# In vitro expression of binary toxin in *C. difficile*

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**Abstract**

*Clostridium difficile* is the world's leading known cause of nosocomial diarrhea. In addition to the two main virulence factors (Toxins A and B) several strains produce a third, binary toxin composed of two unlinked proteins designated CdtA and CdtB. Although the role of binary toxin is still unclear, it is gaining attention as a possible virulence factor. Our aim was to investigate the *in vitro* expression of CdtB across a panel of ribotypes that carry the CDT Operon. As controls, we included ribotypes that do not carry the full operon. Brain-heart infusion broths were inoculated in triplicate with stock *C. difficile* cultures and incubated 24 or 72 hours anaerobically at 37˚C. Culture fluids were assayed using an in-house monoclonal antibody-based ELISA to detect CdtB. We assayed a total of 33 isolates across 29 different ribotypes. Twenty one ribotypes carried genes for binary toxin, and 19 of these made detectable amounts of CdtB *in vitro*. All eight ribotypes that did not carry the genes for binary toxin were negative in the ELISA. As an extension of this study, we inoculated brain-heart infusion broths with 24 hour cultures of *C. difficile*. The broths were harvested after 0, 2, 4, 8, 16, and 24 hour anaerobic incubation at 37˚C. Several ribotypes made detectable amounts of CdtB within four hours of growth. To summarize, our data show that binary toxin is not a ribotype 027-specific phenomenon. Further investigation is needed to learn more about the role of binary toxin as a virulence factor of C. difficile.

## Introduction

Multiple ribotypes of *Clostridium difficile* carry genes coding for the binary toxin (CDT). Unlike the two main virulence factors, toxins A and B, the contribution of binary toxin to *C. difficile* infection is unclear. Original characterization of the toxin showed that when trypsinized, it was cytotoxic to cultured cells *in vitro* (5). Geric et al. showed that Toxin A-/B-/CDT+ isolates were cytotoxic to cultured cells and enterotoxic in the rabbit illeal loop assay. However, they did not cause diarrhea in clindamycin-treated hamsters (3). Conversely, in an unrelated study, four out of five people infected with isolates of the same phenotype (A-/B-/CDT+) required antimicrobial treatment (4). The CDT operon contains three genes; *cdtR*, *cdtA*, and *cdtB*. The *cdtR* gene is thought to code for an up-regulator of *cdtA* and *cdtB* (2). Some CDT+ strains have a truncating SNP in the *cdtR* gene but still express *cdtA* and *cdtB* (7). This suggests either that *cdtR* is not the only regulator or a shortened form of the gene is still functional. CdtA is the enzymatic portion of the toxin that catalyzes the ADP-ribosylation and subsequent disruption of the actin cytoskeleton. CdtB binds to the lipolysis-stimulated lipoprotein receptor on host cells (1) and forms a pore in the cell membrane allowing the translocation of CdtA. "Ghost Isolates" containing a full *cdtR* gene but truncated forms of *cdtA* and *cdtB* have also been described (2).

Previously we showed that the presence of high-level CdtB in fecals correlated with patients infected with ribotype 027 (6). Because binary toxin has been more commonly associated with ribotype 027, which has been associated with worse patient outcome, it seems likely that binary toxin could serve as a predictor of 027 infection and therefore severe disease. However, the interpretation is complicated by our results showing that high-level *in vitro* expression of binary toxin is not specific to ribotype 027.

## Methods

**Bacterial isolates:** Fecal samples were plated onto Cycloserine Cefoxitin Fructose Agar and incubated for 48-72 hours at 37˚C. Colonies with *C. difficile* morphology were subcultured to chopped meat broth and stored until ready for use. Cultures were confirmed as *C. difficile*.

***In vitro* expression of CdtB:** 24 and 72 hour BHI cultures were assayed for CdtB production using an in house ELISA. Cultures were diluted and loaded into micro-titer wells coated with monoclonal antibody raised against CdtB. Following incubation and washing, polyclonal antibodies conjugated to HRP were added. After a second incubation and washing TMB substrate was added. The wells were assayed by 450/620nm dual wavelength.

**CdtB in the Growth Cycle:** Five BHI broths per isolate were inoculated with log phase *C. difficile*. The broths incubated at 37˚C for 0, 2, 4, 8, 16, or 24 hours. Cultures at each time point were assayed in our CdtB ELISA.

**PCR:** DNA was extracted from the overnight BHI cultures using the Qiagen Mini Kit. DNA isolates were standardized to 1ug/mL whole genomic DNA. We amplified the *cdtA* and *cdtB* genes as well as the SNP in *cdtR*.

## Results and Discussion

**PCR:** We analyzed the CDT operon in 33 isolates across 29 ribotypes. Eight ribotypes were *cdtA* and *cdtB*-negative by PCR and did not express CdtB *in vitro*. One out of the eight ribotypes was completely negative for the CDT operon (negative for *cdtR*, *cdtA*, and *cdtB* by PCR) while the other seven had the "ghost operon" (full *cdtR* but only fragments of *cdtA* and *cdtB*.) All seven ghost ribotypes had a WT *cdtR* gene. Three ribotypes had the SNP in *cdtR*, all of which contained full *cdtA* and *cdtB* genes. Strains with a WT *cdtR* made higher amounts of CdtB (Table 1).

**CdtB expression:** All 33 isolates were assayed for CdtB using our ELISA. Twenty two isolates, including two with the *cdtR* SNP, made detectable but in some cases low levels of CdtB. As we had previously seen, 027 strains were among the most potent producers. What is interesting to note is that 033 (a Toxin A-/B-/CDT+ isolate), despite having a wild type *cdtR*, made levels of CdtB comparable to those with the *cdtR* SNP. This may be one reason why Geric et al. did not see symptoms in hamsters infected with this particular strain.

To test whether or not CdtB is produced in log-phase, BHI broths were inoculated with log-phase *C. difficile* and incubated for 0, 2, 4, 8, 16, or 24 hours at 37˚C. Cultures were assayed in our CdtB ELISA. Several strains made detectable levels of CdtB within four hours of growth. The cultures were not standardized to an OD but several non-027 strains made the highest amounts of CdtB in this time study (Fig 2).

Geric et al. showed that when supernatants from 033 (Toxin A-/B-/CDT+) were trypsinized they were both cytotoxic and enterotoxic but the isolates themselves did not cause disease in hamsters. We have data showing that 033 is a weak binary toxin producer. Considering that some strains made 15-20 times more CdtB than 033 (Fig 1), and that binary toxin itself is toxic, it seems reasonable that binary toxin may contribute to disease if sufficient quantities are present.

Using their Clostron technology, Kuehne at el. showed that a Toxin A-/B-/CDT+ ARL 027 was able to cause disease in clindamycin-treated hamsters (8). Here we have shown that other ribotypes can also make levels of CdtB comparable to those made by 027 in *vitro*.

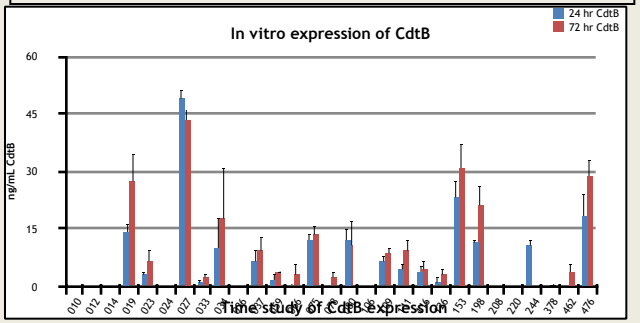


Figure 1. *In vitro* expression of CdtB. Strains are listed by ribotype

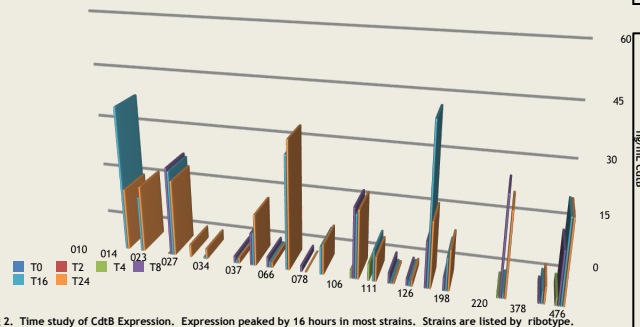


Fig 2. Time study of CdtB Expression. Expression peaked by 16 hours in most strains. Strains are listed by ribotype.

| Ribotype | genes | | | | ng/mL CdtB |
|---|---|---|---|---|---|
| | cdtR | cdtA | cdtB | CDT phenotype | |
| 10 | - | - | - | - | 0.0 |
| 12 | WT | - | - | Ghost | 0.0 |
| 14 | WT | - | - | Ghost | 0.0 |
| 24 | WT | - | - | Ghost | 0.0 |
| 36 | WT | + | + | + | 0.0 |
| 106 | WT | - | - | Ghost | 0.0 |
| 208 | WT | - | - | Ghost | 0.0 |
| 220 | WT | - | - | Ghost | 0.0 |
| 378 | WT | - | - | Ghost | 0.0 |
| 78 | SNP | + | + | + | 2.0 |
| 33 | WT | + | + | + | 2.2 |
| 66 | WT | + | + | + | 3.0 |
| 126 | SNP | + | + | + | 3.0 |
| 59 | WT | + | + | + | 3.4 |
| 462 | SNP | + | + | + | 3.6 |
| 116 | WT | + | + | + | 4.6 |
| 23 | WT | + | + | + | 6.6 |
| 109 | WT | + | + | + | 8.3 |
| 37 | WT | + | + | + | 9.1 |
| 111 | WT | + | + | + | 9.5 |
| 244 | WT | + | + | + | 10.8 |
| 80 | WT | + | + | + | 11.0 |
| 75 | WT | + | + | + | 13.7 |
| 34 | WT | + | + | + | 17.3 |
| 198 | WT | + | + | + | 20.9 |
| 19 | WT | + | + | + | 27.6 |
| 476 | WT | + | + | + | 28.4 |
| 153 | WT | + | + | + | 31.0 |
| 27 | WT | + | + | + | 43.1 |

Table 1. PCR Results of the CDT Operon and CdtB expression levels in different *C. difficile* ribotypes.

## Conclusions

- Strains with a wild type *cdtR* produced more CdtB
- *cdtB* is expressed mostly during log-phase when the organism is actively growing
- Other ribotypes can express levels of CdtB similar to those of 027
- Future study may show that non-027 ribotypes can produce enough binary toxin to contribute to disease

## References

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