Supporting Information

Zhang et al. 10.1073/pnas.1400680111

SI Materials and Methods

Expression and Purification of Recombinant TcdB from *Bacillus megaterium*. The template used for mutagenesis and clone for production of recombinant TcdB wild type and mutant was a *B. megaterium* expression vector pHis1522 encoding the strain VPI10463 obtained from Hangping Feng. Proteins were expressed and purified as previously described (1).

Expression and Purification of Recombinant Codon-Optimized TcdB Constructs. Codon-optimized TcdB sequence was synthesized (GenScript) to increase GC percentage to 47%. The codon-optimized gene was cloned into an *Escherichia coli* expression vector pET28a and transformed into *E. coli* BL21 DE3 competent cells and expressed as C-terminal His-tagged proteins. A total of 50 mL of overnight culture was inoculated into 1 L of LB with 50 μg/mL kanamycin and induced at OD_{600} of 0.6 with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h. Cells were harvested by centrifugation and resuspended with lysis buffer (20 mM Tris pH 8.0, 0.5 M NaCl, protease inhibitor) and lysed by an EmulsiFlex C3 microfluidizer (Avestin) at 15,000 psi. After lysing, lysates were centrifuged at 18,000 × *g* for 20 min. Proteins were purified by Ni-affinity chromatography using a HisTrap FF column (GE Healthcare). Fractions containing TcdB were verified and pooled with a 100,000 MWCO ultrafiltration device. Ten percent of glycerol was added; protein concentration was calculated by densitometry (Image Lab 3.0).

Mutagenesis of TcdB Mutants. Single point mutations were made in the TcdB codon-optimized sequence using QuikChange lightning multimutagenesis kit (Agilent Technologies). Plasmids with correct mutations were transformed and expressed using the same conditions as wild type.

Small-Scale Expression of TcdB Mutants. Plasmids expressing TcdB mutants were transformed into *E. coli* BL21 DE3 cells. Overnight culture were prepared in 24-well blocks (BD Biosciences) in 5 mL of Luria Broth. A total of 250 μL of overnight culture was inoculated into 5 mL of LB with kanamycin and induced at OD_{600} of 0.6 with 0.5 mM IPTG at 37 °C for 4 h. Cells were harvested by centrifugation and resuspended in buffer (20 mM Tris, 500 mM NaCl pH 8.0 and protease inhibitor; Sigma) and lysed by lysozyme (Bioshop) according to the manufacturer's instructions, followed by centrifugation at 4,000 × *g* for 20 min. Supernatants were collected. The concentration of each full-length mutant protein in the lysates was determined by densitometry (Image Lab 3.0).

Cell Viability Assay. Chinese hamster ovary cells CHO-K1 cells were cultured in Ham's F-12 medium (Wisent) with 10% FCS (FBS; Wisent) and 1% penicillin and streptomycin (Wisent). CHO-K1 cells were seeded at a concentration of 8,000 cells per well in 96-well CellBind plates (Corning). The next day, medium was exchanged with serum-free medium and cells were intoxicated by adding TcdB toxins at a serial dilution of 1/3 starting at 1 nM. After intoxication, cells were incubated at 37 °C, 5% CO_2 for 48 h. Serum (FBS) was added back to cells 24 h after intoxication to a final concentration of 10% (vol/vol). The cell viability after 48 h was assessed by PrestoBlue Cell Viability Reagent (Life Technologies). Fluorescence was read on a Spectramax M5 plate reader (Molecular Devices).

Rubidium Release Assay. $^{86}Rb^+$ release assay was performed as previously reported (2) with slight modifications. Briefly, CHO-K1 cells were seeded in 96-well plates in the medium (Ham's F-12 with 10% FBS), supplemented with 1 μCi/mL $^{86}Rb^+$ (PerkinElmer) at a density of 1×10^4 cells per well. Cells were incubated at 37 °C, 5% CO_2 overnight. Medium was exchanged with fresh growth medium with 100 nM bafilomycin A1 (Sigma) and continued to incubate for another 20 min. Then, cells were chilled on ice and ice-cold medium containing TcdB mutants (10 nM) was added. Cells were kept on ice for toxin binding for 1 h at 4 °C before they were washed with ice-cold PBS twice to remove unbound toxins. pH-dependent insertion into the plasma membrane was induced by warm, acidified growth medium (37 °C, pH 4.5 or pH 7.5) for 5 min at 37 °C. After 1 h of further incubation on ice, medium containing released $^{86}Rb^+$ was removed from the cell plate and the amount of $^{86}Rb^+$ released was determined by liquid scintillation counting with TopCount NXT (PerkinElmer).

In Vitro Glucosyltransferase Assay. A total of 10 nM of TcdB mutants was incubated with 0.8 μM GST-Rac1 (0.2 μg/μL; Sigma) in 25 μM UDP-glucose in glucosylation buffer (50 mM Hepes, 100 mM KCl, 2 mM $MgCl_2$ and 1 mM $MnCl_2$, pH 7.5) for 60 min. The reaction was stopped by addition of Laemmli loading buffer with β-mercaptoethanol and boiling at 95 °C for 5 min. The proteins were separated on a 5–12% gradient polyacrylamide gel by SDS/PAGE and then proteins were transferred to nitrocellulose with an iBlot device (Invitrogen). Glucosylated GST-Rac1 was detected by standard Western blotting with an antibody that specifically recognizes the nonglucosylated form of Rac1 (Mab 102; BD Biosciences), anti-GST antibody (GenScript), and HRP-conjugated anti-mouse IgG (GE Healthcare).

2-p-toluidinylnaphthylene-6-sulfonate Fluorescence Assay. pH-induced conformational changes of TcdB were assessed as described previously (3). A total of 2 μg of TcdB was prepared in buffer that pH ranging from 4 to 7.2-(p-toluidiny)-naphthalene-6-sulfonic acid, sodium salt [2,6-2-p-toluidinylnaphthylene-6-sulfonate (TNS); Invitrogen] was added at a final concentration of 150 μM. The final volume was 250 μL and mixed in 96-well black plate (Corning). Mixtures were incubated at 37 °C for 20 min. The plate was analyzed in Spectramax M5 plate reader (Molecular Devices) with excitation of 366 nm and an emission scan of 380–500 nm.

Scintillation Proximity Assay. To determine the glucosyltransferase activity of TcdB mutants, 10 nM of each mutant was added to 1 μM GST-Rac1, 0.25 μM ^3H-UDP-glucose in a buffer containing 50 mM Hepes pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, and 1 mM $MnCl_2$. Reactions were stopped by the addition of 250 μL of PVT glutathione-conjugated beads (2 mg/mL) (PerkinElmer) in 0.5 M EDTA at various time points in a 96-well plate. Plates were incubated at room temperature overnight and read on a Top-Count NXT microplate scintillation counter (PerkinElmer). The resulting data were fit to a one-phase exponential function using GraphPad Prism 6. The linear phase of all progress curves were replotted and analyzed by linear regression to calculate the specific activity of the TcdB mutants.

Black Lipid Bilayer Experiments. Lipid bilayer experiments were performed as described previously with modifications (4). Briefly, membranes were made by painting diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in decane across a 200-μm aperture

in a Delrin cup by using the brush technique. Both *cis*- and *trans*-compartments contained 1 mL of solutions containing universal bilayer buffer (1 M KCl; 10 mM Tris pH 7.4). Translocation was initiated by adding appropriate amounts of 2 M HCl to the *cis*-compartment to lower the pH to 4.5. Each compartment was stirred continuously throughout the experiment with a small stir bar. Agar salt bridges linked Ag/AgCl electrodes in 3 M KCl. The current was amplified through a BC-525C integrating bilayer clamp amplifier (Warner Instruments), filtered at a frequency of 0.1 kHz by a low-pass eight-pole Bessel filter and computer displayed through an analog/digital converter.

Cell Death Assay. Cell death assay was performed as previously described (5). Briefly, IMR-90 cells (cultured in EMEM, 10% FBS, 5% CO_2) were seeded in 96-well Cellbind plates at a concentration of 8,000 cells per well. The next day, the growth medium was exchanged with serum-free EMEM and incubated at 37 °C, 5% CO_2 for 60 min. TcdB toxins were added to cells in dilutions starting at 30 nM. After intoxication, cells were incubated at 37 °C, 5% CO_2 for 3 h. The amount of ATP was assessed with CellTiterGlo as per the manufacturer's instructions (Promega). Plates were read in Spectramax M5 plate reader (Molecular Devices).

1. Yang G, et al. (2008) Expression of recombinant Clostridium difficile toxin A and B in Bacillus megaterium. *BMC Microbiol* 8:192.
2. Genisyuerek S, et al. (2011) Structural determinants for membrane insertion, pore formation and translocation of Clostridium difficile toxin B. *Mol Microbiol* 79(6):1643–1654.
3. Lanis JM, Barua S, Ballard JD (2010) Variations in TcdB activity and the hypervirulence of emerging strains of Clostridium difficile. *PLoS Pathog* 6(8):e1001061.
4. Melnyk RA, Collier RJ (2006) A loop network within the anthrax toxin pore positions the phenylalanine clamp in an active conformation. *Proc Natl Acad Sci USA* 103(26):9802–9807.
5. Chumbler NM, et al. (2012) Clostridium difficile toxin B causes epithelial cell necrosis through an autoprocessing-independent mechanism. *PLoS Pathog* 8(12):e1003072.

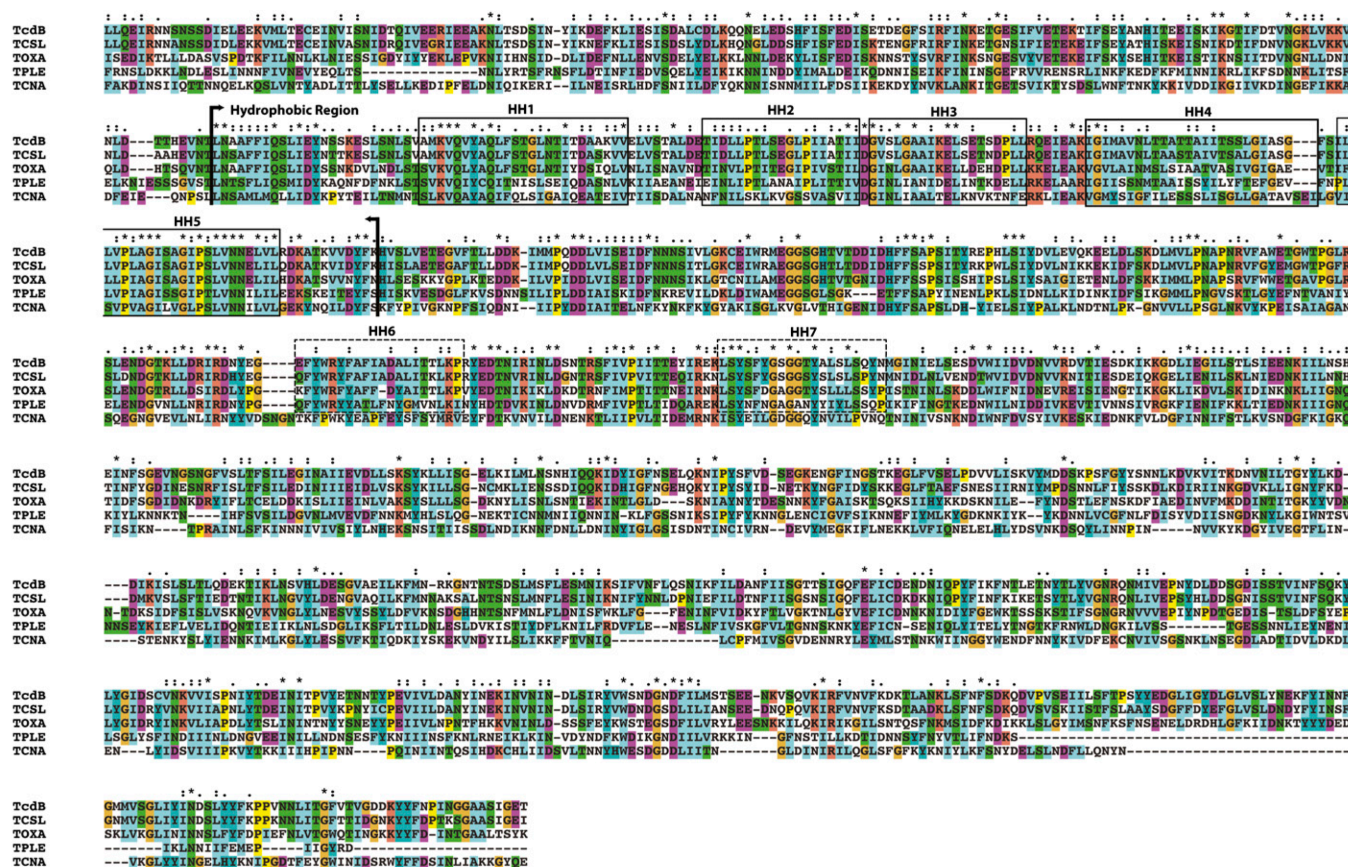

Fig. S1. Alignment of the translocation domain of the large clostridial toxin family using ClustalX2.1. Residues 800–1,880 are shown using TcdB numbering. The hydrophobic region (956–1,128) is highlighted and the regions predicted to be hydrophobic are shown as boxes. Only HH1–HH5 were predicted to be hydrophobic, whereas HH6 and HH7 were predicted in all homologs except for TcnA.

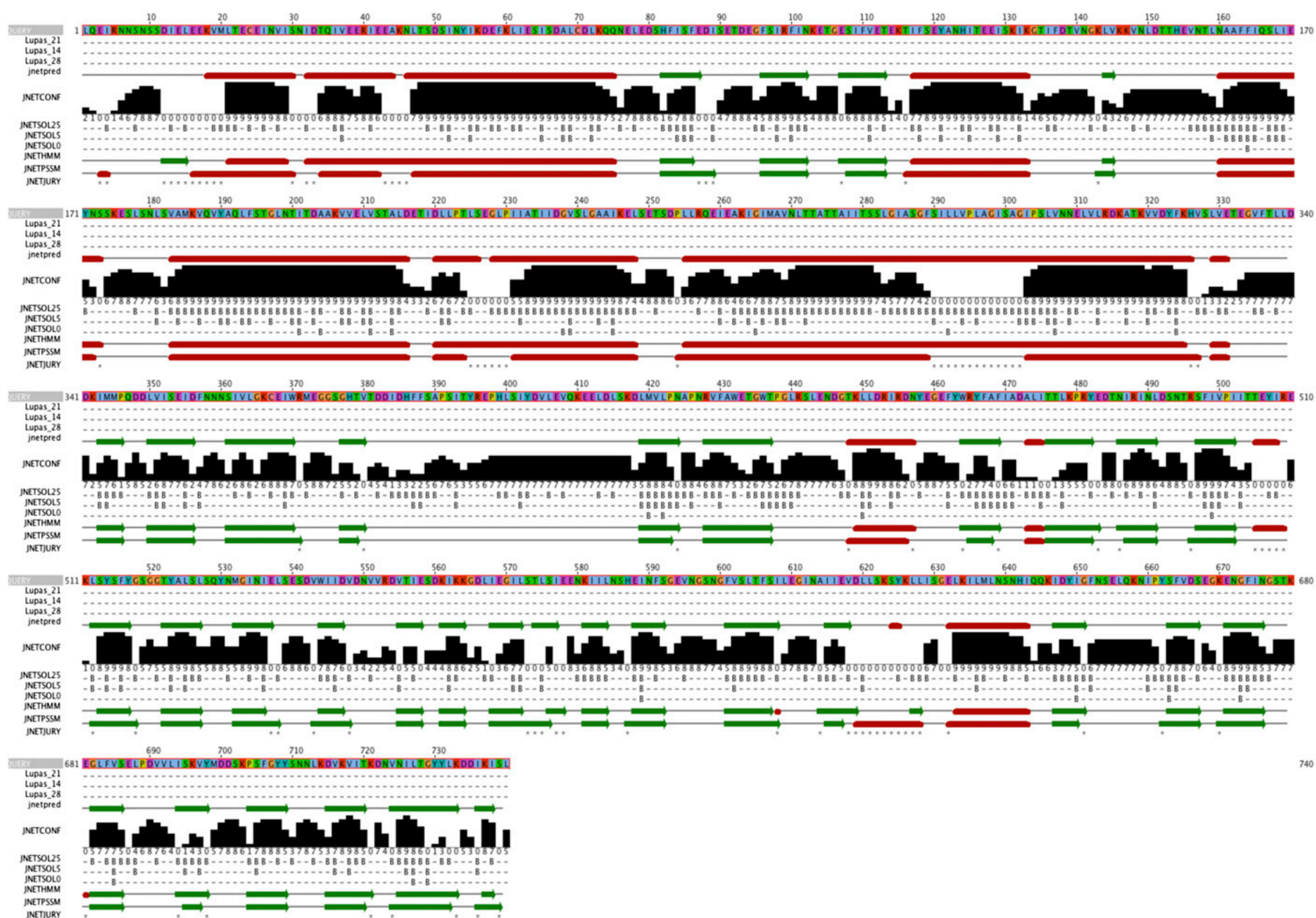

Fig. S2. Secondary structure prediction for the translocation domain of TcdB using JPRED3. Numbering is transposed by 800 residues (i.e., 1 is actually residue 801). Predicted helical regions are shown as red rectangles, beta-sheets as green arrows, and nonstructured areas as lines.

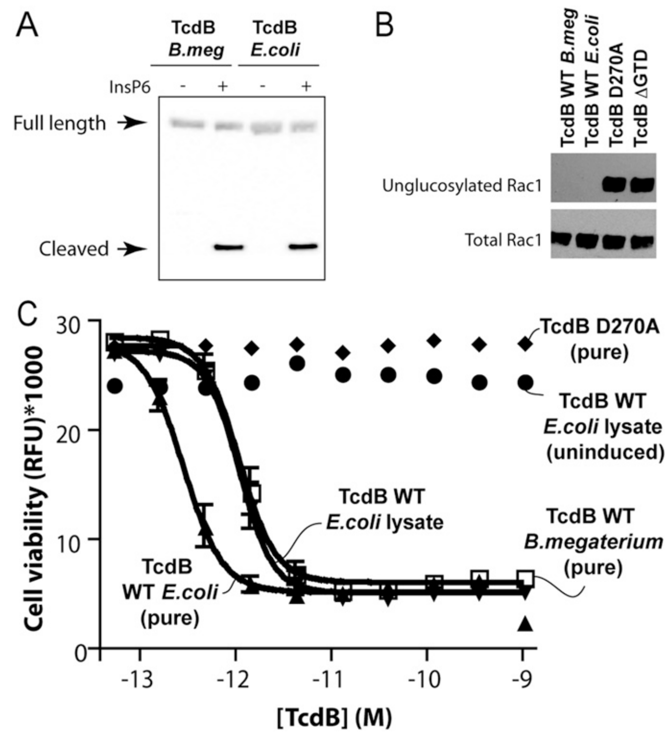

Fig. S3. Validation of GC-enriched, codon-optimized TcdB protein from *E. coli* expression. (*A*) Autoprocessing activity of recombinant toxins. Recombinant TcdB variants were treated with 100 μM InsP6 (+) or PBS (–) for 3 h and cleavage was visualized by Western blot by probing with an anti-glucosyltransferase domain (GTD) antibody. (*B*) GTD activity of recombinant toxins. GST-Rac1 was treated with recombinant toxins, and the level of glucosylation was determined by Western blot analysis using Mab102 that recognizes unglucosyated Rac1 (*Upper*) and an anti-Rac1 antibody to determine total Rac1 (*Lower*). (*C*) Functional activity of recombinant toxins. Recombinant TcdB constructs were added to CHO cells over a range of concentrations. Cellular viability was quantitated 48 h later by measuring the fluorescence of cells treated with the cell viability reagent (PrestoBlue).

Fig. S4. Defective TcdB mutants are folded and display a similar pH-induced unfolding by TNS fluorescence. (*A*) Defective mutants were all folded at neutral pH and began unfolding at pH <5. (*B*) The two most defective mutants (G1098K and L1106K) show a similar unfolding profile to WT TcdB. (*C*) Specific activity of the GTD enzyme for WT and each construct shown using the scintillation proximity assay. Specific activity was measured by calculating the rate of formation of product in the first 10 min of the reaction as outlined in *SI Materials and Methods*.

Fig. S5. Alignment of the hydrophobic region of different forms/strains of TcdB. We aligned five TcdB strains (accession code): R20291 (YP_003217086), CD196 (YP_003213639), 8864 (CAC19891), 630 (YP_001087135), and 1470 (CAA80815). The eight residues that resulted in a greater than 90% decrease in activity upon mutation are indicated below the residues with arrowheads.