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EFFECT OF THE CLOSTRIDIUM DIFFICILE TCDE PROTEIN ON MEMBRANES OF ESCHERICHIA COLI

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ABSTRACT

Clostridium difficile infects millions of hospitalized patients worldwide, causing pseudomembranous colitis, a disease with symptoms attributable to two toxins produced by the bacterium. The PaLoc gene locus in the C. difficile genome is responsible for toxin production and activation, but it is not known how the toxins are released from the pathogen. Within PaLoc is a gene for the TcdE protein, which shares striking similarity to a class of membrane pore-forming proteins called holins. To assess whether TcdE catalyzes toxin release, the engineered tcdE gene was expressed in Escherichia coli. An arabinose promoter upstream of the tcdE gene was manipulated to modulate transcription of the gene. Recombinant E. coli cells showed viability and morphological changes, throughout different phases of growth, consistent with membrane pore formation. Thus, TcdE has the properties required of a toxin release mechanism, and drugs that block TcdE function could be candidates for drug therapy for treating infected patients.
Introduction

Among the Clostridiaceae family, the bacterium Clostridium difficile is the cause of a major nosocomial gastrointestinal infection in the United States, infecting up to 7,000 hospitalized Americans per day (Peery et al., 2012). The bacterium has been recognized as the most important isolated cause of hospital-acquired antibiotic-associated diarrhea and pseudomembranous colitis (Carroll & Bartlett, 2011). The typical risk factors involved with developing pseudomembranous colitis include long-term exposure to a health care environment, antibiotic prescription, and exposure to spores of the pathogen (Carroll & Bartlett, 2011). In addition, it is becoming more common to find a pregnant woman or young adult who has developed an infection due to C. difficile with the absence of risk factors (O’Connor, Johnson, & Gerding, 2009). Symptoms of infection range from an onset of antibiotic-associated diarrhea, or the development of pseudomembranous colitis, and in extreme cases, death.

C. difficile is a gram-positive anaerobe capable of producing spores that are resistant to many chemicals, heat, and even UV light (Carter, Rood, & Lyras, 2012). When patients are treated with certain antibiotics, the normal enteric bacteria are depleted, allowing C. difficile to infect (Borriello, 1998). Spores of the bacterium are transferred from the feces of infected patients and become commonplace in hospitals and nursing homes (Carroll & Bartlett, 2011). The spores are ingested and then pass through the upper alimentary tract to reach the lower gastrointestinal tract. Once residing in an appropriate environment, the now vegetative cells of C. difficile release toxins A and B that cause damage to the mucosal barrier and epithelial cells lining the gut (Carter et al., 2012). The ensuing cascade of cytokines and leukocytes may be responsible for the enhanced permeability, diarrhea, epithelial apoptosis, and ulceration of the digestive tract (Jafari et al., 2013).
The pathogenicity locus in infectious *C. difficile* has been termed PaLoc (Dupuy, Govind, Antunes, & Matamouros, 2008) (Figure 1). Containing five genes, it is solely responsible for transforming a nonvirulent strain of *C. difficile* into an infectious one (Govind & Dupuy, 2012).

**Figure 1. Toxigenic versus non-toxigenic *C. difficile* genomes.** The toxigenic strain of *C. difficile* contains the 19.6-kb pathogenicity locus PaLoc with five virulence genes. Non-toxigenic *C. difficile* contain a short 115bp sequence instead of the PaLoc locus (Govind & Dupuy, 2012).

Enterotoxins A and B are produced from the two genes in the PaLoc location called *tcdA* and *tcdB*. Their mechanism proceeds by glucosylation of the family of small Rho GTP-binding proteins, which disrupts the host cell cytoskeleton and leads to apoptosis (Carter et al., 2012). Since the PaLoc locus is wholly sufficient for virulence, there must exist a gene responsible for toxin release within this locus. One hypothesis is that *tcdE* is that gene, because of its homology to phage holin proteins (Govind & Dupuy, 2012). Bacteriophage lytic infections are characterized by an accumulation of holin proteins within lipid rafts in the plasma membrane, as shown in Figure 2 (Wang, Deaton, & Young, 2003). When a critical concentration of the holin monomers has been reached, an aqueous pore channel is thought to spontaneously form. The depolarization involved with the pore formation causes a conformational change within the holins, and they disperse to form the lesion shown in Figure 2 (Wang et al., 2003). Typically, endolysins accumulate in the cytoplasm until the critical point is reached, and a holin pore allows movement across the membrane. The membrane is then destroyed by degradation of murein (Govind & Dupuy, 2012).

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Figure 2. Pore formation by holing proteins. Each pink circle represents a holing protein. They are thought to accumulate until a critical concentration is reached, coinciding with formation of a port within the membrane.

In a study of the most frequent ribotypes of *C. difficile*, it was found that increased expression of the *tcdE* open reading frame had importance in the release of the toxins (Vohra & Poxton, 2011). Researchers induced cells to express the *tcdE* gene, and then visualized the effect with a transmission electron microscope (Tan, Wee, & Song, 2001). The results demonstrated a lack of membrane vesicles as well as fusion of the periplasm and cytosol in the cells with expressed *tcdE* gene. This evidence, compared to a control without *tcdE* expression, showed that the former cell was dying because of membrane disturbance. The authors posited that the fact that the *tcdE* gene is polycistronically transcribed with the *tcdA* and *tcdB* genes supports the critical pathogenic role of TcdE. Neither toxin A nor B have the capacity to allow for extracellular secretion, thus there must be another factor causing such excretion (Tan et al., 2001).

The full 19 kDa TcdE protein is suspected to be composed of three transmembrane helices that are depicted in Figure 3. The $S^{105}$ is a form of the phage $\gamma$ S protein that has holin activity, and is depicted to the left of the TcdE protein in Figure 3 (Govind & Dupuy, 2012). The similarities in structure contribute to the evidence for the holin activity of TcdE. In particular, it could be a member of the class I holins, of which phage $\gamma$ S is a member. It has been demonstrated that the full 19 kDa translation product of *tcdE*, as shown in Figure 3, could be in...
an inactive state (Olling et al., 2012). Previous *in vivo* experiments have suggested that the

![Figure 3](image1.png)

*Figure 3*. Structural similarity between TcdE and $\gamma$ S holin protein. This illustration shows that the TcdE structure composed of three hydrophobic transmembrane domains is similar to the structure of a form of a phage $\gamma$ S holin protein (Govind & Dupuy, 2012).

19 kDa TcdE isoform functions as an antiholin-like inhibitor of the smaller translation product, 16 kDa. So, this inhibition of the active isoform occurs until a precise time during which the functional holins, the 16 kDa isoform, are produced to initiate holin-mediated cell lysis. The 16 kDa TcdE isoform is thought to produce the holin activity, triggering membrane disruption by the formation of a pore through which toxins A and B may be released. The expression of the active 16 kDa isoform can occur by initiating translation at either of two alternative internal start codons, located at positions 72 and 79 of the *tcdE* open reading frame (Olling et al., 2012). These internal codons are depicted in Figure 4, along with the first start codon that may be used to create the entire 19 kDa inactive isoform.

![Figure 4](image2.png)

*Figure 4*. N-terminal section of TcdE DNA. The DNA sequence harbors three alternative start codons that are shown in the figure. The first methionine codon may be translated to produce the 19 kDa isoform of TcdE (thought to be the inactive, regulated form). The interior alternative start codons located at positions 73 and 79 of the open reading frame may also be start points for translation, thus creating the suspected active holin 16kDa isoform of TcdE (Olling et al., 2012).
Since persistent antibiotic treatment is not always effective in treating patients infected with \textit{C. difficile}, and is in fact a source of the infection, the search for a proper treatment is a priority. Currently, the standard approach is to discontinue antibiotic therapy, and instead prescribe a different antibiotic. Although this method reduces symptoms initially, there is a 20-25\% chance that the patient will suffer a relapse of symptoms (Surawicz et al., 2000). The development of useful drugs has been impeded by the ambiguity surrounding the mechanism by which toxins are released. If the theory behind the holin activity of the TcdE protein in \textit{C. difficile} is proved correct, then significant advances toward treatment of antibiotic-associated diarrhea and pseudomembranous colitis can be made. Therefore, the primary goals of the present study were to analyze the morphological effects that gradual amounts of both the inactive and active TcdE protein have on host cell membranes, and to create a growth curve consistent with bacterial growth and subsequent synthesis of TcdE proteins.

\textbf{Materials and Methods}

\textbf{Media and growth conditions}

The \textit{E. coli} strains BL21 [pLysS] (\textit{F\textsuperscript{-}ompT gal dcm lon hsdSB(rB- mB-) }\lambda\text{(DE3)} pLysS(CmR)) and UA381, a chloramphenicol-resistant derivative of \textit{E. coli} BL21 star (\textit{F\textsuperscript{-}ompT gal dcm lon hsdSB(rB- mB-) rne131 }\lambda\text{(DE3)} pRARE), were maintained on Luria-Bertani (LB) broth and agar supplemented with chloramphenicol at 30 \textmu g/ml. Plasmids used in this study conferred ampicillin resistance, and were selected with ampicillin (100 \textmu g/ml) or carbenicillin (50\textmu g/ml).

\textbf{Recombinant DNA procedures}

DNA purification, restriction endonuclease digestions, ligations, transformations with chemically competent cells, and polymerase chain reaction (PCR) was performed using standard
molecular biology methods (Bouillaut, McBride, & Sorg, 2011). Electroporation employed a Bio-Rad Gene Pulser apparatus, set to deliver 1.7 kV at 200 ohms and 25 μfarads. Electroporated cells were incubated with shaking at 37°C for 1 hour prior to plating. Site-directed mutagenesis was performed using the Quickchange kit from Agilent (Santa Clara, CA).

**Construction of the arabinose-inducible tcdE gene**

A pGEM5Zf(+) -based plasmid containing the promoter-less tcdE gene, made in previous studies and designated pUA573, was used to insert the gene into the pBAD-HisA plasmid. Plasmid pUA573 was cut with restriction enzymes NcoI and HindIII, around the tcdE gene, thus isolating the linear tcdE fragment. The tcdE fragment was then added to NcoI/HindIII-treated pBAD-HisA, and recombinant plasmid was ligated in such a way that the tcdE gene was under the control of the arabinose promoter. The resultant plasmid is referred to as pUA575. The plasmid pUA575 was then transformed into E. coli strain UA381, a BL21-based strain suitable for expression studies. The transformed product is referred to as strain UA577.

To test the importance of the two potential internal start codons in the tcdE gene, a mutant form of strain UA577 was made. The mutant plasmid was made via site-directed mutagenesis. The Quikchange procedure was utilized to mutate the internal alternative start codons from the start codon methionine (AUG) to the codon ACG. The mutated plasmid transformed in the UA381 E. coli strain is referred to as UA585.

**Induction of arabinose promoter**

Various concentrations of the monosaccharide L-arabinose were made by diluting a 10% stock into LB broth. The final concentrations were 2%, 0.2%, 0.02%, 0.002%, and 0.0002%. Cultures to be induced were shaken at 37°C until they were at the beginning of the log phase of growth. Ten milliliters of the cultures were aliquotted into each of five tubes, and 100 μl of each
arabinose concentration was added to the tubes. The tubes were then shaken at 37°C, and samples were analyzed tubidimetrically and by fluorescence microscopy at various time points after arabinose addition.

**Microscopy**

Slides prepared by wet mount or by pipetting cells onto agarose pads were examined by phase contrast and fluorescence microscopy, using a Zeiss Axio Imager M1 microscope.

**Results**

The primary aim of the present study was to assess the effect that the TcdE protein has on the cell membranes of *E. coli* cells. Because the likely effect of high levels of expression of a holin-like protein is cell lysis, it proved necessary to control the dosage of TcdE in order to obtain viable *E. coli* cells. The route determined to be the most effective involved the use of the arabinose promoter of plasmid pBAD/HisA, in a host *E. coli* strain derived from the BL21 (DE3) strains typically used for T7 promoter-driven expression. The arabinose promoter should not allow transcription of the *tcdE* gene whatsoever until there is a sufficient amount of arabinose present (Khlebnikov, Risa, Skaug, Carrier, & Keasling, 2000). We used this arabinose-inducible system to study the effect of TcdE expression on host *E. coli* cells. First, we measured the optical density of an exponentially growing culture of *E. coli* cells both before and after the addition of varying concentrations of arabinose (see Methods). We found that increasing arabinose concentrations decreased culture turbidity (Figure 5), suggesting the TcdE expression may be causing cellular lysis. Cells containing the control plasmid pUA579, lacking the *tcdE*
gene, showed no change in turbidity with increasing arabinose concentrations, suggesting that the effect was indeed due to TcdE expression.

![Image of turbidity decreases of strain UA577, associated with varying arabinose concentrations.](image)

**Figure 5.** Turbidity decreases of strain UA577, associated with varying arabinose concentrations. In the five left-most tubes, arabinose concentrations decrease as a 10-fold serial dilution, left to right, from 2% to 0%. The tube on the far right contains cells the tcdE-minus control plasmid, exposed to 2% arabinose.

In order to have a more quantitative assessment the difference in growth between cells that express the putative holin protein versus cells that do not, growth curves were constructed of cells containing the wild-type and mutant tcdE alleles. For UA577 cells expressing wild-type tcdE, adding 0.2% arabinose to mid-log phase cells causes an immediate decline in cell number. The decline persists for approximately 1 hour, after which the cell numbers remain constant for at least another 4 hours. Based on comparisons of turbidity, the arabinose-treated cultures are estimated to have an 80% reduction in the number of intact cells relative to untreated cultures. Arabinose at 0.002%, which likely induces lower levels of expression of tcdE, causes a less pronounced decline in cell numbers, and turbidity begins to increase toward the end of the 4-hour induction period. In contrast, UA585 cells expressing the mutated tcdE gene do not show a
significant decline in cell numbers in response to arabinose, at even the highest concentration (0.2%). Low concentrations (0.002%) have almost no effect on growth, while 0.2% arabinose triggers a slight decrease in growth rate during the first hour of induction, followed by a 3-4 hour period during which the cell population remains constant or increases slightly.

![Arabinose Induction](image)

*Figure 6. Effects of arabinose induced expression of wild-type and mutant tcdE alleles on E. coli growth.*

Next, we visually inspected *E. coli* cells expressing TcdE when exposed to these various amounts of arabinose. This approach allowed us to examine cellular morphology to better understand why TcdE expression decreased turbidity. As a control, we also used a mutant allele of *tcdE*, where two internal methionine codons were changed to threonine codons. This mutant allele is unable to produce the active 16 kDa isoform, as a result of disrupting the alternative translation initiation sites at the internal methionine/start codons. We examined *E. coli* carrying either wild-type or mutant *tcdE* following the addition of 0.002% arabinose. Cells were visualized 1 and 2 hours after arabinose addition (Figure 7). Cells expressing wild-type *tcdE*
following 1-hour induction with 0.002% arabinose were mostly the characteristic *E. coli* rod shape, with some cells having a rounded or oval shape. A 2-hour induction led to a higher percentage of rounded cells (Figure 7B). At higher magnification the cells show a clumping of rounded, enlarged, less opaque morphology.

The control cells containing the mutant *tcdE* allele (which only can translate the 19 kDa isoform) were also exposed to 0.002% arabinose and visualized microscopically (Figure 7). Figure 7C shows the *E. coli* cells one hour after induction with arabinose, which demonstrates a characteristic clumping of the *E. coli* cells. In contrast with the wild-type form, they are almost

**Figures 7A-7D.** A) Wild-type UA577 one hour after induction with arabinose of concentration 0.002%. B) Wild-type UA577 two hours after induction with arabinose concentration of 0.002%. C) Mutant UA585 one hour after induction with arabinose of concentration 0.002%. D) Mutant UA585 two hours after induction with arabinose of concentration 0.002%.
all the typical rod-shaped *E. coli* cells. After an additional hour, more isolated *E. coli* cells can be seen in Figure 7D that retain their characteristic rod shape more so than the wild-type strain that had been exposed to the same amount of arabinose and after the same length of time (Figure 7B).

The morphological differences due to the effect of adding arabinose to the wild-type versus mutant cells can be better seen in magnifications. This was done with the cells exposed to 0.002% arabinose two hours prior to visualizing them under the microscope (Figure 8).

We also used fluorescence microscopy to directly compare morphological differences between cells containing wild-type versus mutant *tcdE*. Arabinose induction was performed on cells containing *tcdE*, along with an *E. coli* strain expressing green fluorescent protein (GFP), but lacking *tcdE*. Fluorescent and phase contrast images of each field were superimposed to compare the differences in cell shape. GFP-containing cells showed the typical morphology of log-phase *E. coli*, while the non-fluorescent cells were larger and mostly flattened or otherwise misshapened (Figure 9). The results show that the dramatic morphological changes seen in response to arabinose induction were the result of TcdE protein accumulation, and not simply caused by activation of the arabinose promoter *per se*.
Figure 9. Superimposed fluorescence and phase contrast image of arabinose-treated E. coli cells. GFP containing cells do not contain the tcdE gene.

Discussion

An essential feature in future drug developments for pseudomembranous colitis and antibiotic-associated diarrhea is to understand the mechanism by which toxins A and B are released from C. difficile. The TcdE protein could be targeted in drugs developed for treating these nosocomial diseases if it can be identified as the source by which the symptom-carrying toxins are released. An additional topic of interest with regards to drug development is the potential inhibitory function that the 19 kDa isoform of the TcdE protein may possess. The progress of such drug development is a priority within the medical community due to the ineffectiveness of current therapy. Existing antibiotics are actually both part of the source of the disease and the treatment for it. Although different types of antibiotics are prescribed following infection by C. difficile, this therapy does not prove to be sustainable with recurrance of symptoms occuring one fourth of the time, and within two to three weeks after discontinuing the original antibiotic (Kelly & LaMont, 1998).
Overall, the results of the present study support the prediction that there would be alterations or disruption to the plasma membrane of host cells that express the TcdE protein (Tan et al., 2001). The modulated induction of the arabinose promoter that was used for expressing the \textit{tcdE} gene proved to be effective. Such expression has proved to be a problem in the past when excessive amounts of TcdE would lyse the cells and no analyses could be made on the host cells (Tan et al., 2001). The effect of \textit{tcdE} expression on growth, as measured by turbidity, suggests that wild-type TcdE protein expression causes dose-dependent lysis of cells. It is likely that a threshold concentration of TcdE proteins is required in order for permanent damage to the cytoplasmic membrane to occur. Lower levels of induction of the wild-type \textit{tcdE} gene, or even high levels of induction of the mutated \textit{tcdE} gene, lead to fewer cells with the threshold concentration of TcdE protein. In those cases, the effects on turbidity and cell morphology are less pronounced, and/or require longer incubations in order to be observed.

The morphology of the wild-type cells compared to the mutant cells after induction with arabinose also presented substantial evidence for the role that TcdE plays in the state of the host membrane. As demonstrated with the use of microscopy, the wild-type cells exhibited an unusual round shape that is very different than the mutant strain that retained the rod-shape typical of \textit{E. coli} cells. The wild-type cells also presented a more translucent form, being less opaque and generally more hazy in appearance compared to the distinct mutant cells that had viable, intact membranes. This phenomenon could possibly be explained as being due to the disruption of wild-type membranes. Another study (Tan et al., 2001) produced similar results showing a lack of membrane vesicles, merging of the cytosol and periplasm, and membrane foldings in the cells expressing TcdE. Compared to the control that had no TcdE expressed, it maintained a differentiated cytosol and periplasm and a distinct, smooth membrane outline. This is what was
seen in the mutant *E. coli* cells in the present study, while the less opaque membrane outline of
the wild-type strain could be due to similar reasons that the researchers previously discovered.

The wild-type UA577 cells also demonstrated an increase in clustering at the two hour
mark after arabinose induction while the mutant cells were individually isolated from each other.
A potential explanation for this finding could be due to the clustering that holin proteins undergo
prior to pore formation and holin lesion. Most of the clustered cells also have the irregular shape
previously discussed, so these wild-type cells could be aggregating and also undergoing
membrane disruption due to the holin lesion whereas the mutant cells that have the inactive TcdE
protein could be retaining their morphology and spatial independence.

These studies suggest that potential source of drug advancement could be aimed at the
oligimerization of the holin-like TcdE proteins. If the proteins are unable to congregate and
interact, then they could not oligomerize and create the pore in the membrane that ultimately
leads to release of toxins A and B, causing symptoms of pseudomembranous colitis and
antibiotic-associated diarrhea. In order to progress in this direction, however, the TcdE protein
must be confirmed as a holin protein. Future studies designed to directly test the holin function
of TcdE include the construction of His/3XFlag-tagged TcdE. If such a protein, bound to
fluorescent antibodies, exhibits a clustering or halo shape, then such a finding would contribute
to evidence supporting the holin theory of TcdE.
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