Supplementary Information

Title: Heterologous expression of *Salmonella typhi* - Cytolethal distending toxin subunit B in *E. coli* and comparative viable bioactivity characterization.

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Supplementary Methods

SM1: *Bacterial strains, Media and growth conditions*

Restriction enzymes, *BamH*I & *Xho*I (New England Biolabs, USA). Luria-Bertani (LB) and 2X TY media (Difco, USA) were used for growing bacterial strains overnight at 37°C. Ampicillin (100 µg mL^{-1}) and/or chloramphenicol (50 µg mL^{-1}) (Merck, Darmstadt, Germany) were supplemented where appropriate. Protein concentration estimation was performed using Pierce™ BCA Protein Assay Kit (Thermo Scientific; USA). Ni-NTA resin was purchased from Qiagen and GE, Isopropyl-β-D-thiogalactopyranoside (IPTG) from Amresco. All the chemicals for reagents preparation were of analytical grade and obtained either from Amresco, Merck-Sigma or MP Laboratories.

SM2: *CdtB and ΔCdtB amplification and expression Plasmid construction*

PCR amplification of truncated CdtB (ΔCdtB) from the *S. typhi* genomic DNA was performed using Phusion-HF Taq DNA polymerase (NEB) and specific *primers* with *Bam*HI or *Xho*I restriction sites [*S. typhi* CdtB gene (Accession No.AL513382.1.) ΔCdtB-For 5'-GC*GGATCC*ATGACCTGGAATCTTCAGG-3' and ΔCdtB-Rev 5'-GC*CTCGAG*TTAGCTTCGTGCCAAAAAGGC-3'], PCR conditions were: 98°C for 2 m, followed by 34 cycles of 98°C for 10 s; 55°C for 10 s; 72°C for 30 s; followed by a final extension at 72°C for 10 m. The amplicon ΔCdtB ~736 bp was gel-eluted and in (*Bam*HI/*Xho*I) double digested, pPROEX^{TM}-HTB and pET32a(+) vectors, further, transformed into chemically competent DH5α or BL21 (DE3) or Rosetta gami^{TM} (DE3) *E.coli* cells by heat-shock method.

SM3: *Expression, Purification and Refolding of recombinant CdtB and ΔCdtB in E. coli*

For the Protein expression, the overnight culture was inoculated in 1 L of fresh 2X TY media (1:100) supplemented with 100 µg mL^{-1} of ampicillin, 50 µg mL^{-1} chloramphenicol, and allowed to grow until the optical density at 600 nm reached ~0.4. The culture was then induced with 0.5 mM IPTG and allowed to grow for overnight at 37°C. Cells were harvested by centrifugation at 6000 × g for 10 m, lysed in appropriate buffer and checked for expression along with the fraction solubility while heterologous expression of protein forms (CdtB and ΔCdtB) in soluble and insoluble fractions on 15% Tri-Glycine, SDS-PAGE followed by Coomassie brilliant blue (CBB) staining. Most of the target protein was present in the pellet as inclusion bodies (IBs). Isolation of pure IBs containing different forms of CdtB proteins were performed by sonication and several washing steps [1]. The homogenous suspensions of IBs (CdtB/ΔCdtB) were disaggregated overnight in 9 mL of solubilization buffer (50 mM Tris-HCl, pH 8.0, 6M Gu-HCl, 500 mM NaCl, 2 mM DTT, 0.1% CHAPS) at RT followed by centrifugation at 15,000 × g for 20 m at 10°C. The supernatant obtained post-centrifugation was used for purification recombinant CdtB/ΔCdtB protein under denaturing conditions. Purification of CdtB/ΔCdtB protein was performed by immobilized metal ion affinity chromatography (IMAC)

using HisTrap FF column (GE Healthcare Buckinghamshire, UK), in denaturing condition. Protein was eluted using buffer [50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2 mM DTT, 0.1% CHAPS, 8 M Urea, 100-500 mM Imidazole]. Fractions were collected and analyzed on 15% Tris-glycine, SDS-PAGE followed by CBB staining. Denatured purified CdtB/ΔCdtB protein was refolded as described by Sharma et al., [1]. Briefly, the three buffers, B1 (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM DTT, 0.1% (w/v) CHAPS), B2 (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10% (v/v) Glycerol, 0.4 M L-arginine), B3 (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10% (v/v) Glycerol, 50 mM L-arginine, 50 mM Glutamic acid) were used in dialysis to sequentially remove Urea (i.e. 8, 6,…, 0.5, 0 M). The refolded target protein was centrifuged at 24,000 × g for 30 m at 4°C and the supernatant containing refolded active protein was concentrated using 10 kDa cut-off centrifugal filters (Vivaspin, GE). Protein was quantitated by bicinchoninic acid assay (Thermo Scientific Pierce, Rockford, IL, USA) and analyzed with SDS-PAGE, and confirmed by Western blotting, using anti-His monoclonal antibody (Cell Signaling Technology, Inc., MA, USA).

SM4: The hydropathy plots were generated for CdtB and ΔCdtB using Expasy webserver (http://web.expasy.org/protscale/) (Supplementary), the removal of an N-terminus hydrophobic region (AA 1-26) from the CdtB sequence, exhibiting a very high hydrophobicity index, reduced the Grand average of hydrophobicity (GRAVY) of CdtB from G=0.061 (CdtB) to G= -0.050 (ΔCdtB)

SM5: ***Cell Cytotoxicity Assay***

HeLa Cells were seeded at the density of 5×10^4 cells per well in 96-well plates using 200 µL Dulbecco's modified Eagle's medium (Gibco™ DMEM) containing 10% (v/v) fetal bovine serum and incubated in 5% CO2 atmosphere at 37°C for 24 h. At 70% confluency CdtB/Δ CdtB proteins (1.5 µM & 3 µM) were supplemented in culture medium. Cell viability after 24 h was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega) using manufacturer's protocol. The percentage of surviving cells was calculated by comparison with non-treated cells.

SM6: ***Binding of recombinant CdtB proteins to HeLa cells***

Poly-D-Lysine (0.01% solution) treated coverslips were seeded with HeLa cells were at a density of 2×10^4 cells per well in DMEM supplemented with 10% FBS and 10 µg mL^{-1} Gentamycin, and allowed to grow for 24 h at 37°C in a humidified CO_2 incubator. The cells were washed thrice with PBS (1X) and then incubated for 4 h in serum free DMEM. The protein was diluted in serum free DMEM, and 200 µL of it was added to each well, and allowed to incubate for 24 h. The coverslips were washed thrice with PBS (1X). The treated cells were fixed with 4% PFA for 20 m at RT (400 µL each), and Permeabilized using 0.1% Triton-X100 (200 µL each). The cells were washed again and blocked with 1% BSA (in PBS) for 1 h, and again washed thrice with 1x PBS., followed by incubation with anti-His tag antibody (1:1000) for 1 h at RT. The cells were washed thrice with PBS (1X) and Incubated with anti-mouse-Alexa488 conjugated antibody (1:1000) for 1 h at RT. The coverslip was washed thrice with PBS (1X) and then mounted onto a slide using Fluoroshield® mounting medium containing DAPI. The slides were examined under a fluorescent microscope and images recorded with a digital camera.

Supplementary Figures:

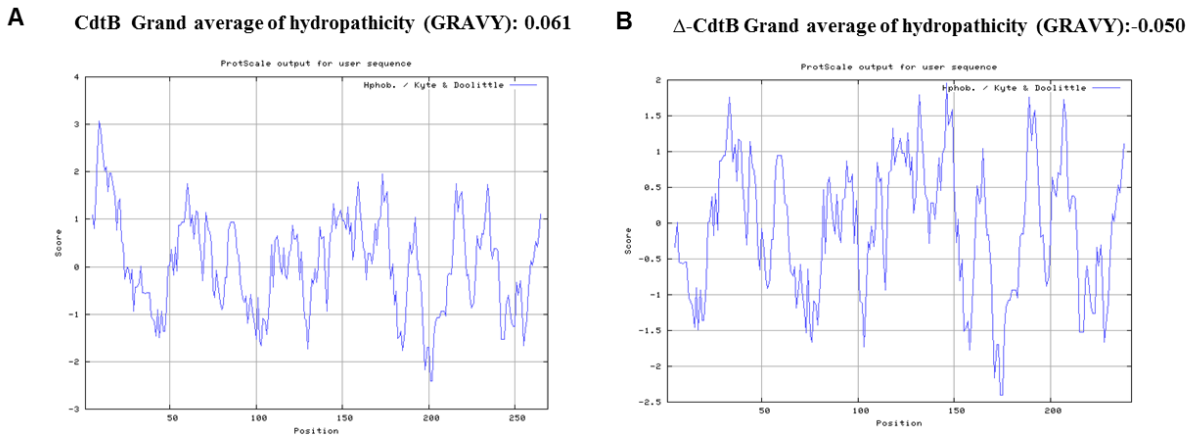

Figure S1. Grand average of hydropathy (GRAVY) indices and Kyte-Doolittle hydropathy plots; for CdtB (A), as well as for ΔCdtB (B). Due to the removal of a hydrophobic region (1-29 AA) from the CdtB sequence, the Grand average of hydrophobicity of CdtB was reduced from 0.061 (CdtB) to 0.050 (ΔCdtB) http://web.expasy.org/protscale/

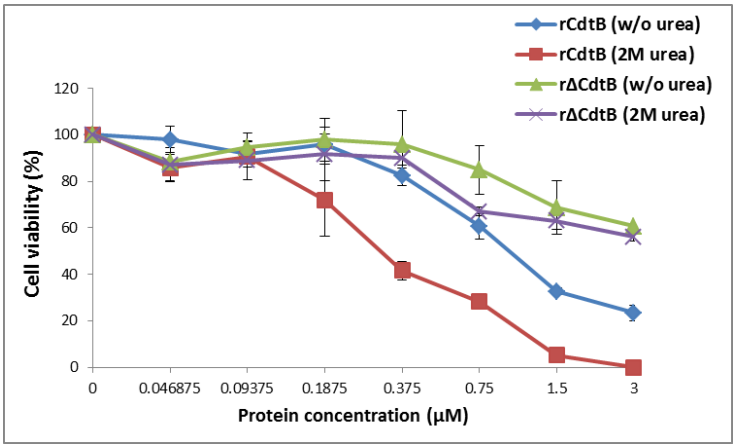

Figure S2. Viability of the HeLa cells treated with purified CdtB and ΔCdtB of *S typhi* (both refolded and denatured forms).Viability of HeLa cell line was determined by the XTT assay. Each measure was made in triplicate. Error bars represent standard deviation.

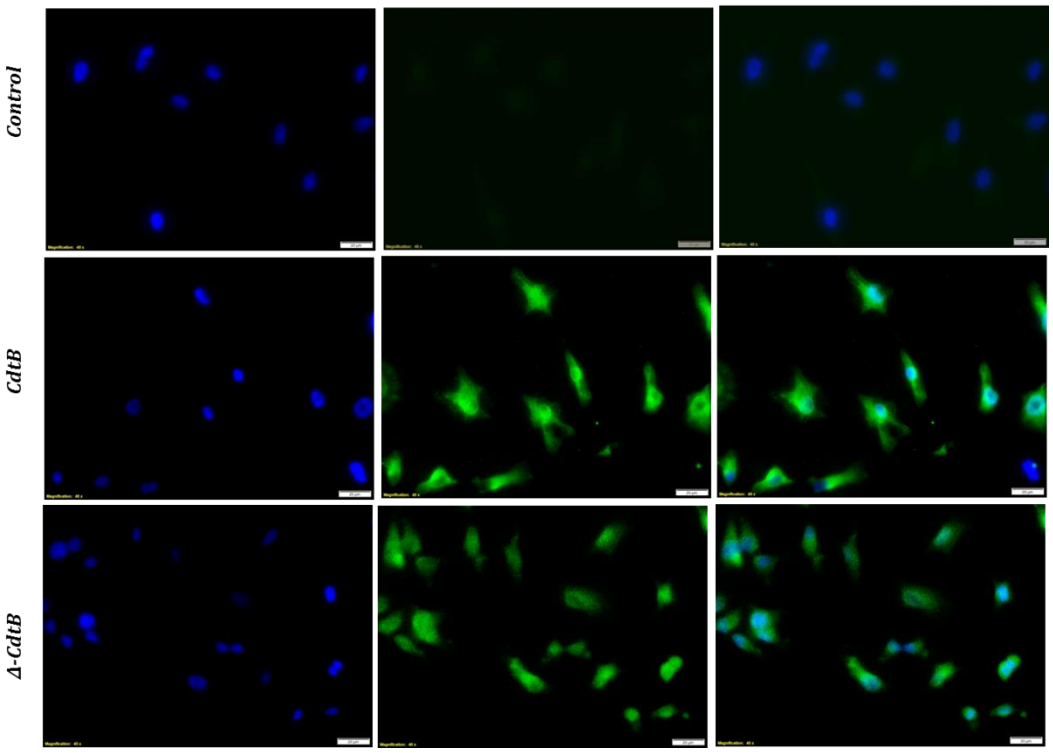

Figure S3. Binding of recombinant His-tagged CdtB proteins to HeLa Cells. When Compared to untreated cells (A), cells treated with purified His-tagged CdtB (B) or ΔCdtB (C) of *S typhi* showed cell surface binding and internalization when detected with anti-His-Tag monoclonal antibody (1:1000 dilution) and fluorescein conjugated secondary antibody (1:1000 dilution). The slides were examined under a fluorescent microscope and images recorded with a digital camera.

References

1. Sharma T, Sharma C, Sankhyan A, Bedi SP, Bhatnagar S, Khanna N et al. Serodiagnostic evaluation of recombinant CdtB of S. Typhi as a potential candidate for acute typhoid. Immunologic research. 2018;66(4):503-12. doi:10.1007/s12026-018-9009-4.