Supplementary Methods

V_HH expression and purification

Selected V_HH coding sequences were cloned into the pET32b expression vector (Novagen) for cytosolic expression of V_HHs fused to thioredoxin in *E. coli* Rosetta-gami 2 (DE3)pLacI cells (Novagen). V_HH monomers contain a carboxyl terminal epitope tag (E-tag) [1] for detection and a hexahistidine tag (His-tag) for purification. The *E. coli* culture pellet (from 100 ml of culture) was resuspended in 5 ml of lysis buffer and cells were disrupted by One-Shot cell disrupter (Constant Systems, Kennesaw GA). The supernatant was passed through a 0.2 μm sterile syringe filter (VWR) before passing through a nickel-charged Hi Trap chelating HP column (GE Heathcare). Purification of recombinant His-tagged V_HHs from bacterial lysate was performed by Ni-affinity chromatography as described previously [2].

Surface plasmon resonance (SPR) analysis

SPR analysis of affinity of V_HHs: TcdA or TcdB was coupled to the surface of a BIAcore CM5 sensor chips by direct immobilization in HBS-P buffer from Biacore (Biacore Inc., New Jersey), containing 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant p20, pH 7.4. The carboxymethyl-dextran surface of chip (flow cell 2 and 3) was activated with a 35 μl injection of a mixture of 0.1 M NHS and 0.1 M EDC in water. An aliquot of 100 μl of 10 μg/ml TcdA and TcdB proteins in 10 mM sodium acetate, pH 5.0, were injected into flow cells 2 and 3 of one CM5 chip, to levels of 600 resonance units (RU). The remaining NHS-ester active sites in the dextran surface flow cell-2 and 3 were blocked with 35 μl 1M ethanolamine, pH 8.2, and washed at 100 μl/min with one pulse of 50 μl 2 M NaCl/20 mM NaOH and one pulse of 100 μl

of HBS-P buffer, pH 7.4. Flow cell-1 was used as reference, and was activated with 0.1 M NHS and 0.1 M EDC in water and blocked with 35 µl of 1 M ethanolamine, pH 8.2), without protein coupling.

The affinity of V_HHs to each toxin was assessed via kinetics analysis of binding. In order to minimize mass transport effects, the binding analyses were performed at flow rate of 30 µl/ min at 25° C. The analytes (60 µl each of analytes, 0-500 nM, in HBS-EP buffer) were injected and the association was recorded by SPR with a Biacore 3000 (Biacore, Inc., New Jersey). The surface was then washed with buffer for 180 seconds and the dissociation phase was recorded similarly. The signal from the blank channel (flow cell-1) was subtracted from the channel containing TcdA and TcdB proteins. Before repeating the studies, all remaining proteins bound non-covalently to the chip surface were removed with 20 µl 2 M NaCl/20 mM NaOH followed by 100 µl HBS-P, pH 7.4.

SPR analysis of affinity of ABA interaction with toxins: ABA was amine coupled to the carboxy-methyl surface of Biacore CM5 chip (GE Healthcare) on a Biacore T100. *Streptococcus pyogenes* Endoglycosidase S (EndoS) was coupled in another flow cell as a control surface. First Toxin A was titrated over the surface using a kinetic titration with concentrations in twofold dilutions ranging from 12.5 nM to 200 nM. The surface was then regenerated with a solution of 2M NaCl, 20 mM NaOH (50 ml flowed at 100 µl/min). TcdB was then titrated over the surface in a kinetic titration with concentrations in twofold dilutions from 3.25 nM to 60 nM. An additional injection of 120 nM TcdB was then used to ensure that the surface was completely saturated with Toxin B. Immediately, TcdA was titrated across the surface again, in a manner identical to the first kinetic titration. All titrations were performed in HBS-EP+ buffer (10 mM HEPES, 3 mM EDTA, 0.05% P20, pH 7.4) at 25 C and 100 µl/min.

Data analyses: Sensorgrams of the interaction generated by the instrument were analyzed using the software BIAeval 3.2 (Biacore Inc., New Jersey). The reference surface data were subtracted from the reaction surface data to correct for changes in the refractive index of the solution, injection noise and non-specific binding to the blank surface. A blank injection with buffer alone was subtracted from the resulting data. Data were globally fitted to the Lagmuir model for a 1:1 binding.

Figure legends

sFigure 1. Purification of anti-TcdA and anti-TcdB V_HHs. A) SDS-PAGE gel images illustrate ~ 30 kDa of single bands of the purified anti-Tcd thioredoxin-V_HH fusion proteins in pET-32 expression system; B) Culture supernatants (Pre) from stable-transfected 293 cells were collected and passed through (FT) a HisTag affinity column. After washes, ABA was eluted by 500 mM of imidazole (E1-E5). SDS-PAGE gel images illustrate a ~66 kDa band of purified ABA.

sFigure 2: SPR binding analysis of ABA toxin interactions. Flow cells of an SPR chip were coupled with ABA and EndoS, as a reference surface. A) Toxin A was titrated over the two surfaces with the referenced sensorgram shown. B) After regenerating the ABA surface, Toxin B was titrated across the surface in an identical manner. C) Instead of regenerating the surface, additional Toxin B was bound to ensure that the surface was completely saturated with Toxin B. Toxin A was then titrated across the surface again. D) Schematic showing each step of the experiment i) ABA coupled to surface, ii) 1st Toxin A titration, iii) surface regenerated, iv) Toxin B titration, v) surface saturated with Toxin B, vi) 2nd Toxin A titration.

sTable 1: SRP analysis of affinities of V_HHs.

	VHHs	K_{on} (Ms^{-1})	K_{off} (s^{-1})	K_D (nM)
TcdA	AH3	2.20×10^{4}	7.10×10^{-4}	32.0
	AA6	3.52×10^{4}	6.92×10^{-4}	19.7
	A3H	1.51×10^{5}	4.77×10^{-3}	31.5
	AC1	1.07×10^{4}	7.54×10^{-4}	70.6
TcdB	5D	1.52×10^{6}	9.94×10^{-4}	0.65
	E3	2.95×10^{6}	9.4×10^{-5}	0.03
	7F	3.96×10^{4}	3.48×10^{-4}	8.80
	B12	1.5×10^{6}	1×10^{-4}	0.07
	C6	1.11×10^{6}	9.84×10^{-4}	0.89
	5E	1.16×10^{6}	3.8×10^{-5}	0.03

1. Mukherjee J, Tremblay JM, Leysath CE, et al. A novel strategy for development of recombinant antitoxin therapeutics tested in a mouse botulism model. PLoS One **2012**; 7:e29941.
2. Yang G, Zhou B, Wang J, et al. Expression of recombinant Clostridium difficile toxin A and B in Bacillus megaterium. BMC Microbiol **2008**; 8:192.

sTable 1

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TcdA	AH3	2.20×10^4	7.10×10^{-4}	32.0
	AA6	3.52×10^4	6.92×10^{-4}	19.7
	A3H	1.51×10^5	4.77×10^{-3}	31.5
	AC1	1.07×10^4	7.54×10^{-4}	70.6
	AB8	1.99×10^4	1.53×10^{-3}	76.6
TcdB	5D	1.52×10^6	9.94×10^{-4}	0.65
	E3	2.95×10^6	9.4×10^{-5}	0.03
	7F	3.96×10^4	3.48×10^{-4}	8.80
	B12	1.5×10^6	1×10^{-4}	0.07
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A

Anti-TcdA Anti-TcdB

M AA6 A11G A3H B12 B7 E3 5E 5D

kDa

50

37

25

B

ABA purification

M Pre FT E1 E2 E3 E4 E5

250kDa

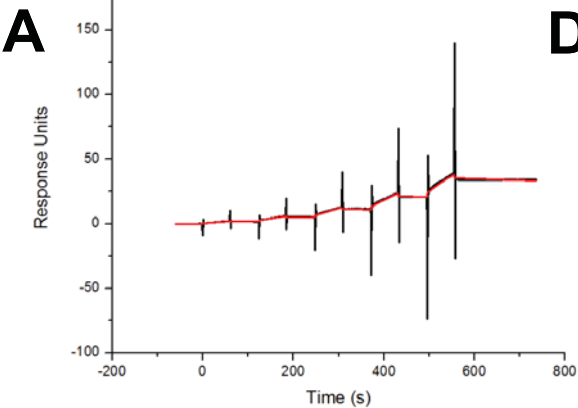
150kDa

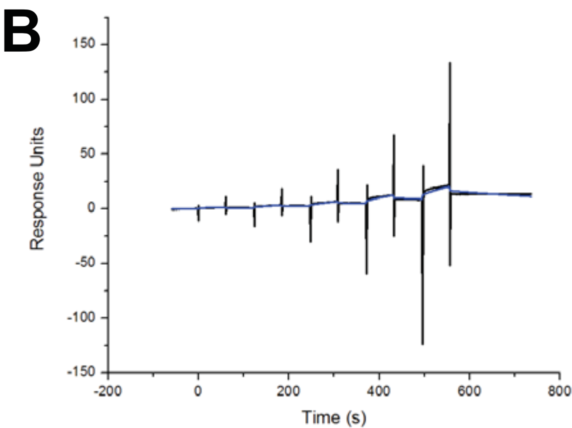
75kDa

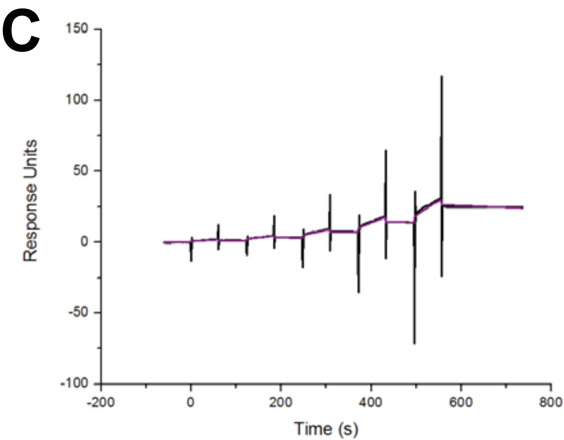
50kDa

25kDa

ABA







D

ABA

Toxin A

Toxin B

i)

ii)

iii)

iv)

v)

vi)