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The C-terminal domain of *Clostridioides difficile* TcdC is exposed on the bacterial cell surface

Introduction

Clostridioides difficile is an anaerobic gram-positive bacterium that can produce the large clostridial toxins, Toxin A and Toxin B, encoded within the pathogenicity locus (PaLoc). The PaLoc also encodes the sigma factor TcdR, that positively regulates toxin gene expression, and TcdC, a putative negative regulator of toxin expression. TcdC is proposed to be an anti-sigma factor, however, several studies failed to show an association between tcdC genotype and toxin production. Consequently, TcdC function is not yet fully understood. Previous studies have characterized TcdC as a membrane-associated protein with the ability to bind G-quadruplex structures. The binding to the DNA secondary structures is mediated through the OB-fold domain present at the C-terminus of the protein. This domain was previously also proposed to be responsible for the inhibitory effect on toxin gene expression, implicating a cytoplasmic localization of the OB-fold.
In this study we aimed to obtain topological information on the C-terminus of TcdC , through two independent assays and show that the C-terminus of TcdC is exposed on the bacterial cell surface.

TcdC C-terminal domain is predicted to be extracellular

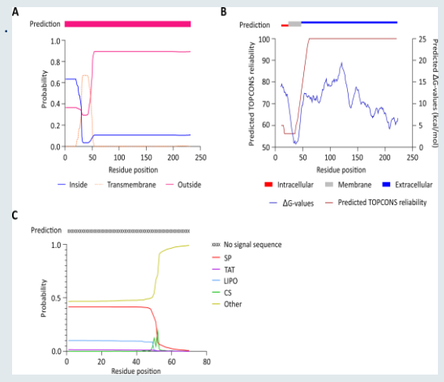
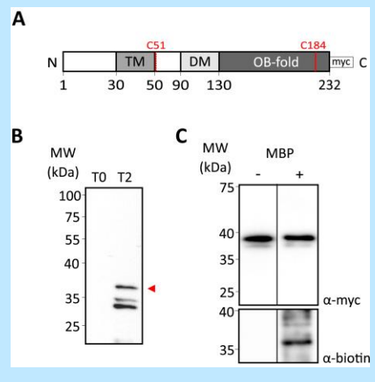


Figure 1 - Prediction of a transmembrane helix in TcdC. A) Output from the prediction by TMHMM 2.0 software (24) through 1-best algorithm (pink bar) and probability plot: inside the cell (blue line), transmembrane region (orange dotted line) and outside the cell (pink line). B) Prediction by TOPCONS software (25), with consensus in residues 1-26 inside the cell (red box), a transmembrane helix (residues 26-46, grey box) and residues 47-232 on the outside of the cell (blue box). TOPCONS reliability score (brown line) and predicted ΔG-values for each residue (blue line) are shown. C) Output from the SignalP 5.0 (26) web-server for the TcdC amino acid sequence. No signal sequence was detected (X). Probabilities of signal peptides presence from the systems Sec (SP, red line), Tat (purple line), and lipoprotein (LIPO, blue line) are shown. Predicted cleavage site score (CS, green line) and no signal sequence probability is depicted (OTHER, light green line).

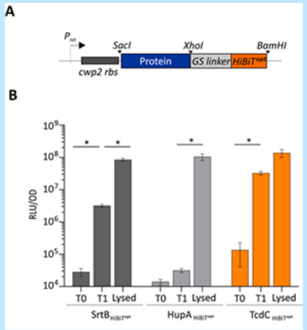
Cysteines of TcdC are exposed on the bacterial surface

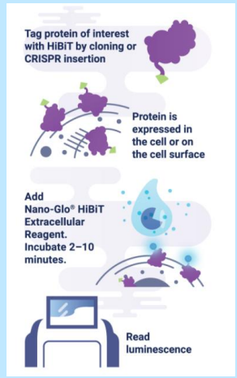
Figure 2 - Mapping the location of the TcdC C-terminus with cysteine accessibility analysis. A) Schematic representation of the 3xmyc-tagged TcdC construct used for the cysteine accessibility analysis. The different domains of TcdC are represented: transmembrane domain (TM, grey box), the dimerization domain (DM, light grey box) and the OB-fold (dark grey box). The 3xmyc-tag is represented as a white box, the cysteines residues present on TcdC are represented (zigzag red line). B) Western-blot analysis of α-TcdC antibody specificity in C. difficile 630Δerm lysates harbouring pLDJ1 (Ptet-tcdC-3xmyc), before (T0) and after induction with 200ng/ml anhydrotetracycline for 2 hours (T2). Full-length TcdC is indicated with a red arrow. C) Cysteine labelling analysis of the different TcdC-3xmyc construct. The strain harbouring the myc-tagged TcdC construct (38 kDa) was induced for 2 hours. Samples were collected and not treated with MPB (-) or treated with 1 mM MPB (+). Samples were immunoprecipitated and immunoblotted with α-myc for TcdC-3xmyc protein detection (upper panel), and α-biotin for detecting biotinylated proteins (bottom panel). Cysteine biotinylation of TcdC-3xmyc was observed.

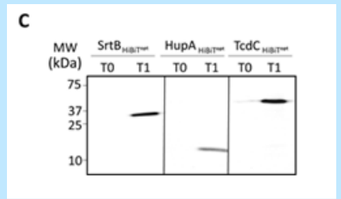


TcdC C-terminus is located extracellularly

Figure 3 – Detection of C-terminal HiBiT^{opt} tags. A) Representation of the HiBit^{opt} modular cassette. The protein of interest (blue box) fused at the C-terminus to the HiBitopt (orange box) through the GS linker (grey box) are indicated. The positions of used restriction sites are marked (ScaI, XhoI and BamHI) and the cwp2 ribosomal binding site (dark grey box) are represented. B) Proteins of interest were C-terminally fused to a HiBiT protein tag and induced with 50 ng/mL ATc for 45 minutes. Optical density-normalized luciferase activity (RLU/OD) is shown right before induction (T0), after 45 min of induction (T1) and subsequent lyses of T1 samples (lysed). HiBiT^{opt}-tagged sortase (dark grey bars) and HupA (light grey bars) proteins were used as extracellular and intracellular controls, respectively. TcdC-HiBiT^{opt} associated luciferase activity is displayed in the orange bars. The averages of biological quadruplicate measurements are shown, with error bars indicating the standard deviation from the mean. Significance was defined as higher than p<0.001(*) by two-way ANOVA. C) Blot detection of HiBiTopt- tagged proteins resolved on a 12% SDS-PAGE. Sample volumes were normalized for optical density of the cultures from which they were derived. Expression of HiBiTopt fused proteins was observed at 0 (T0) and 45 minutes after induction (T1).

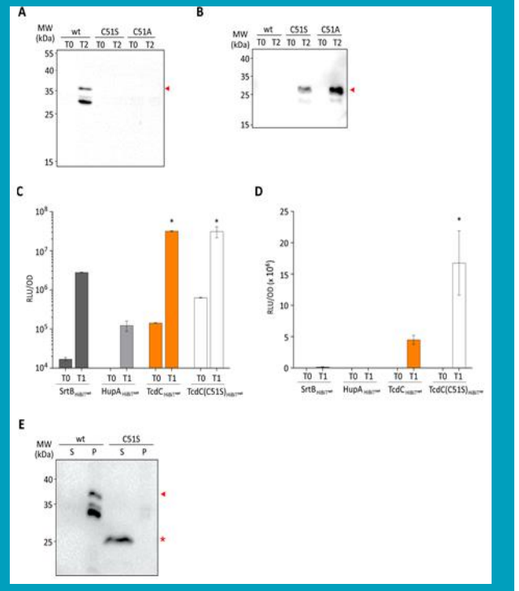






TcdC C51 is important for membrane association

Figure 4 – TcdC C51S affects membrane-bound localization. A) Western-blot analysis, with α-TcdC, of C. difficile 630Δerm lysates harbouring pLDJ1 (Ptet-tcdC-3xmyc), pLDJ2 (Ptet-tcdC(C51S)-3xmyc) and pJC129 (Ptet-tcdC(C51A)-3xmyc), before (T0) and after induction with 200ng/ml anhydrotetracycline for 2 hours (T2). TcdC is indicated with a red arrow. B) Western-blot analysis, with α-TcdC, of C. difficile 630Δerm culture supernatants harbouring pLDJ1 (Ptet-tcdC-3xmyc), pLDJ2 (Ptet-tcdC(C51S)-3xmyc) and pJC129 (Ptet-tcdC(C51A)-3xmyc), before (T0) and after induction with 200ng/ml anhydrotetracycline for 2 hours (T2). Secreted/released TcdC is indicated with a red arrow. C) Proteins of interest were C-terminally fused to a HiBiT protein tag and induced with 50 ng/mL ATc for 45 minutes. Optical density-normalized luciferase activity (RLU/OD) of the culture (cells plus medium) is shown right before induction (T0), after 45 min of induction (T1). HiBiTopt-tagged sortase (dark grey bars) and HupA (light grey bars) proteins were used as extracellular and intracellular controls, respectively. TcdC-HiBiTopt associated luciferase activity is displayed in the orange bars and TcdC-HiBiT(C51S)opt in the white bars. The averages of biological triplicate measurements are shown, with error bars indicating the standard deviation from the mean. Significance was defined as higher than p<0.001(*) by two-way ANOVA. D) Observed luciferase activity (RLU) in supernatants only, from the cells in C). E) Comparison of cell-associated and cell-released TcdC. Same samples as in A) and B) were run next to each other for a fair comparison of the size of the proteins. Cell-associated TcdC is indicated with a red arrow, cell-released TcdC is indicated with a red star.



Conclusions

- TcdC C-terminus is exposed on the bacterial surface
- Membrane localization of TcdC is dependent on C51
- Prevailing model of TcdC as a cytoplasmic anti-sigma factor is challenged
- HiBiT^{opt} can be used to determine the topology of C-terminal domains of membrane proteins in cells grown under anaerobic conditions, without complex sample processing

Possible topologies of TcdC

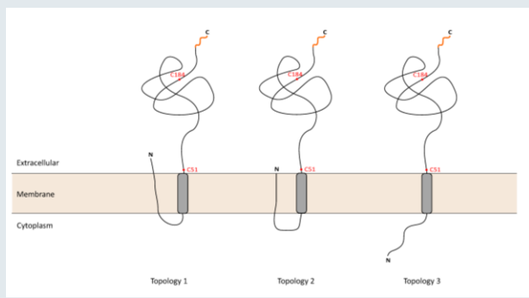


Figure 5 – TcdC topology models. TcdC is located in the cell membrane with an extracellular C-terminus region. The 50-aminoacid N-terminal of TcdC cellular location is unknown. In the topology model 1 N-terminus can cross the cell membrane exposing the N-terminus to extracellular environment. Another possibility, topology model 2, is the N-terminus present in the cell membrane and not accessible extracellularly or intracellular. Finally, in the topology model 3 the N-terminus is present on the intracellular environment of the cell. Cysteines residues used for the cysteine accessibility analysis (red dots) and the HiBiTopt C-terminus locations (orange line) are indicated.

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