolated with increasing frequency. Rapid and accurate diagnosis is

of critical importance because of the changing epidemiology and increased virulence of *C. difficile* strains^{1,2}, and because this organism is now causing disease in individuals previously thought not to be at risk, such as children and pregnant women²⁸. In summary, it is clear that our findings represents a paradigm shift in our understanding of the pathogenesis of *C. difficile*-mediated disease and will have major implications for the diagnosis, control and treatment of these syndromes.

METHODS SUMMARY

The toxin A- and toxin B-specific recombination vectors pJIR3051 and pJIR3050 were constructed by cloning a 566 bp *tda* (accession M30307) PCR product (nt 1071–1637) and the equivalent *tdb* (accession X53138) region, respectively, into the shuttle vector pJIR1456 (accession U90554). These plasmids were transferred by conjugation to *C. difficile* strain JIR8094 and the resultant chromosomal mutants analyzed by PCR, Southern blotting and plasmid rescue¹³.

Filtered supernatants of 2TY broth cultures (72 h) were used for Western blots, cytotoxicity and toxin neutralization assays. Serial two-fold dilutions of these supernatants were used in Vero and HT-29 cell cytotoxicity assays, with the end-point scored as the last dilution yielding complete CPE. Toxins were neutralized with affinity purified polyclonal antibodies specific for toxin A and toxin B; neutralization was carried out at the last dilution giving complete CPE. Partially purified ammonium sulphate-precipitated toxin proteins were Western blotted using toxin A-specific affinity purified polyclonal goat antibodies.

Syrian hamsters (n=10) were infected with *C. difficile* spores for virulence testing²³. Clindamycin was administered orogastrically to render the hamsters susceptible to infection (day zero). On day five hamsters received 1000 cfu of spores by gastric inoculation. Fecal pellets were collected daily for 12 days, then weekly until day 30 and inoculated onto selective TCCFA plates to monitor colonization. Colonies were typed by REA to confirm the identity of the infecting isolate. Fecal pellets were analyzed for the presence of toxin A using a toxin A-specific ELISA and for the presence of toxin B using a human foreskin fibroblast cytotoxicity assay.

Full methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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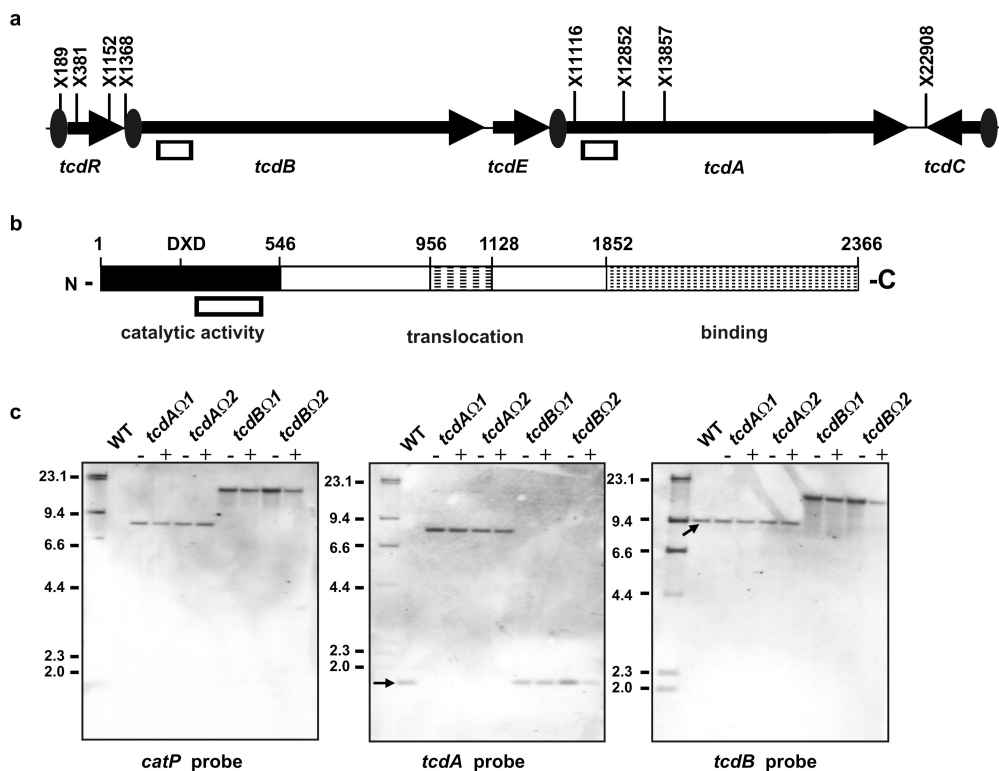


Fig. 1. Genetic organization of the *C. difficile* PaLoc and analysis of toxin mutants. **(a)** PaLoc genetic organization. *Xba*I sites (X) are shown, with the PaLoc co-ordinate indicated. Boxes indicate regions used in the recombination vectors and the *tcdB*- and *tcdA*-specific probes. Ovals show the gene-specific promoters and arrows the direction of transcription. **(b)** Structural organization of toxin B (toxin A is similar). Co-ordinates indicate the amino acid number. Relevant domains are shown. The box indicates the region in the recombination vectors. **(c)** Southern hybridization of mutants. Genomic DNA was *Xba*I-digested and blots probed as shown. λ -*Hind*III markers are indicated (kb). WT represents strain JIR8094, the mutant lanes are indicated. DNA was purified from cultures grown in the absence (-) or presence (+) of thiamphenicol selection.

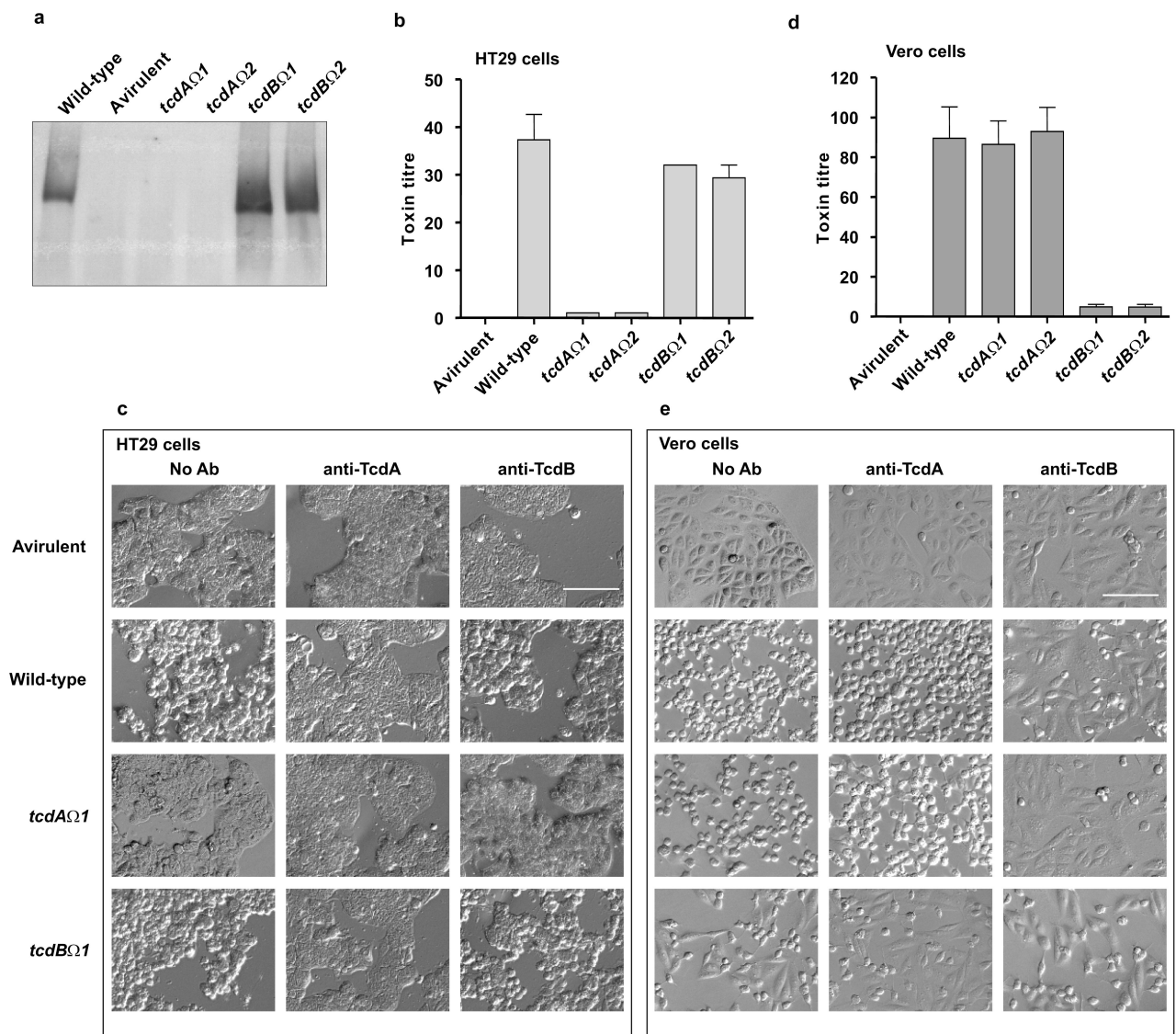


Fig. 2. Comparative analysis of toxin production by wild-type and mutants. The wild-type is JIR8094 and the avirulent strain is CD37, which does not produce toxins²⁹. **(a)** Western blot using toxin A-specific antibodies. **(b)** and **(d)** Toxin A and toxin B cytotoxicity assays using HT-29 and Vero cells, respectively. Data represent the mean \pm SEM. $n=3$. **(c)** and **(e)** Neutralization of toxicity. Culture supernatants were added to the cells and morphological changes observed after 24 h (No Ab) or pretreated with toxin A (anti-TcdA) and toxin B (anti-TcdB) neutralizing antibodies prior to their addition to the cells. Representative images are shown. No neutralization was observed when supernatants were pretreated with anti-ErmB methylase antibodies (data not shown). Bar indicates 1 mm.

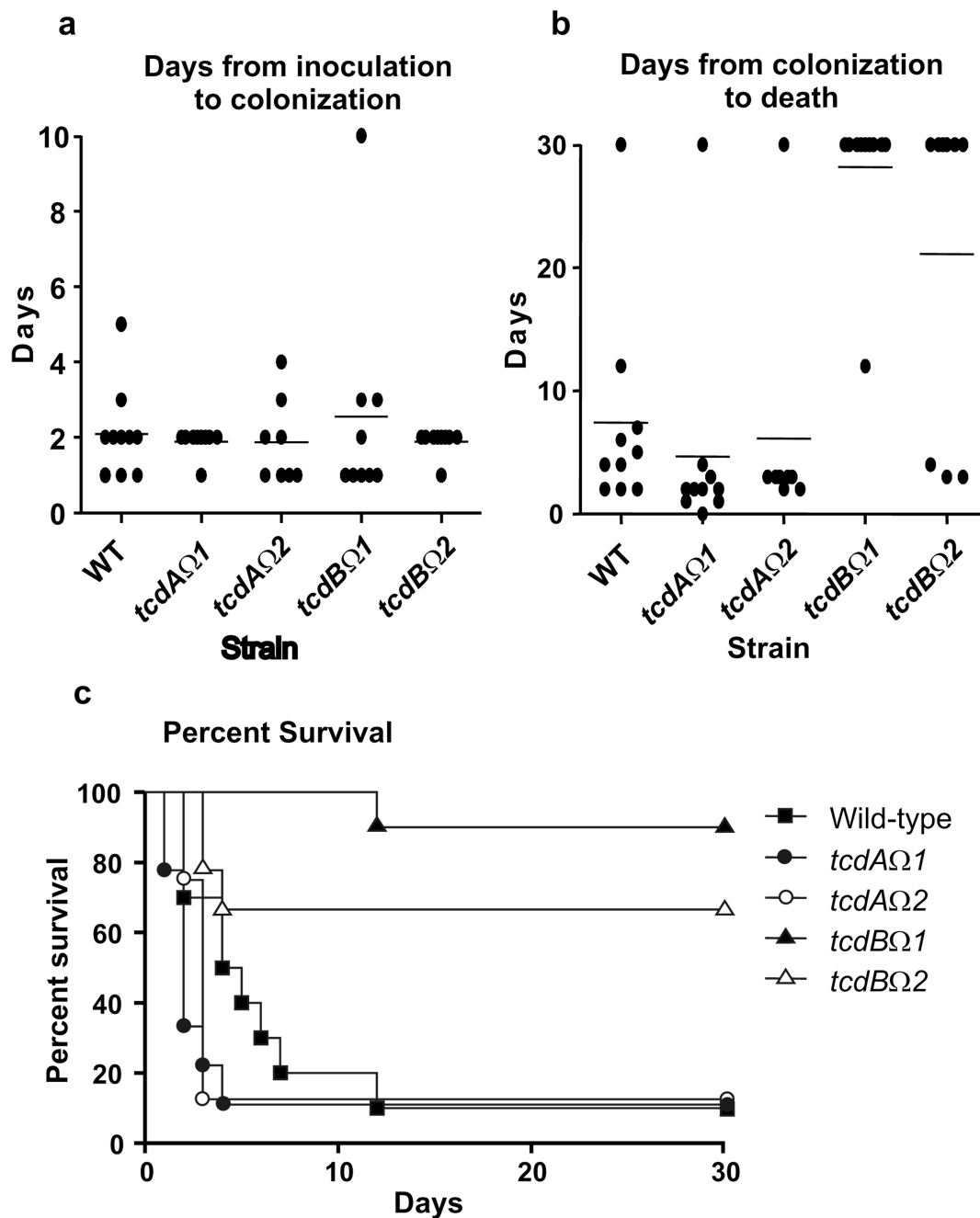


Fig. 3. Virulence of *C. difficile* wild-type and mutant strains in hamsters. **(a)** Days from inoculation of *C. difficile* to colonization of clindamycin treated hamsters. The rate of colonization was 10/10 for WT, 9/10 for *tcdA*Δ1, 8/10 for *tcdA*Δ2, 9/10 for *tcdB*Δ1 and 9/10 for *tcdB*Δ2. *n*=10. **(b)** Days from colonization to death or sacrifice. All hamsters that survived were sacrificed at day 30. *n*=10. **(c)** Kaplan-Meier survival estimate demonstrating days from colonization with *C. difficile* strains to death. *n*=10.