BACTERIAL PATHOGENICITY

Evidence for holin function of *tcdE* gene in the pathogenicity of *Clostridium difficile*

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Toxigenic strains of *Clostridium difficile* produce two large bacterial toxins called toxins A (TcdA) and B (TcdB). *tcdA* and *tcdB* genes are located on the pathogenicity locus of *C. difficile*, a unique characteristic of toxigenic strains of this species. Intergenic to the two toxin genes is *tcdE*, a small 501-bp open reading frame of unknown function. Expression of the *tcdE* gene in *Escherichia coli* caused bacterial cell death. Computational analysis of the amino acid sequence of TcdE revealed structural features that are strikingly similar to a class of bacteriophage proteins called holins. Holins are cytolytic proteins that cause lysis of bacterial hosts to effect the release of progeny phages. Further analysis of the recombinant clone expressing TcdE by transmission electron microscopy confirmed that the site of action of TcdE is on the bacterial cell membrane. The results provide evidence that TcdE is structurally and functionally similar to holin proteins. TcdE may function as a lytic protein to facilitate the release of TcdA and TcdB to the extracellular environment, as these toxins lack signal peptide.

Introduction

Clostridium difficile is a gram-positive, spore-forming anaerobic bacterium responsible for pseudomembranous colitis and a significant proportion of antibiotic-associated diarrhoea [1]. Recently, this bacterial species has been recognised as a major nosocomial diarrhoeagenic pathogen in industrialised parts of the world [2]. *C. difficile* produces two large toxins designated toxins A (TcdA) and B (TcdB) which are held to be largely responsible for the disease symptoms [3–6]. The genes coding for the toxins are located on a large 19.6-kb chromosomal fragment called the pathogenicity locus (PaLoc). The PaLoc is found in toxigenic strains of *C. difficile*. In non-toxigenic strains, the PaLoc is replaced by a short fragment of 127 bp [7].

tcdE is a 501-bp open reading frame (ORF) between the *tcdA* and *tcdB* genes. This ORF is located 122 bp downstream of the stop codon of *tcdB* and 727 bp upstream of the start codon of *tcdA* [7]. The putative protein encoded, TcdE, contains 166 amino acids with a deduced mol. wt of 19 kDa and is predicted to be highly hydrophobic. Although the sequence of *tcdE* has

been known since 1990 [5], to the best of our knowledge, there is no published report on any study done on this ORF. Evidence showing that *tcdE* is polycistronically transcribed together with the *tcdA* and *tcdB* genes supports the idea that the putative protein, TcdE, may have a crucial role in the pathogenicity of *C. difficile* [8, 9]. Dove *et al.* speculated that TcdE could be a regulatory protein for the toxin genes [5]. Eichel-Streiber and Sauerborn suggested otherwise, and proposed that the function of this putative protein is to facilitate the release of *C. difficile* toxins to the extracellular environment, as both TcdA and TcdB lack the necessary signal peptide for extracellular secretion [10]. This controversial view and the lack of supportive investigation regarding the function of this elusive ORF prompted this study. This report describes the cloning, expression and efforts to determine the function of TcdE. It also presents the evidence for and proposes the involvement of TcdE in facilitating the release of *C. difficile* toxins to the extracellular environment.

Materials and methods

Bacterial strains and plasmids

C. difficile strain VPI 10463 was used to provide the genomic DNA for PCR. The bacteria were cultured overnight in Brain Heart Infusion (BHI) Broth (Difco

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Laboratories, Detroit, MI, USA) under anaerobic conditions. *Escherichia coli* strains JM109 [11] and M15[pREP4] (Qiagen, Valencia, CA, USA) were used as cloning and expression hosts, respectively. The bacteria were cultured either in Luria-Bertani (LB) (Life Technologies, Gibco BRL, Rockville, MD, USA) or BHI broth, supplemented with kanamycin 50 mg/L or ampicillin 100 mg/L. The plasmid vector pQE-30 (Qiagen) was used both as a cloning and expression vector.

DNA manipulations

Restriction endonucleases, T4 DNA ligase, alkaline phosphatase and DNA polymerase were purchased from New England Biolabs (Beverly, MA, USA) and used as recommended by the manufacturer. All molecular techniques were performed as described previously [12].

C. difficile genomic DNA was extracted with DNAzolTM reagent (Life Technologies). Plasmids were extracted and purified by Wizard[®] Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). PCR amplified products were purified by Wizard[®] PCR Preps DNA Purification System (Promega). All procedures were done according to the manufacturer's instructions. The DNA fragment containing the *tcdE* gene was generated by PCR. The sequences of the forward and reverse primers used were 5'-CGCGGATCCATGCACAGTAGTTCACCTT-3' and 5'-CCCCCAAGCTTCCAAGTACCATG CACC-3', respectively. The 5'-end of each of the forward and reverse primer contained a *Bam*HI and *Hind*III restriction enzyme site, respectively (underlined). PCR reaction was performed in a mixture of 100 µl containing 50 ng of DNA, 100 pmoles of each primer, 200 µM dNTP, 2 mM MgSO₄, 1× Thermopol buffer and 2U Vent[®] DNA polymerase. The conditions for the PCR were: one initial cycle of denaturation at 94°C for 2 min, annealing at 49°C for 45 s and extension at 72°C for 2 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 50 s; and a final

extension at 72°C for 2 min. The *Bam*HI- and *Hind*III-created sites of the *tcdE* fragment were ligated into the the *Bam*HI and *Hind*III sites of the digested pQE-30 vector. The pQE-30 vector allowed the *tcdE* fragment to be cloned in-frame with the fusion histidine counterpart of the vector. The ligated sample was transformed into *E. coli* M15 by electroporation with a gene pulser apparatus (BioRad, Hercules, CA, USA). The presence of a β-lactamase gene on the vector allowed screening of the transformants by antibiotic resistance. Recombinant plasmids were sequenced by an ABI Prism[®] BigDyeTM cycle sequencing ready reaction kit (PE Applied Biosystems, CA, USA) to ensure that no mutations had occurred during DNA manipulations or cloning procedures.

Expression of recombinant clone

Bacterial cultures (50 ml) in LB or BHI broth supplemented with the appropriate antibiotics were grown at 37°C with shaking at 200 rpm until the OD₆₀₀ was 0.6. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). A time-course analysis of protein expression was performed to determine the effects of TcdE on host cells.

Preparation of cell extracts and protein purification

Cells were harvested 5 h after induction. Bacterial cells were pelleted by centrifugation at 3500 *g* for 20 min and the supernate was discarded. The cells were resuspended and washed in 5 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH₂PO₄·7H₂O, 1.4 mM KH₂PO₄). The washing step was repeated once. The pellet was finally resuspended in 2 ml of PBS and transferred to a new tube for protein extraction. The controls used were the non-induced *E. coli* M15E and *E. coli* M15NC (Table 1).

For preparative-scale purification of the expressed protein, the culture size was scaled up to 500 ml and the bacteria were grown to OD₆₀₀ of 0.8 before they

Table 1. Plasmid vectors and bacterial strains

Plasmids/ strains	Descriptions	Source/ Reference
Plasmids		
pREP4	Repressor plasmid carrying the <i>lacI^q</i> and kanamycin-resistance gene	Qiagen
pQE-30	Expression vector with N-terminal 6X His-tag and ampicillin-resistance gene	Qiagen
ptcdE	pQE-30 expression vector with <i>tcdE</i> cloned into the <i>Bam</i> HI and <i>Hind</i> III restriction sites of the vector	This study
<i>E. coli</i> strains		
M15[pREP4]	<i>E. coli</i> K12 possessing the pREP4 repressor plasmid	Qiagen
M15E	M15[pREP4] harbouring the ptcdE recombinant vector	This study
M15NC	M15[pREP4] harbouring the pQE-30 parental vector	This study
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB) F(traD36 proAB⁺ lacI^q lacZM15)</i>	[11]
JM109E	JM109 harbouring the ptcdE recombinant vector	This study
JM109NC	JM109 harbouring the pQE-30 parental vector	This study

were harvested. The cells were lysed by six cycles of sonication with a $\frac{1}{8}$ " microtip probe. The cell lysate was centrifuged at 15 000 *g* for 30 min at 4°C to pellet the cellular debris. The supernate that contained the soluble protein fraction was transferred to a new tube and stored at -20°C before use. The remaining pellet that contained the insoluble protein was resuspended and washed with cold PBS and centrifuged at 15 000 *g* for 30 min at 4°C. The supernate was discarded, and this washing step was repeated twice. The insoluble protein was solubilised with 8 M urea (Sigma), Triton X-100 (Sigma) 2% and β -mercaptoethanol (Sigma) 3%. The fusion protein was purified following the protocol recommended by the manufacturer (Qiagen). Cells were grown to an OD₆₀₀ of 0.8 before induction with IPTG. Both the soluble and insoluble protein fractions were purified. The samples were analysed by SDS-PAGE with a polyacrylamide 12% gel.

Transmission electron microscopy

After the induction with IPTG, the bacteria were cultured for a further 30 min before the transmission electron microscopy (TEM) studies. The procedures for TEM were performed as described previously [13]. Thin sections of the cells were processed and examined at 100 000 $\times$ magnification with a Philip EM208S electron microscope operating at 100 kV.

Computational analyses

Preliminary sequence analyses of the DNA and protein were done with the computer programmes MacVector 5.02 (Oxford Molecular Group, Campbell, CA, USA) and Strider 1.1 (Commissariat à l'Energie Atomique, France). The amino acid sequence homology search was done by the Basic Local Alignment Search Tool

(BLAST) and Pairwise Sequence Alignment services using the Protein Information Resource (PIR) database [14]. The transmembrane helix and signal peptide predictions were performed by the TMHMM and SignalP prediction servers at the Centre for Biological Sequence Analysis (CBS) website at <http://www.cbs.dtu.dk> (The Technical University of Denmark, Denmark).

Results

Expression of TcdE in *E. coli* resulted in a sudden halt of bacterial growth following induction with IPTG. Gradual loss of culture turbidity was observed thereafter. This phenomenon was not observed in non-induced *E. coli* M15E. In the negative control cells, *E. coli* M15NC, the cells continued to grow even after the addition of IPTG (Fig. 1). The loss of culture turbidity upon induction of TcdE expression with IPTG was still observed even with cultures in BHI, an enriched medium. Performing the protein expression at lower temperatures also could not sustain cell growth (data not shown). Expression of TcdE in *E. coli* JM109 also gave the same phenomenon. Attempts to visualise the expressed product on SDS-PAGE gels were unsuccessful. The expected 19-kDa protein band of TcdE was not observed for either the crude (soluble and insoluble), or the purified protein extracts (data not shown).

TEM study

A TEM study was performed to observe the effect of TcdE on the cell after its expression is induced. As shown in Fig. 2a, cells containing only the parental vector showed distinct and smooth membrane outline

Fig. 1. Growth profile of bacteria incubated at 37°C. OD₆₀₀ reading of bacterial growth was determined after the bacteria were induced with 1 mM IPTG at 0 min. ■, *E. coli* M15E (IPTG-induced); ▲, *E. coli* M15E (non-induced); ●, *E. coli* M15NC (IPTG-induced).

Fig. 2. Electron micrographs of *E. coli* strain M15E harbouring *ptcdE* recombinant vector. **(a)** Thin section of *E. coli* cell before protein induction. The cell has an abundance of membrane vesicles (a) on the outer membrane. Also, the cytosol and periplasm of the bacteria are clearly differentiated. **(b)** Thin section of *E. coli* expressing TcdE after induction by IPTG. Numerous membrane foldings (b) with the merging of cytosol and periplasm were clearly visible. There was an obvious lack of membrane vesicles.

with the periplasmic and cytosolic regions of the cells clearly segregated from each other. Moreover, a number of membrane vesicles could be clearly seen on the surface of the cell. However, the phenotypic characteristics of the cells expressing TcdE were markedly different. As shown in Fig. 2b, there was an apparent membrane folding in the cells with the fusion of periplasm and cytosol, and a lack of membrane vesicles on the surface of the cells. These were indications that the bacterial cells were dying due to membrane disruption.

Computational analyses

The amino acid sequence homology search performed by BLAST with the PIR database revealed significant homology of TcdE with holin homologue *yqxH* of *Bacillus subtilis* (23% identity and 54% similarity) [15]. Global alignment of TcdE amino acid sequence with the sequence of other holin proteins revealed that TcdE has 32% identity and 50% similarity to holin 141 and 23% identity and 54% similarity to holin 87 of *Streptococcus thermophilus* bacteriophage Sf19 [16]. TcdE also exhibited 23% identity and 77% similarity to putative holins of *S. pneumoniae* phage Cp-1 [17], *B. subtilis* phage phi-105 holin (18% identity and 55% similarity), *B. subtilis* bacteriophage sk-1 holin (24% identity and 74% similarity) [18] and *B. subtilis* bacteriophage B103 holin (25% identity and 52% similarity) [19]. The greatest number of amino acid identities and similarities between TcdE and holins were found within a C-terminal stretch of 46 residues between Leu 121 and Lys 166 of TcdE (Fig. 3). Striking resemblance in the distribution of the hydrophilic and hydrophobic amino acids was also observed when the hydropathy plots [20] of TcdE and holins were compared (Fig. 4).

		#	##	#	#	#		#	#	
TcdE	121	LF-ILYESVSILKNMCLGLVVPK	----	RL---	KEKIAILLDAMTDEMNA-KDEK	166				
		** * *	** * *	** * *	** * *	** * *				
yqxH	92	LFIYIANEGLSITENLAQIGVKIPS	-----	SI---	TDRLQTIENEKEQSKNN-ADKA	138				
		* * * * *	* * *	* * *	* * * * *	* * *				
h-141	94	MM-AAYGS-SILANLSSSLGVKFPYIDRYIRLNIDKEKF-ILLDEEEEEEND								
		*		* * *		* * *		*		
h-87	28	VF-LMLQQF-----GLHVPN-----NI---		QEGINTLVGILVILGII-TDPT	64					
		** * *	* * *	* * *		* * *		**		
phi-105	87	LFIYIANEGLSITENLAQIGVKIPA	-----	VI---	TDRLHVI-ESDNDQKTE-KDDQ	132				
		** * * *	* * *	* * *		* * * * *				
Cp-1	80	II-NLYYALSIMENLAVMGVFI	PK-----	FM---	TARVQEELQKYTAQLDAGKDLL	127				

Fig. 3. Amino acid sequence similarity of TcdE and holins from bacteriophages of gram-positive and -negative bacteria. Single letter code for amino acid sequence is used throughout. Residues identical to TcdE are indicated by an asterisk (*) above the amino acid sequence. The # sign above the TcdE amino acid sequence indicates common residues in at least four of six proteins examined. The numbers next to the letters indicate the position of amino acid residues of the proteins. The partial location for one of the transmembrane helices of the protein predicted by the TMHMM server is underlined. *yqxH*, holin homologue of *B. subtilis*; h-141, holin 141 of *S. thermophilus* bacteriophage Sf19; h-87, hdn 87 of *S. thermophilus* bacteriophage Sf19; phi-105, holin of *B. subtilis* phage phi-105; Cp-1, holin of *S. pneumoniae* phage Cp-1.

Fig. 4. Hydropathy plot of TcdE and two of the holin homologues. The hydropathy plot was predicted by use of the DNA Strider software. The positive and negative numbers on the y-axis indicate the hydrophobic and hydrophilic regions of the proteins, respectively. The x-axis represents the amino acid residues (a) TcdE, (b) Cp-1, (c) phi-105.

Discussion

Expression of TcdE in *E. coli* M15E resulted in loss of culture turbidity, as represented by the decrease in OD₆₀₀ readings. The loss of culture turbidity could be interpreted as a measure of bacterial cell lysis (Fig. 1). The negative controls consisting of non-induced *E. coli* M15E cells and *E. coli* M15NC cells did not exhibit

the cell lysis phenomenon. These results suggest that the expression of TcdE from the recombinant plasmid *ptcdE* was responsible for the lysis of bacteria. This conclusion is further supported by the fact that the lysis of bacterial cells was not related to nutrients, bacterial strain or temperature. Protein extraction and purification were attempted despite the inability to sustain cell growth during protein expression. Unfortunately, even with the use of higher cell density and a shortened induction time, the TcdE protein band was not visible on the SDS-PAGE gel. Cell growth was affected just 30 min after induction (Fig. 1). Thus it could be inferred that small quantities of the TcdE protein were sufficient to affect cell growth, most likely by causing membrane damage, leading to the arrest of all cellular metabolism, including further production of the heterologous protein. These factors could have resulted in protein amounts that were below the resolution of the SDS-PAGE gel.

Further evidence to support the lytic activity of TcdE comes from the observation that significant structural similarities were observed between TcdE and holin proteins. Holins identified to date are small, integral membrane proteins with membrane-spanning domains. These proteins usually possess a hydrophilic N-terminus and a highly charged C-terminus [21]. Consistent with this, TcdE was predicted to contain membrane-spanning domains from the hydropathy plot peaks and the TMHMM prediction server. Amino acid sequence analysis of TcdE also revealed a short hydrophilic stretch at the N-terminus of the protein and a series of charge-rich residues at the C-terminus of the protein (Fig. 3).

When the nucleic acid sequence of *tcdE* was analysed in detail, three adjacent ATG start codons were found. Other than the initially presumed start codon of *tcdE* that is located 122 bp downstream of the stop codon of *tcdB* gene [6], two further start codons were found located at amino acid residues 25 and 27 of TcdE. Holins of bacteriophages λ S, P22, 21S, PA-2S and ϕ 80S contain a second start codon (ATG) at the third or fourth amino acid residue relative to the first start codon of the proteins [22, 23]. The importance of this dual-start affinity has been studied in detail for λ S phage. Both the ATG start sites in λ S phage are used *in vivo* for translation initiations, resulting in the formation of two products, S107 and S105 [24]. It has been demonstrated that the two proteins exhibit opposing functions *in vivo* with the shorter product, S105, acting as the functional holin while the longer product, S107, plays the role of an inhibitor of S105 function. Thus, at a precisely scheduled time, functional holins are produced to cause holin-mediated cell lysis. Interestingly, a putative ribosome binding site was found upstream of both the first (GGTGG) and third (GGAGG) start codons of TcdE [5]. The deduced mol. wts of the proteins initiating from the first and third start codon are 19 kDa and 16 kDa, respectively.

Thus, whether these two start codons are used *in vivo* to produce two versions of proteins involved in the similar temporal regulation of protein function as in λ S phage remains to be determined. The dual-affinity ATG start sites of phage holins are often separated by positively charged amino acids. However, the residue separating the two start sites of TcdE was a polar amino acid asparagine. Mutagenesis experiments are being done at this laboratory to determine the significance of this residue.

In-vitro studies involving the expression of holins have yielded similar discouraging results. It has been shown that expression of holin genes in either a heterologous or a homologous host system caused cell death. Expression of holins in the eukaryotic host *Saccharomyces cerevisiae* also gave lethal outcomes [21]. Attempts to visualise the holin proteins on SDS-PAGE were also unsuccessful [25]. This may be due to the small quantities of holin protein required to cause sufficient membrane damage to arrest all cytoplasmic activities, including further production of holin. Purification of the small, membrane-embedded, low abundance holin protein in its native form has been considered to be technically difficult, if not impractical.

To further confirm the hypothesis that the site of action of TcdE is on the bacterial cell membrane, TEM (which allows visualisation of membranes at high magnification) was performed. In physiologically healthy bacterial cells, the membrane outline was distinct and smooth, with the periplasmic and cytosolic regions of the cells clearly segregated from each other (Fig. 2a). The electron microscopic profile of the cells expressing TcdE showed irregular membrane folding together with fusion of the bacterial cytosol and periplasm, suggesting loss of cell membrane integrity (Fig. 2b). The occurrence of these abnormal features around the entire cell surface also suggested that TcdE was probably not transported to the cell surface by any specific pathway. Furthermore, membrane-trafficking vesicles that were observed in physiologically healthy bacteria cells were missing [26]. These observations provide evidence that the bacterial cells were dying as a result of membrane disruption.

The suggestion that both toxins A and B of *C. difficile* lack any recognisable signal sequence [27] was confirmed by the computational analyses performed with the SignalP prediction server. Therefore, once synthesised, these toxins will probably accumulate in the cytoplasm of bacteria. The mechanism of toxin release from *C. difficile* is not known. Ketley *et al.* [28] reported that both toxins A and B appeared to be produced and released during the death phase of bacterial growth. In their study, no toxins were detected during the logarithmic phase of growth. The toxin level began to increase gradually during the early bacterial death phase [28]. In the present study, expression of TcdE resulted in lysis of the bacterial host (Fig. 1). Genetic evidence

from in-vivo transcript analysis performed by Hundsberger *et al.* showed that the mRNAs of *tcdA*, *tcdB* and *tcdE* were detectable only during the stationary phase of *C. difficile* growth [9]. Presumably, TcdE protein together with toxins A and B will only be synthesised after the stationary phase of growth. As mentioned earlier, toxins A and B lack signal peptide for extracellular secretion and no export mechanisms for these toxins are known. Thus, TcdE as a putative holin, could function as the lytic protein that creates membrane lesions to facilitate the release of toxins A and B to the extracellular environment. It is premature at this point to predict whether membrane lesions formed by TcdE alone are sufficient for the lysis of *C. difficile*. Although it may appear puzzling why bacterial toxins are excreted at the expense of the bacterial life, this altruistic mechanism of toxin release may be a key to *C. difficile* survival through sporulation. Duncan reported that the formation and release of type A enterotoxin of *C. perfringens* coincided well with the sporulation time of the organism [29]. Correlation between cytotoxin production and sporulation has also been reported in *C. difficile*. Kamiya *et al.* showed that when sporulation of *C. difficile* was inhibited, a marked decrease in both sporulation and cytotoxin production was observed [30]. Thus, sporulation appears to be closely related to cytotoxin production.

Both the experimental and computational analyses provided evidence that TcdE is structurally and functionally similar to a holin protein. The entire sequence of events, starting from the polycistronic transcription of *tcdA*, *tcdB* and *tcdE*, the putative holin function of TcdE, the lack of signal sequences and export mechanism for the toxins, fit extremely well (more than by chance) to suggest that TcdE may function as a lytic protein to facilitate the release of toxins from *C. difficile*. Despite discouraging results obtained while attempting to isolate the TcdE protein for more extensive characterisation of its activity, this study has undoubtedly provided an insight into the structure and function of what was previously an enigmatic ORF.

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