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Release of TcdA and TcdB from *Clostridium difficile* cdi 630 is not affected by functional inactivation of the *tcdE* gene

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ABSTRACT

The small open reading frame *tcdE* is located between the genes *tcdA* and *tcdB* which encode toxin A (TcdA) and B (TcdB), respectively, within the pathogenicity locus of *Clostridium difficile*. Sequence and structure similarities to bacteriophage-encoded holins have led to the assumption that TcdE mediates the release of the toxins from *C. difficile* into the extracellular environment. A TcdE-deficient *C. difficile* 630 strain was generated by insertional inactivation of the *tcdE* gene. Data revealed that TcdE does not regulate or affect growth or sporogenesis. TcdE-deficiency was accompanied by a moderately increased accumulation of TcdA and TcdB prior to sporulation in this microorganism. Interestingly, this observation did not correlate with a delayed or inhibited toxin release: inactivation of TcdE neither significantly altered kinetics of release nor the absolute level of secreted TcdA and TcdB, indicating that TcdE does not account for the pathogenicity of *C. difficile* strain 630. Furthermore, mass spectrometry analysis could not reveal differences in the secretome of wild type and TcdE-deficient *C. difficile*, indicating that TcdE did not function as a secretion system for protein release. TcdE was expressed as a 19 kDa protein in *C. difficile*, whereas TcdE expressed in *Escherichia coli* appeared as a 19 and 16 kDa protein. Expression of the short 16 kDa TcdE correlated with bacterial cell death. We conclude that TcdE does not exhibit pore-forming function in *C. difficile* since in these cells only the non-lytic full length 19 kDa protein is expressed.

1. Introduction

Pathogenic *Clostridium difficile* strains possess a 19.6 kb chromosomal genetic locus, designated as the pathogenicity locus, PaLoc. It encompasses the genes (*tcdA* and *tcdB*) encoding the respective pathogenicity factors TcdA and TcdB and is a prerequisite for virulence. In addition to the *tcdA* and *tcdB* genes, the pathogenicity locus harbors another three genes. The *tcdR* gene encodes an alternative sigma factor responsible for transcription of the toxin genes [1–3]. Conversely, a negative regulatory function has been proposed for *tcdC* [4–6]. The small open reading frame (ORF) *tcdE* is located between *tcdB* and *tcdA* (122 bp downstream of *tcdB* and 727 bp upstream of *tcdA*) and encodes the putative protein TcdE with a deduced molecular weight of 19 kDa [7]. TcdE was predicted to be highly hydrophobic and to possess three transmembrane domains [7]. Although TcdE sequence was analyzed in 1990 by Dove and coworkers, the function of the hydrophobic protein is yet to be defined [8]. Structural similarities led to the suggestion that this protein has a holin-like function in *C. difficile* [7,9].

Holins are small, bacteriophage-encoded membrane proteins that control the length of an infection cycle of tailed phages. They trigger disruption of the cytoplasmic membrane by oligomerizing and forming lethal holes within the membranes. Holins are a diverse grouping, but on the basis of primary structure and membrane topology two classes are distinguishable. Class I holins, exemplified by lambda holin (S^{λ}), are typically 90 or more amino acid residues in size and possess two, or more usually three, transmembrane domains (TMD), Class II holins, exemplified by the S-protein of lambdoid phage 21 (S^{21}), are usually 75 amino acid residues or less in length and possess two putative TMDs [10–12]. A dual start motif was described for S^{λ} where both Met codons are utilized for translation initiation, resulting in proteins of different length and function, designated S107 and S105 [9,13,14]. S105 is the active holin of λ-phage, whereas the two amino acids longer S107 acts as its inhibitor (antiholin) [15]. Interestingly, *tcdE* mRNA also exhibits alternative start codons within the N-terminal sequence

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and a putative ribosome binding site upstream of both the first and third start codons of *tcdE* [8]. The deduced molecular weights of the resulting TcdE forms are 19 kDa and 16 kDa, respectively.

The aim of the present study was to characterize *C. difficile* TcdE with respect to toxin release and sporulation to elucidate an assumed function as a holin-like protein.

2. Material and methods

2.1. Antibodies

Polyclonal rabbit anti-TcdA and anti-TcdB antibodies (Institute of Toxicology, Hannover Medical School); Antibodies recognizing non-glucosylated (clone 102) and total Rac1 (clone 23A8) purchased from BD Biosciences; β-Actin (clone AC15) was from Sigma; horseradish-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Rockland Immunochemicals). All chemicals were of the highest purity available.

2.2. Bacterial strains and culture

C. difficile strains VPI10463 (Accession-No. X92982), 630Δ*erm* (Hussain et al., 2005), 630*tcdE::erm* (this study), 1470 (Pasteur Institute, Paris, France) and the ribotype 027 strain 196 (Accession-No. L76081), as well as *Clostridium sordellii* strains 6018 and CN6 (Pasteur Institute, Paris, France), were grown in Brain Heart Infusion (BHI) broth at 37 °C in an anaerobic gas jar.

T7 Express *lysY/I*^{q} Competent *Escherichia coli* (New England Biolabs) serving as cloning and expression hosts were grown in Luria-Bertani (LB) medium supplemented with 100 mg/L ampicillin. Transformed *E. coli* or different *C. difficile* strains were grown in their respective media. At the indicated time points, 750 μl of liquid culture were taken and subjected to photometry to determine the optical density at 600 nm (OD_{600}). The photometer was calibrated to $OD_{600} = 1$ corresponding to 5×10^8 cells per ml.

2.3. Generation of a TcdE deficient C. difficile mutant

The *tcdE* gene in *C. difficile* strain 630Δ*erm* was disrupted by directed insertional inactivation (ClosTron) using a bacterial group II intron according to [16]. In brief, one-tube Splicing by Overlap Extension PCR was performed to amplify a 353 bp product containing the modified IBS, EBS1d and EBS2 sequences which are specific for the target gene *tcdE* and responsible for intron targeting. The target sites were identified and specific intron re-targeting primers were designed by means of a computer algorithm [17] which is part of the Targetron Gene Knockout system kit (http://www.sigmaaldrich.com). The following primers were used: IBS: AAAAAAGCTTATAATTATCCTTAAATATCCATGCTGTGCGCCCAGATAG GGTG; EBS1d: CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATGC TATTAACTTACCTTTCTTTGT; EBS2: TGAACGCAAGTTTCTAATTTCGAT TATATTTCGATAGAGGAAAGTGTCT. The amplified targeting region was ligated via HindIII and BsrGI restriction sites into the plasmid pMTL007C-E2 and transformed in *E. coli* donor CA434. The retargeted intron, named pMTL007C-E2:cdi-*tcdE*-234a, was then transferred via conjugation into *C. difficile* 630*Δerm*. Finally, the transconjugants were re-streaked on BHI plates supplemented with 2.5 μg/ml erythromycin to select for integrants and 250 μg/ml cycloserine and 8 μg/ml cefoxitin to select against the *E. coli* donor. Positive selected clones were checked by specific PCR for disrupted *tcdE* gene and termed *C. difficile* strain 630*tcdE::erm*. In addition, Western blot analysis was performed to check for TcdE expression. The following experiments were performed with two independent clones. The presented results are based on clone 1.

2.4. C difficile cultivation and detection of toxins

Overnight cultures of *C. difficile* were pelleted and resuspended in fresh BHI broth. The main culture was then inoculated with a bacterial suspension calibrated to the respective optical density. The different strains were cultivated in triplicate and 5 ml of culture were pelleted at the indicated time points by centrifugation for 10 min at 4000 g. The supernatants were collected and subjected to ELISA and cytotoxicity assay, respectively. The bacteria pellet was solubilized in PBS supplemented with 5 mg/ml lysozyme and 1 mM AEBSF and incubated for 1 h at 37 °C. Following sonication, the lysates were centrifuged for 5 min at 16,000 g to eliminate cell debris and non-lysed bacteria. Finally, after calibration to the respective optical densities at time point of harvest, the soluble fractions were subjected to SDS-PAGE and Western blot analyses to determine the level of non-released TcdA and TcdB. In order to confirm *tcdE* inactivation on protein level, 500 ml of cdi630Δ*erm* and cdi630*tcdE::erm* cultivated for 48 h were lysed using French Press and centrifuged for 20 min at 8000 g. The resulting pellet was resuspended in 2 ml PBS containing 2% Triton X-100 and incubated 30 min at 4 °C to solubilise membrane-associated TcdE. Suspensions were subjected to SDS-PAGE and Western blot analyses to detect TcdE.

For MS analysis, after onset of exponential growth cdi630Δ*erm* and cdi630*tcdE::erm* were cultivated for 10, 20 and 30 h, respectively, and culture supernatants were obtained by centrifugation at 4000 g for 8 min at 4 °C. Supernatant was removed and the centrifugation step was repeated. The protein concentration was determined using the Bradford-assay and bovine serum albumin (BSA) as a standard in a Smartspec 3000 photometer. Cell free culture supernatant was directly used or stored at −20 °C. To control the protein content of the BHI medium, 1 ml of the autoclaved medium was dried by vacuum evaporation and subjected to SDS-PAGE. A similar amount of medium was dissolved in 100 μl of 5% ACN and 0.1% FA and directly analyzed by LC-MS/MS.

To investigate TcdE of different clostridia species and strains on protein level, 1 ml of the respective bacterial suspension grown for 20 h in BHI broth were pelleted by centrifugation for 10 min at 4000 g and solubilized in Lämmli buffer. After sonication, lysates were directly subjected to SDS-PAGE and Western blot analyses to detect TcdE.

2.5. Generation of TcdE constructs

C. difficile VPI10463 genomic DNA was used for amplification of *tcdE* (base pairs 1-498) and *tcdE*$^{27-166}$ (base pairs 79-498) applying the forward primers 5′AGTCGGATCCATGCAC AGTAGTTCACC-TTTTTATATTTC and 5′AGTCGGATCCATGACAATATCTTTTTTATCAG for tcdE and *tcdE*$^{27-166}$, respectively, and reverse primer 5′AGTC-AAGCTTCTTTTCAC CCTTAGCATTCATTTCATC for both constructs. The PCR was performed in 50 μl with 200 ng DNA, 300 nM of appropriate sense and antisense primer, 250 μM dNTPs, 1x complete reaction buffer for Pwo polymerase and 2.5 U Pwo DNA Polymerase. The PCR conditions were the following: one initial cycle for denaturation at 95 °C for 1 min, 28 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 70 °C for 1 min followed by a final extension phase at 70 °C for 10 min. The PCR products were digested by BamHI and HindIII followed by insertion into pQE30 plasmid vector. Vector amplification and protein expression was performed in T7 Express *lysY/I*^{q} competent *E. coli*.

2.6. Expression of recombinant protein

For protein expression 50 ml *E. coli* cultures were grown at 37 °C until the OD_{600} was 0.5. Protein expression was induced by the