toxins

# Supplementary Materials: A Streamlined Method to Obtain Biologically Active TcdA and TcdB Toxins from *Clostridioides difficile*

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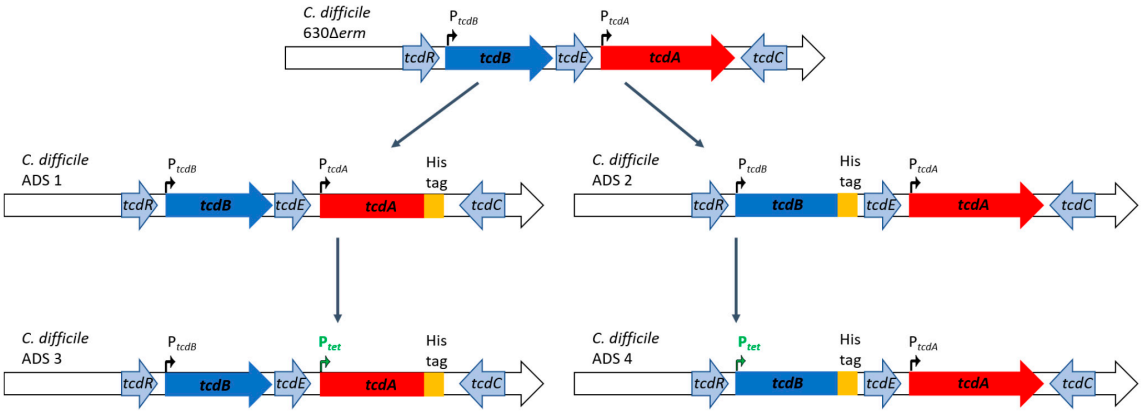



**Figure S1.** Strain construction for the production of the recombinant toxins rTcdA and rTcdB of *C. difficile*. The strains were obtained in two steps using the allele exchange method. Strains ADS1 and ADS2 were obtained by adding a His-tag at the 3' end of the *tcdA* or *tcdB* genes of the *C. difficile* strain 630Δ*erm*. Strains ADS3 and ADS4 were obtained by replacing the respective promoter of *tcdA* and *tcdB* by the promoter Ptet inducible to anhydro-tetracycline (ATc). AD3 and ADS4 were used for the purification of recombinant toxin A (rTcdA) and B (rTcdB).

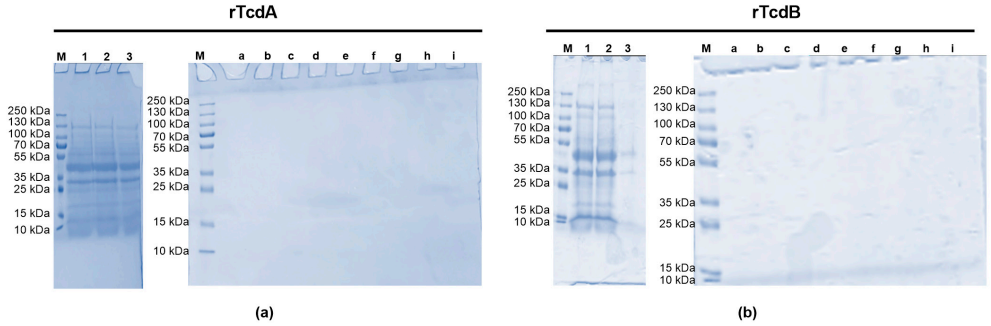
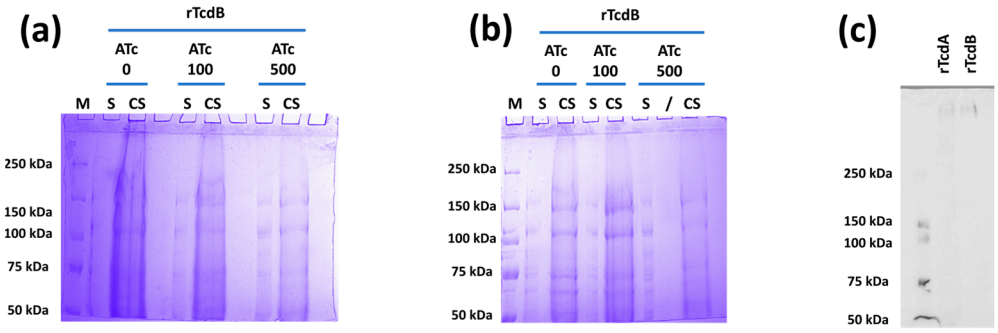



**Figure S2.** Purification of recombinant His-tagged toxins by Ni-NTA affinity chromatography under denaturing conditions from the genetic construction without the Ptet promoter. Toxins were visualized by Coomassie staining for (**A**) recombinant toxin A (rTcdA); (**B**) recombinant toxin B (rTcdB). M: PageRuler Plus marker (ThermoFisher) / 1: raw protein samples / 2: flow through / 3: wash. Imidazole eluted fractions: a: 10 mM / b: 20 mM / c: 30 mM / d: 40 mM / e: 60 mM / f: 80 mM / g: 100 mM / h: 200 mM / i: 1 M.




(d)

| DNA quantification (ng/µL) | rTcdA | rTcdB |
|---|---|---|
| Recombinant protein | 12.5 | 18.6 |
| DNAse-treated recombinant protein | 9.8 | 9.7 |
| Recombinant protein + DNA spike in | 23 | 38.3 |

**Figure S3.** Validation of the recombinant protein extraction method. Absence of recombinant proteins in culture supernatant was confirmed for rTcdA and rTcdB (panels **a** and **b** respectively). Specificity of extracted recombinant proteins was confirmed by His-tag directed western-blot (panel **c**). DNA contamination in the extracted protein samples was estimated by DNA quantification using the nanodrop before and after DNAse treatment, using spiked-in DNA as a control (panel **d**). For experimental details, see Supplemental File S2. M: Page Ruler Plus ladder (ThermoFisher), S: raw culture supernatant, CS: Concentrated supernatant after protein precipitation.

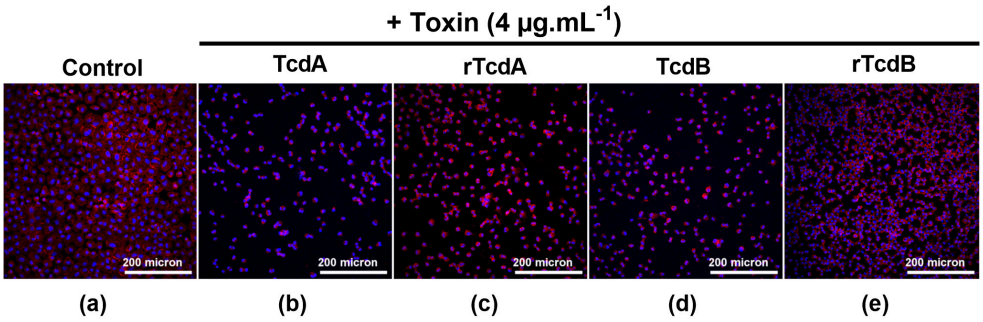



**Figure S4.** Disruption of cytoskeleton on Vero cells with 20x objective. Cells in 24 wells plate were in the absence of toxin (panel a = control) or in the presence of 4 µg.ml$^{-1}$ of either native or recombinant toxins **A** (panel b = native / panel c = recombinant) and **B** (panel d = native / panel e = recombinant). Cells were incubated for 18 hours, fixed, and stained with Rhodamine Phalloidin (actin staining in red) and DAPI (nuclei staining in blue) then observed under confocal microscopy with 20× objective to quantify the effect of toxins on the cells.

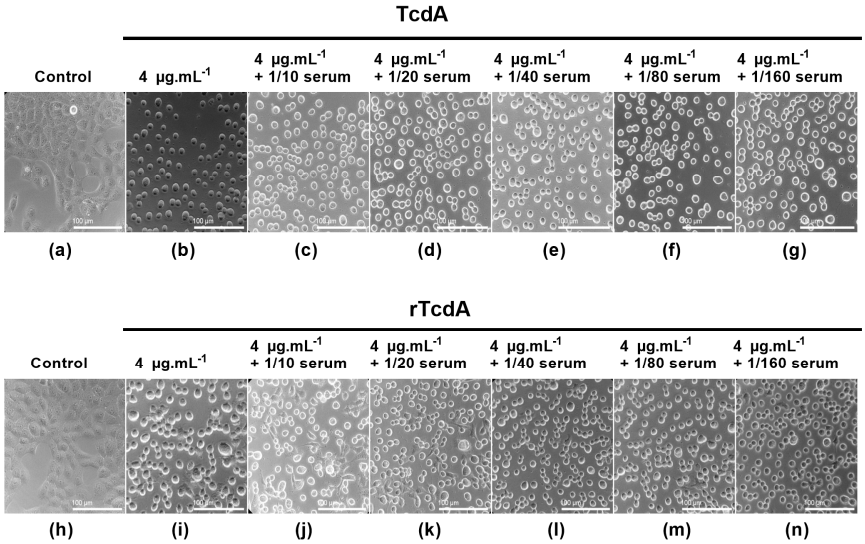



**Figure S5.** Serum without anti-TcdA neutralizing IgG does not neutralize TcdA toxins activity. Toxins were preincubated with several sera from a CDI-convalescent patient using dilutions ranging from 1/10 (panels **c** and **j**) to 1/160 (panels **g** and **n**) before addition to Vero cell monolayers. Native VPI 10463 TcdA and recombinant TcdA were compared (panels **b**-**g** and **i**-**n** respectively). Cells incubated without toxins (panels **a** and **h**) were included as a negative control. Cells incubated with non-neutralized toxins were included as a positive control (panels **b** and **i**). Morphological changes were observed under phase contrast microscopy after 18 h at 37 °C.

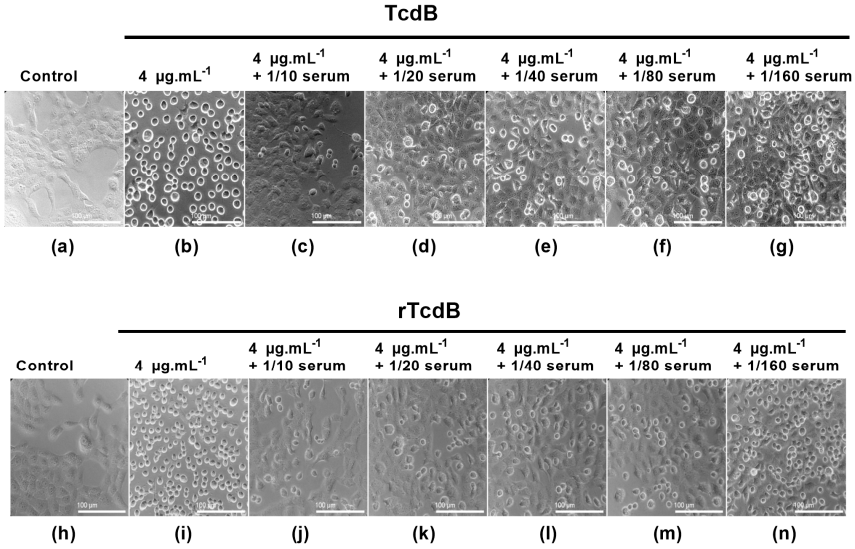



**Figure S6.** Serum with anti-TcdB neutralizing IgG neutralizes both native and recombinant TcdB activity. Toxins were preincubated with several sera from a CDI-convalescent patient using dilutions ranging from 1/10 (panels **c** and **j**) to 1/160 (panels **g** and **n**) before addition to Vero cell monolayers. Native VPI 10463 TcdB and recombinant TcdB were compared (panels **b**-**g** and **i**-**n** respectively). Cells incubated without toxins (panels **a** and **h**) were included as a negative control. Cells incubated with non-neutralized toxins were included as a positive control (panels **b** and **i**). Morphological changes were observed under phase contrast microscopy after 18 h at 37 °C.

**Table S1.** List of bacterial strains used in this study.

| Strains | Characteristics | Reference(s)/source |
|---|---|---|
| TG1 | *E.coli* SupE *hsd*Δ5 thiΔ(lac-proAB) *F'*[*tra36 pro AB*$^{+}$*lacI*$^{q}$ *lac*ZΔM15] | [59] |
| HB101*tra* | *E. coli,* F-Δ (gpt-proA) 62 LEU B6 gln V44 ara-14 gal-K2 lacY1 delta (mcrC-mrr) rps L20 (srtr) xyl-5 mLt-1 recA13, pRK24 (transfer function) | Laboratory |
| 630Δ*erm* | *C. difficile*, Wild-type strain sensible to erythromycin | Laboratory |
| AD1 | *C. difficile,* 630Δ*erm* with 6xhis-tag in the 3′ end of *tcdA* | This study |
| AD2 | *C. difficile,* 630Δ*erm* with 6xhis-tag in the 3′end of *tcdB* | This study |
| AD3 | *C. difficile* strain AD1 with *tcdA* native promoter replaced with $P_{tet}$ promoter (630Δ*erm* $P_{tet}$-TcdA_his tag) | This study |
| AD4 | *C. difficile* strain AD2 with *tcdB* native promoter replaced with $P_{tet}$ promoter (630Δ*erm* $P_{tet}$-TcdB_his tag) | This study |

**Table S2.** List of primers used in this study.

| Name | Sequence 5′ – 3′ | Utilization |
|---|---|---|
| 2836 | ctgcacccgggGAGACCGGAAAAGATCCGGGG-GATCGATCCTCTAG | Primer for amplification of the spectinomycin cassette |
| 2837 | catgtcgcatgcGAGACCTTAGCCTAATTGAGA-GAAGTTTCTATAG | Primer for amplification of the spectinomycin cassette |
| 2854 | GGCTACGGTCTCCCCGGGgcctcaactggttata-caagtattaatgg | Primer for the amplification of PCR fragment 1 for pADS1 construction |
| 2851 | GGCTACGGTCTCCGGCAttaG-tGGTGATGGTGATGGTGATGgccatatatcccaggggct ttttactcc | Primer for the amplification of PCR fragment 1 for pADS1 construction |
| 2852 | GGCTACGGTCTCCTGCCaatatatgtttga-taaaaaattattcctgtg | Primer for for amplification of PCR fragment 2 for pADS1 construction |
| 2853 | GGCTACGGTCTCGCATGCggtaacgaatttag-taatgaaggaaaagg | Primer for amplification of PCR fragment 2 for pADS1 construction |
| 2858 | GGCTACGGTCTCCCCGGGgctgaaaacgga-gaaatgcaaataggag | Primer for amplification of PCR fragment 1 for pADS2 construction |
| 2859 | GGCTACGGTCTCCGGCAttaG-tGGTGATGGTGATGGTGATGttcactaatcactaatt-gagctgtatcaggatc | Primer for amplification of PCR fragment 1 for pADS2 construction |
| 2860 | GGCTACGGTCTCCTGCCataaaaa-tatgttaaatatatcctcttatacttaa | Primer for amplification of PCR fragment 2 for pADS2 construction |
| 2861 | GGCTACGGTCTCGCATGCgtccttcatcacgtatttttt-gtgtttttg | Primer for amplification of PCR fragment 2 for pADS2 construction |
| 3046 | gatgagaagggcataatgggctacggtctctttgc | Forward primer for CO1 fragment amplification for pADS3 construction |
| 3047 | ttacttttcatccttagcattcggctacggtctcttcttatttttatgctagc | Reverse primer for CO1 fragment amplification for pADS3 construction |
| 3048 | taagaagcctgcatttgcagggctacggtctc | Forward primer for $P_{tet}$ fragment amplification for pADS3 construction |
| 3049 | aaagacataaaattttctcctttactgcaggggctacggtctcg | Reverse primer for $P_{tet}$ fragment amplification for pADS3 construction |
| 3050 | ctttaatatctaaagaagagttaataaaacggctacggtctcg | Forward primer for CO2 fragment amplification for pADS3 construction |
| 3051 | ctgccgcctctaattttatcatttccggctacggtctc | Reverse primer for CO2 fragment amplification for pADS3 construction |

| | | |
|---|---|---|
| 3052 | ttatgtaattgttacttgaaaattgatcggctacggtctcattgc | Forward primer for CO1 fragment amplification for pADS4 construction |
| 3053 | ttacaagttaaaataattttcatagtctttttttatttttttaaatgggctacggtctcgtcttatttttatgctagc | Reverse primer for CO1 fragment amplification for pADS4 construction |
| 3054 | aagaagcctgcatttgcagggctacggtctcg | Forward primer for $P_{tet}$ fragment amplification for pADS4 construction |
| 3055 | aaaattttctcctttactgcaggggctacggtctcgactaaactcat | Reverse primer for $P_{tet}$ fragment amplification for pADS4 construction |
| 3056 | tagttaatagaaaacagttagaaaaaatgggctacggtctcg | Forward primer for CO2 fragment amplification for pADS4 construction |
| 3057 | tgttctgaggtatattctggggctacggtctcttgcc | Reverse primer for CO2 fragment amplification for pADS4 construction |
| 3064 | tatacaattgagactggatggat | Forward primer outside CO1 fragment to verify $P_{tet}$ promoter's insertion in front of *tcdA* in *C. difficile* |
| 3065 | aatatctcagatttttcactttt | Reverse primer outside CO2 to verify $P_{tet}$ promoter's insertion in front of *tcdA* in *C. difficile* |
| 3066 | gtcttgaaaatatttagtttgaa | Forward primer outside CO1 fragment to verify $P_{tet}$ promoter's insertion in front of *tcdB* in *C. difficile* |
| 3067 | attttaacttctagtggtgatgc | Reverse primer outside CO2 to verify $P_{tet}$ promoter's insertion in front of *tcdB* in *C. difficile* |
| 3068 | ctgtttttctgtaggccgtgt | Forward primer inside $P_{tet}$ promoter to verify $P_{tet}$'s insertion in front of *tcdA* and *tcdB* in *C. difficile* |
| 3069 | acacggcctacagaaaaacag | Reverse primer inside $P_{tet}$ promoter to verify $P_{tet}$'s insertion in front of *tcdA* and *tcdB* in *C. difficile* |
| 3070 | gctgttaataaggctaaaaatagagca | Forward primer inside CO1 fragment to verify $P_{tet}$ promoter's insertion in front of *tcdB* in *C. difficile* |
| 3071 | aaggctttatttctaccagattttt | Reverse primer inside CO2 to verify $P_{tet}$ promoter's insertion in front of *tcdB* in *C. difficile* |
| 3072 | gaaagtgtaagtatattaaaaaatat | Forward primer inside CO1 fragment to verify $P_{tet}$ promoter's insertion in front of *tcdA* in *C. difficile* |
| 3073 | cattattgtttgtagttaacttat | Reverse primer inside CO2 to verify $P_{tet}$ promoter's insertion in front of *tcdA* in *C. difficile* |
| 3143 | tcaaagcggcaactaagagt | Forward primer outside CO1 fragment in pJV7 vector to characterize the first Crossing-over between pADS3 and gDNA in AD3 and between pADS4 and gDNA in AD4 |
| 3144 | tgtaaaacgacggccagt | Reverse primer outside CO2 fragment in pJV7 vector to characterize the first Crossing-over between pADS3 and gDNA in AD3 and between pADS4 and gDNA in AD4 |
| 3145 | tggcatctgctccctcgttttcat | Reverse primer inside *tcdA* native promoter in front of CO2 fragment to characterize the first Crossing-over between pADS3 and gDNAin AD3 |
| 3146 | aggagtgtataagatttatttc | Forward primer inside *tcdA* native promoter in front of CO2 fragment to characterize the first Crossing-over between pADS3 and gDNA in AD3 |
| 3147 | aagacactcagttgattaatttgc | Reverse primer inside *tcdB* native promoter in front of CO2 fragment to characterize the first Crossing-over between pADS4 and gDNA in AD4 |
| 3148 DS30 | gcaaattaatcaactgagtgtctt | Forward primer inside *tcdB* native promoter in front of CO2 fragment to characterize the first Crossing-over between pADS4 and gDNA in AD4 |

**Table S3.** List of Plasmids used in this study.

| Name | Characteristics | Reference (s)/Source |
|---|---|---|
| pAT28 | Spec$^r$ ori pUC ori pAmβ1 (resistance spc) | [43] |
| pTC129 | pBlunt+ spc (BsaI) (resistance Kn+spc) | This study |
| pTC130 | pMSR BamH1/XhoI +pTC129 BamH1/xhoI (resistance Cm+spc) | This study |
| pADS1 | pTC130 with 6xhis - tag for allele exchange in *C. difficile* 630Δ*erm* to generate strain AD1 | This study |
| pADS2 | pTC130 with 6xhis - tag for allele exchange in *C. difficile* 630Δ*erm* to generate strain AD2 | This study |
| pRPF185 | pRPF177 with fdx transcriptional terminator added after *tetR, gusA* | [46] |
| pJV7 | pMSR spc (BsaI) (resistance Cm+spc) | [47] |
| pADS3 | $P_{tet}$ promoter and homologous regions cloned in pJV7 vector for allele exchange upstream of *tcdA* in *C. difficile* strain AD1 to generate strain AD3 | This study |
| pADS4 | $P_{tet}$ promoter and homologous regions cloned in pJV7 vector for allele exchange upstream of *tcdB* in *C. difficile* strain AD2 to generate strain AD4 | This study |

**Table S4.** List of Genome accession numbers used for Figure 1.

| Genome Name / Sample Name | Genome ID |
|---|---|
| *Clostridioides difficile* E12 | CAMZ00000000 |
| *Clostridioides difficile* E28 | CAMX00000000 |
| *Clostridioides difficile* T11 | CAML00000000 |
| *Clostridioides difficile* NAP08 | ADNX00000000 |
| *Clostridioides difficile* NAP07 | ADVM00000000 |
| *Clostridioides difficile* QCD-66c26 | CM000441 |
| *Clostridioides difficile* 630 delta erm | LN614756 |
| *Clostridioides difficile* T19 | CANA00000000 |
| *Clostridioides difficile* T61 | CAND00000000 |
| *Clostridioides difficile* QCD-97b34 | CM000657 |
| *Clostridioides difficile* T17 | CAMT00000000 |
| *Clostridioides difficile* E15 | CAMM00000000 |
| *Paeniclostridium sordellii* ATCC 9714 | APWR00000000 |
| *Clostridioides difficile* T6 | CAMR00000000 |
| *Clostridioides difficile* E24 | CAMP00000000 |
| *Clostridioides difficile* 630 | AM180355, AM180356 |
| *Clostridioides difficile* E14 | CAMS00000000 |
| *Clostridioides difficile* QCD-23m63 | CM000660 |
| *Clostridioides difficile* CD15 | FUUU00000000 |
| *Clostridioides difficile* T15 | CAMK00000000 |
| *Clostridioides difficile* CIP 107932 | CM000659 |
| *Clostridioides difficile* E25 | CAMJ00000000 |
| *Clostridioides difficile* E16 | CAMH00000000 |
| *Clostridioides difficile* QCD-32g58 | CM000287 |
| *Clostridioides difficile* T42 | CAMQ00000000 |
| *Clostridioides difficile* QCD-63q42 | CM000637 |
| *Clostridioides difficile* E23 | CAMY00000000 |
| *Clostridioides difficile* QCD-37x79 | CM000658 |
| *Clostridioides difficile* R20291 | FN545816 |
| *Clostridioides difficile* T22 | CAMI00000000 |
| *Clostridioides difficile* T10 | CANB00000000 |

| | |
|---|---|
| *Clostridioides difficile* E1 | CAMD00000000 |
| *Clostridioides difficile* QCD-76w55 | CM000661 |
| *Clostridioides difficile* T5 | CAMB00000000 |
| *Clostridioides difficile* T20 | CAMC00000000 |
| *Clostridioides difficile* E7 | CAMV00000000 |
| *Clostridioides difficile* T23 | CAMN00000000 |
| *Clostridioides difficile* T3 | CAMW00000000 |
| *Clostridioides difficile* CD196 | FN538970 |
| *Clostridioides difficile* A685 | https://mage.genoscope.cns.fr/microscope/search/exportForm.php?O_id=1276 |