The holin like protein TcdE mediates *Clostridium difficile* toxin secretion

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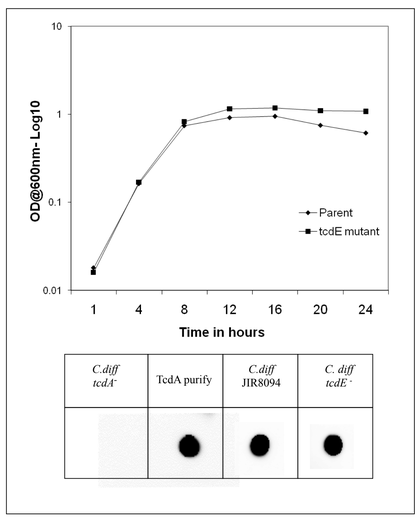
Abstract

Clostridium difficile toxins A and B have no export signature and their secretion is not explained by cell lysis, suggesting that they might be secreted by an unusual mechanism. The TcdE protein encoded within the *C. difficile* pathogenicity locus (PaLoc) has structural features similar to those of bacteriophage holin proteins. During many types of phage infection, host lysis is driven by an endolysin that crosses the cytoplasmic membrane through a pore formed by holin oligomerization. We demonstrated that TcdE has a holin-like activity by functionally complementing a λ phage deprived of its holin. Similar to λ holin, TcdE expressed in *E. coli* and *C. difficile* formed oligomers in the cytoplamic membrane. A *C. difficile tcdE* mutant strain grew at the same rate as the wild-type strain, but secreted a dramatically reduced amount of toxin proteins into the medium. There was no difference in the levels of *tcdA* and *tcdB* gene expression and several cytoplasmic proteins were equally abundant in the mutant and the wild-type strains. In addition, TcdE did not affect the membrane integrity of *C. difficile*. Thus, TcdE acts as a holin-like protein to facilitate the release of *C. difficile* toxins to the extracellular environment but, unlike the phage holins, does not cause the non-specific release of cytosolic contents. TcdE appears to be the first example of a bacterial protein that releases toxins into the environment by a phage-like system.

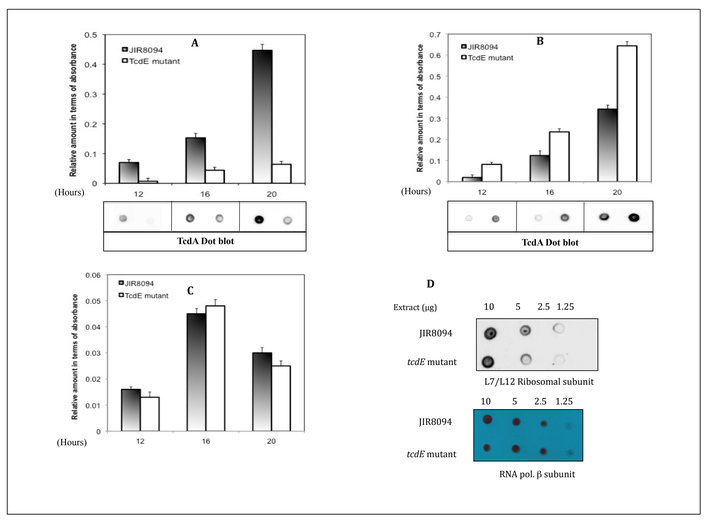
Material and Methods *C. difficile strains* JIR8094 and its *tcdE mutant* were grown under anaerobic conditions in TY broth. *tcdE m*utant was generated by the Clostron technology. To test TcdE holin activity, we used as host lysogens of *E. coli* strain MC1061 for a defective λ prophage bearing a nonsense mutation in its holin gene (Sam7) or carrying a deletion in holin and endolysin genes [λCmrΔ(SR)] to performed complementation assays. Cytosolic proteins were prepared by sonication, spotted on nitrocellulose filters and probed with monoclonal antibodies against TcdA (pGC4), RNA pol β subunit or ribosomal subunits. Toxins were measured using Enzyme linked immunoassay (Meridian diags., Inc) and LDH activity using CytoTox 96™ kit (Promega). Cell membrane integrity was assay by flow cytometry analysis (FACS calibur, Becton Dickinson) and analyzed with the CellQuest software. Localization of TcdE in *C. difficile* and *E. coli* was performed from membrane and cytosolic preparation using antibodies against 6xHis-Tag (BioDesign Inter.), ATP β subunit and ribosomal proteins.

Results

TcdE is required for efficient secretion of TCDA/B

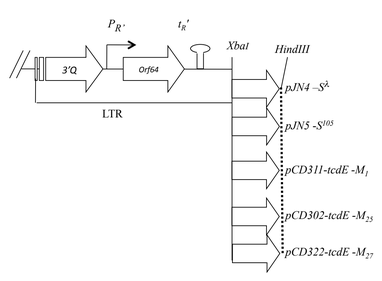


Growth curve and TcdA expression of the strain JIR8095 and its derivative *tcdE* mutant. **Both strains grew similarly** in TY but a slightly higher OD600 was seen for the mutant at 16 hrs. **The quantity of TcdA detected in total crude extract of both strains is the same** and was confirmed by qRT-PCR for the levels of tcdA, *tcdB, tcdR* and *tcdC* transcripts.

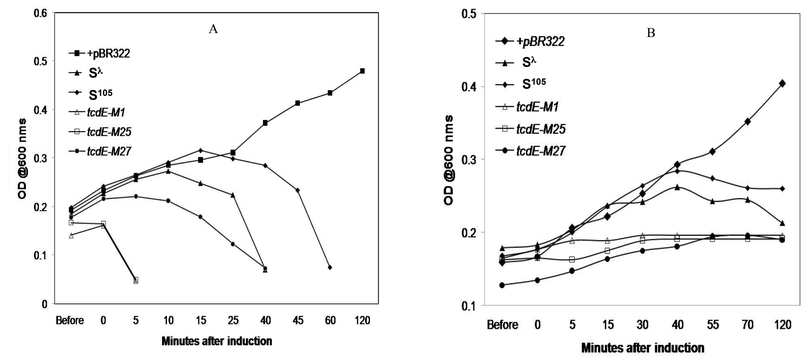


Quantification of toxins and lactate dehydrogenase (LDH) acitivity in JIR8095 and *tcdE* mutant strains. Toxin titers was determined in the culture supernatant (A) and in cytoplasmic proteins (B). The cytoplasmic proteins was determined for LDH activity (C) and through dot blots using monoclonal antibodies against ribosomal subunits and RNA poly. β subunits (D). **We observed a dramatic reduction in the amount of toxin secreted by the mutant when compared to the wild type strain. However there is no significant difference between the parent strain and the mutant in their levels of cytosolic LDH activity as well as the other cytosolic maker proteins (RNA poly and Ribosomal subunits).**

TcdE functions as a phage holin

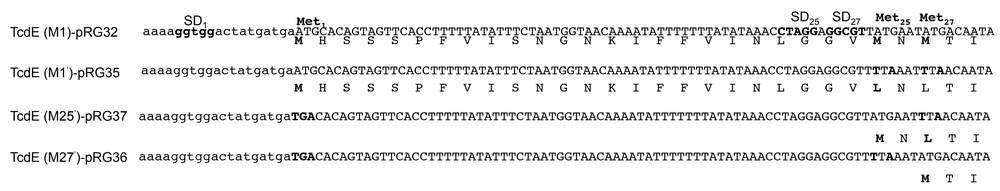


Relevant features of the pBR322 derivative plasmids used for testing holin function of *tcdE*: holin expression is under the control of the late transcription regulatory (LTR) elements of phage λ.



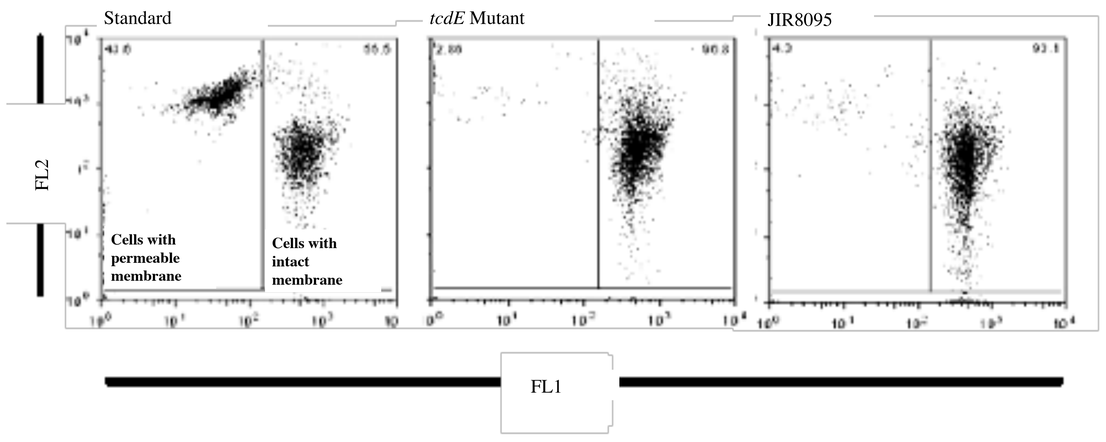
Testing holin function of TcdE in *E. coli*: Holin-like activity of TcdE was tested by following a λ lysogen lysis curves after thermoinduction of lysogenic cultures of λcI_{857}*Sam7* **(A)** or λCmrΔ(SR) **(B)** carrying pJN4, pJN5, pCD311, pCD302, and pCD322, expressing in trans *S', S^{105}*,TcdE-M_1, TcdE-M_{25}, and TcdE-M_{27}, respectively. **When expressed in *E. coli* MC1063 λ (cI_{857}Sam7), the TcdE-M1 and TcdE-M25 forms induced bacterial lysis within five minutes after thermo-induction whereas for the TcdE-M27 form induced lysis at about 40 min (A). No lysis was observed after heat induction of expression plasmid in λCmrΔ(SR) in absence of the λ endolysin (B)**

Alternative translational (dual start) motifs in the t*cdE*

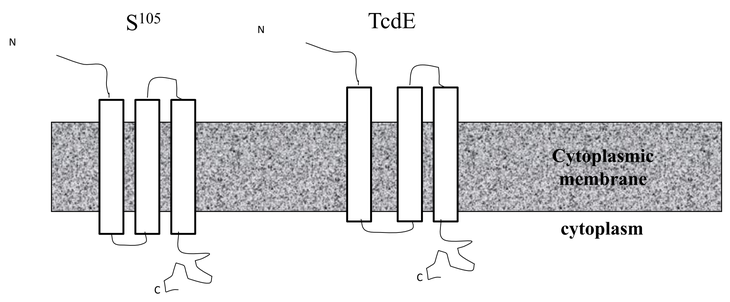


TcdE sequence: all the possible translational starts are indicated as M_1, M_{25} and M_{27} and the potential Shine-Delgarno sequences are underlined. Construct of pRG35 was created by converting methionines M25 and M27 to leucine residues designated as TcdE-M1'. Constructs pRG37 and pRG36 expressing TcdE-M25' and TcdE-M27', respectively, were created by converting methionine M1 into a stop codon and then by changing methionines M27 to L27 in pRG37 and M25 to L25 in pRG36.

... without affect membrane integrity

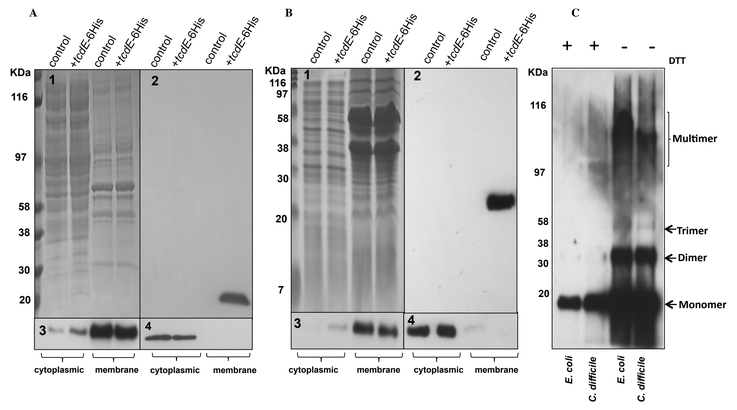


FACs analysis of *C. difficile* cells for membrane permeability through propidium iodide (PI) and SYTO staining. The standard sample containing the mixture of heat killed and actively growing *C. difficile* cells at 1:1 ratio. The wild type and the *tcdE* mutant strains were collected from the overnight (16h) cultures, and subjected to FACs analysis following PI, SYTO staining. **No significant difference could be observed in the intact vs. membrane-permeable cell populations of the mutant and parental strains indicating that in both cases the membrane-permeable fraction was minimal**

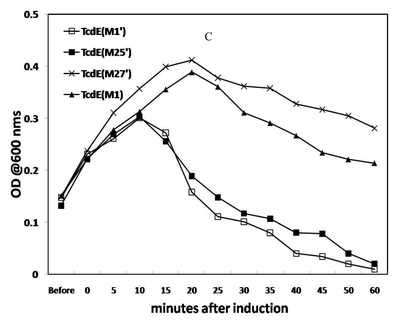


Predicted topology of TcdE and Lambda S holin. TcdE is a hydrophobic protein of 116 amino acids with a short strech at the N-terminus and a serie of charge residues at the C-terminus. TcdE is predicted to contain three transmembrane domains. All these structural features and primary sequence similiraties strongly suggested that **TcdE could be a member of the class I holins of which phage λ S protein is a member.**

TcdE is a membrane protein which forms oligomers



Localization of TcdE in *E. coli* and *C. difficile. E. coli* lysogen cultures of λCmrΔ(SR) carrying pBR322 (control) or pCD463 (+*tcdE*-6His) were thermo induced **A** and. *C. difficile* strains carrying either pMTL84151 (control) or pRG46 (+*tcdE*-6His) were grown overnight **B.** Cytoplasmic and inner membrane proteins were analyzed by WB probed with His Tag (2), ATPase B subunit (3), and Ribosomal subunits LI/L2 (4) monoclonal antibodies. The same membrane protein samples were treated **C** with and without 100 mM DTT and were analyzed using His Tag antibody . **TcdE is only detected in the cell membrane fraction, indicating that TcdE is exclusively membrane-bound. Moreover TcdE forms oligomers both in *E. coli* and in *C. difficile* which is consistent with the status of membrane-associated holins, such as λ holin S^{105} when they are forming holes.**



Differentiation of lytic activity of TcdE-M1 from that of TcdE-M25 and TcdE-M27. Each versions of TcdE encode only a single form of the protein. When expressed in *E. coli* MC1063 λ (cI_{857}Sam7), The M1'- and M25'- expressing clones induced much more rapid lysis than did M27'-expressing clone. **M27 may be both relatively inactive and a potential antiholin analogous to λ S^{107} when multiple forms of TcdE are produced simultaneously.**

Conclusion

We provide here the first genetic evidence that TcdE is required for efficient secretion of toxins. We also showed that TcdE has properties similar to those of λ S protein, a holin needed for phage release. Surprisingly, TcdE facilitated toxin secretion without inducing cell lysis or general membrane permeability. Several models for the TcdE-dependent secretion of TcdA/TcdB may be proposed. If toxins are secreted in an unfolded state, only a narrow channel in the cytoplasmic membrane would be needed. Such a channel would not allow cytoplasmic protein leakage. if toxins are secreted as fully folded proteins, they could, for instance, act as plugs in the TcdE pore to prevent loss of solutes or proteins from the cells. Finally, we cannot exclude the possibility that TcdE forms a specific, gated channel that only opens in the presence of TcdA/TcdB, without inducing cell lysis. **The detailed, mechanistic understanding of the holin-dependent systems that mediate toxin secretion by *C. difficile*. will be an important advance in understanding how important proteins are released outside the cell in the absence of known secretion and export signals.**