Characterization of *Clostridium perfringens* TpeL Toxin Gene Carriage, Production, Cytotoxic Contributions, and Trypsin Sensitivity

Jianming Chen, Bruce A. McClane

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Large clostridial toxins (LCTs) are produced by at least four pathogenic clostridial species, and several LCTs are proven pivotal virulence factors for both human and veterinary diseases. TpeL is a recently identified LCT produced by *Clostridium perfringens* that has received relatively limited study. In response, the current study surveyed carriage of the *tpeL* gene among different *C. perfringens* strains, detecting this toxin gene in some type A, B, and C strains but not in any type D or E strains. This study also determined that all tested strains maximally produce, and extracellularly release, TpeL at the late-log or early-stationary growth stage during *in vitro* culture, which is different from the maximal late-stationary-phase production reported previously for other LCTs and for TpeL production by *C. perfringens* strain JIR12688. In addition, the present study found that TpeL levels in culture supernatants can be repressed by either glucose or sucrose. It was also shown that, at natural production levels, TpeL is a significant contributor to the cytotoxic activity of supernatants from cultures of *tpeL*-positive strain CN3685. Lastly, this study identified TpeL, which presumably is produced in the intestines during diseases caused by TpeL-positive type B and C strains, as a toxin whose cytotoxicity decreases after treatment with trypsin; this finding may have pathophysiologic relevance by suggesting that, like beta toxin, TpeL contributes to type B and C infections in hosts with decreased trypsin levels due to disease, diet, or age.

*C*lostridium perfringens is a Gram-positive, spore-forming, anaerobic, and toxigenic bacterium (1). This microorganism is widely distributed, with a presence in soil, sewage, feces, foods (such as raw meat, fish, and poultry), and surfaces of residential homes. Although *C. perfringens* is present in the intestines of some healthy humans and animals (1), it is also a pathogen causing a range of diseases, spanning from histotoxic infections, such as gas gangrene, to infections originating in the intestines, e.g., hemorrhagic or nonhemorrhagic diarrhea and often-fatal enterotoxemia. *C. perfringens* virulence is due largely to its abundant toxin production, which varies by strain (2). Different *C. perfringens* isolates are assigned to one of five types (A to E) based upon their production of four typing toxins (alpha toxin [CPA], beta toxin [CPB], epsilon toxin [ETX], and iota toxin [ITX]) (3, 4). Besides these four typing toxins, this bacterium can produce at least 13 other toxins, including the enterotoxin (CPE) (1, 5), although no single strain produces all of these toxins.

TpeL, a recently discovered toxin produced by some *C. perfringens* strains, is a member of the large clostridial toxin (LCT) family (6). Other LCTs include toxins A (TcdA) and B (TcdB) from *C. difficile*, the hemorrhagic toxin TcsH and the lethal toxin TcsL from *C. sordellii*, and alpha toxin (TcnA) from *C. novyi* (7, 8). This toxin group consists of proteins ranging in size from ~195 to 310 kDa that share primary amino acid sequence identities spanning from 36% to 90% (9). Most LCTs contain four functional domains, including the biologically active domain located in the N terminus, the cysteine protease domain, the putative pore-forming and delivery domain, and a C-terminal receptor binding domain that contains combined repetitive oligopeptides (CROPs) (9–11). Like other LCTs, TpeL possesses the glycosyltransferase activity domain, autocatalytic activity domain, and the transmembrane domain; however, it lacks the CROP sequences present in all other LCTs (12).

Despite the absence of CROP sequences, TpeL exerts cytotoxic effects on Vero cells, HeLa cells, and rat pheochromocytoma PC12 cells (6, 12–15). A recent study identified the low-density lipopro-

tein receptor-related protein 1 (LRP1) as the TpeL receptor and also demonstrated the existence of a second, CROP-independent receptor-binding domain in LCTs, suggesting a two-receptor model for the cellular uptake of these toxins (15). TpeL is the largest toxin among the ≥17 toxins produced by *C. perfringens*, with a typical size of ~205 kDa (12). However, at least one strain produces a truncated, 15-kDa-smaller TpeL variant that is less active (6). Nagahama et al. reported *in vitro* studies indicating that a recombinant N-terminal TpeL fragment containing the glycosyltransferase domain uses both UDP-GlcNAc and UDP-Glc as donor cosubstrates and mainly modifies Ras subfamily proteins via glycosylation to mediate its cytotoxic effects (13). A later study confirmed that TpeL preferentially glycosylates Ras and, to a lesser extent, Rap1a and R-Ras3, but it only very weakly modifies Rac1 (14). However, Guttenberg et al. found that TpeL preferentially utilizes UDP-GlcNAc as a sugar donor (12). Whether, at natural production levels, TpeL contributes to the cytotoxic or virulence properties of TpeL-positive *C. perfringens* strains is largely unclear at present.

Some toxins produced by *C. perfringens* in the intestines are exceptionally sensitive to trypsin, while other toxins made by this bacterium must be activated by trypsin or other proteases (2, 16–18). CPB, produced by *C. perfringens* type B and type C strains, is

very sensitive to endogenous trypsin degradation in the intestines of natural host animals (18, 19). In contrast, a major toxin of both type B and D strains, i.e., ETX, is produced as an inactive protoxin that must be proteolytically activated by intestinal proteases (such as trypsin, chymotrypsin, and carboxypeptidases) or, perhaps, proteases produced by *C. perfringens* (20–25). Until now, no information has been available regarding whether TpeL, which can be encoded by strains causing diseases originating in the intestines (26, 27), is trypsin sensitive.

Since TpeL is a recently identified toxin of *C. perfringens*, many of its features remain poorly understood, including the carriage of the *tpeL* gene among different *C. perfringens* types or subtypes, whether natural production levels of TpeL contribute to strain cytotoxicity, the timing of TpeL production, and TpeL sensitivity to trypsin. This study has addressed each of those important topics.

MATERIALS AND METHODS

Bacterial strains and growth media. Wild-type *C. perfringens* type A to E isolates used in this study are listed and described in Table 1. The toxin genotypes (A to E) and some phenotypic characteristics of these isolates were determined previously (23, 24, 26–34). All *C. perfringens* isolates used in this study were maintained as stock cultures in cooked meat medium (Oxoid) and stored at −20°C. All type E isolates were maintained as stock cultures in 15% glycerol and stored at −80°C. FTG medium (fluid thioglycolate medium; Difco Laboratories), TH medium (Bacto Todd Hewitt broth [Becton-Dickinson], with 0.1% sodium thioglycolate [Sigma-Aldrich]), TGY medium (3% tryptic soy broth [Becton-Dickinson] containing 2% glucose [Fisher Scientific], 1% yeast extract [Becton-Dickinson], and 0.1% thioglycolate [Sigma-Aldrich]), or TY medium (3% tryptic soy broth [Becton-Dickinson], 1% yeast extract [Becton-Dickinson], and 0.1% thioglycolate [Sigma-Aldrich]) was used to grow broth cultures of *C. perfringens*. *E. coli* strain TOP 10 was grown in Luria-Bertani (LB) broth and used for constructing toxin gene mutagenesis plasmids.

DNA extraction. Genomic DNA from *C. perfringens* was purified from overnight TGY broth cultures using the MasterPure Gram-positive DNA purification kit (Epicentre Biotechnologies, WI).

PCR analyses of *tpeL* carriage by *C. perfringens* strains. PCR was performed to evaluate *tpeL* gene carriage among *C. perfringens* isolates of different toxin types. The primers used for these PCR analyses were described previously (27). Template DNA for these short-range PCRs was obtained from colony lysates or purified genomic DNA from these strains. Each PCR mixture contained 2 μl of template DNA, 10 μl of 2× *Taq* master mix (New England BioLabs), 1 μl of each primer pair (1 μM final concentration), and 7 μl of H$_2$O. PCR conditions used for these amplifications included 94°C for 3 min and 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 90 s and a final extension for 10 min at 68°C. PCR products were run on a 1% agarose gel and stained with ethidium bromide for visualization.

PCR analyses to link the *cpb* and *tpeL* genes in type C strain CN3685. To evaluate a linkage between the *tpeL* and *cpb* genes in CN3685, overlapping PCR and long-range PCR were performed using previously described primers and conditions (26, 27). PCR products then were electrophoresed on a 1% agarose gel, which was stained with ethidium bromide for product visualization.

Southern blotting. Purified DNA (3 μg) was digested overnight with EcoRI at 37°C (New England BioLabs) and then separated by electrophoresis on a 0.8% agarose gel. The separated DNA fragments were transferred onto a positively charged nylon membrane (Roche) for hybridization with digoxigenin (DIG)-labeled specific intron or *tpeL* probes, which were prepared as previously described using primer pairs specific for intron or *tpeL* genes (27). To detect probe hybridization, CSPD substrate (Roche Applied Science) was utilized in accordance with the manufacturer's instructions.

Construction of CN3685::*tpeL* and CN3685::αβθ*tpeL* null mutants. To evaluate TpeL contributions to the cytotoxic properties of *C. perfringens* supernatants, the *tpeL* gene of type C isolate CN3685 was inactivated by the targeted insertion of a group II intron using the *Clostridium*-modified Targetron system (35). Briefly, the *tpeL* gene sequence was entered into the Sigma-Aldrich Targetron website. Three primers were generated to target insertion of an intron, in the sense orientation, into the *tpeL* open reading frame (ORF) between nucleotides 1426 and 1427. The primers used for PCR targeting of the intron were TpeL-IBS (5-AAAAA AGCTTATAATTATCCTTATATATCCAGCTGGTGCGCCCAGATAG GGTG-3), TpeL-EBS1d (5-CAGATTGTACAAATGTGGTGATAACAG ATAAGTCCAGCTGCTTAACTTACCTTTCTTTGT-3), TpeL-EBS2 (5-TGAACGCAAGTTTCTAATTTCGGTTATATATCGATAGAGGAAAG TGTCT-3), and the EBS universal primer provided in the kit (Sigma-Aldrich). The 350-bp PCR product was inserted into pJIR750ai to construct a *tpeL*-specific Targetron plasmid (pJIR750*tpeL*i). Mutant preparation and selection then were performed as described previously (35). Transformants were selected on brain heart infusion (BHI) agar plates containing 15 μg/ml of chloramphenicol, and colonies carrying an intron insertion were screened by PCR using primers *tpeL*-KOF (5-AATGTTTA TCTTCGGCTAT-3) and *tpeL*-KOR (5-TTTGAAAACTCCTTTACTG-3) for detecting the *tpeL* null mutation.

Each reaction mixture was subjected to the following PCR amplification conditions: cycle 1, 94°C for 5 min; cycles 2 through 35, 94°C for 30 s, 55°C for 30 s, and 68°C for 90 s; and a final extension for 10 min at 68°C. An aliquot (20 μl) of each PCR sample was electrophoresed on a 1.5% agarose gel and then visualized by staining with ethidium bromide.

The pJIR750*tpeL*i Targetron plasmid was similarly transformed into a previously constructed CN3685::αβθ mutant named BMC107 (19) to create the CN3685::αβθ*tpeL* null mutant. This mutant was selected and characterized as described above for the CN3685::*tpeL* mutant.

Reversal of the intron insertion to restore TpeL production by CN3685::*tpeL* mutant. A previously described procedure (18, 36) was used to partially restore TpeL production in the CN3685::*tpeL* strain. Briefly, this mutant was electroporated to receive the Targetron plasmid pJIR750*tpeL*i and then grown in TH medium (with 15 μg/ml chloramphenicol) for 8 h at 37°C. Those cultures then were transferred to a 30°C water bath for another 16 h of growth to facilitate excision of the intron insertion from some *tpeL* mRNA.

Preparation of anti-TpeL antibody. Two predicted antigenic peptides (peptide 1, CPGIKKHIFKDINKPT; peptide 2, CDSIQFDAIPEILKGK) from the translated *tpeL* DNA ORF were identified by bioinformatics analysis and then synthesized and purified by high-performance liquid chromatography (HPLC). The highly purified peptides then were conjugated to the carrier protein keyhole limpet hemocyanin (KLH). Serum against these TpeL antigenic peptides was raised in rabbits by the Pocono Rabbit Farm and Laboratory using the conjugated peptides as the immunogenic antigen. This immunization followed the standard protocol of Pocono Rabbit Farm and Laboratory under their approved IACUC permit (PRF2A). Briefly, the rabbits were injected subcutaneously with 200 μg of conjugated antigens per rabbit in complete Freund's adjuvant (CFA). After 2, 4, and 8 weeks, 50 to 100 μg of conjugated antigens mixed with incomplete Freund's adjuvant (IFA) were given subcutaneously. At day 91, rabbits were terminally bled from the carotid artery.

Western blotting of *C. perfringens* culture supernatants or cell lysates. *C. perfringens* culture supernatants or boiled lysates of washed *C. perfringens* cells were mixed with 5× loading buffer and electrophoresed on an SDS-containing 8% polyacrylamide gel for detecting TpeL or an SDS-containing 12% polyacrylamide gel for detecting other toxins. The separated proteins then were transferred onto a nitrocellulose membrane and the blot was blocked for 1 h with washing buffer (20 mM Tris-HCl [pH 8.0], 0.3 M NaCl, 0.5% [vol/vol] Tween 20) containing 5% (wt/vol) nonfat dry milk before incubation with primary TpeL antibody overnight at 4°C. Blots then were rinsed three times with washing buffer and incubated with rabbit anti-mouse IgG-horseradish peroxidase (HRP) or goat

TABLE 1 Description of wild-type *C. perfringens* strains used in this study

Strain type and name	Origin (reference)	*tpeL* PCR	*tpeL* Southern blotting
A			
JGS5369	Chicken necrotic enteritis (24)	+	+
JGS4143	Chicken necrotic enteritis (24)	−	−
JGS4140	Chicken necrotic enteritis (24)	+	+
F4396	Sporadic diarrhea (29)	−	−
B38	Antibiotic associated diarrhea (30)	−	−
ATCC3624	Gas gangrene (30)	−	−
F5603	Sporadic diarrhea (29)	−	−
PS16	Unknown, Centers for Disease Control and Prevention (31)	−	−
B2	Antibiotic associated diarrhea (30)	−	−
NCTC10239	Food poisoning (29)	−	−
NCTC8235	Food poisoning (30)	−	ND
NCTC8238	Food poisoning (30)	−	ND
NCTC8239	Food poisoning (29)	−	ND
NCTC8679	Food poisoning (30)	−	ND
NCTC8799	Food poisoning (30)	−	ND
NCTC8798	Food poisoning (30)	−	ND
NCTC8359	Food poisoning (30)	−	ND
FD1041	Food poisoning (30)	−	ND
042-03	Intestines of healthy North Americans (29)	−	ND
00-803	Intestines of healthy North Americans (29)	−	ND
168	Intestines of healthy North Americans (29)	−	ND
011-03	Intestines of healthy North Americans (29)	−	ND
B			
CN1795	Veterinary laboratory, toxigenic (27)	+	+
CN2416	Stomach, 5-day-old lamb (27)	+	+
CN677	Acute lamb dysentery (27)	+	+
CN684	Lamb dysentery (39)	+	+
CN1301	Lamb dysentery[a]	−	−
NCTC3110	Unknown (27)	+	+
NCTC8533	Lamb dysentery (27)	+	+
PS49	Unknown (27)	+	+
Bar6	Lamb dysentery (32)	−	−
Bar2	Sheep (27)	+	+
C			
CN882	Sheep with struck (26)	+	+
CN3955	Ewe peritoneal fluid (39)	+	+
CN3763	Animal (species unknown) (26)	−	−
Bar3	Human pigbel (26)	+	+
CN3685	Sheep with struck (39)	+	+
CN2065	Ewe with struck[a]	+	+
CN885	Veterinary laboratory (39)	−	−
CN5388	Human pigbel (39)	+	+
CN3708	Calf[a]	+	+
CN3727	Calf[a]	+	+
D			
CN462	Goat (28)	−	−
CN1634	Lamb with suspected dysentery (28)	−	−
CN3718	Guinea pig heart blood (23)	−	−
CN3842	Ewe, small intestine (28)	−	−
CN2068	Lamb stomach (28)	−	−
JGS1948	Caprine, enterotoxemia (61)	−	−
JGS1945	Caprine, Diarrhea (33)	−	−
JGS1240	Sheep, bronchopneumonia, and enterotoxemia (33)	−	−
NCTC8346	Sheep (34)	−	−
JGS1705	Ovine (33)	−	−

(Continued on following page)

TABLE 1 (Continued)

Strain type and name	Origin (reference)	tpeL PCR	tpeL Southern blotting
E			
NCIB10748	Unknown (50)	−	−
51	Neonatal calves, hemorrhagic enteritis (50)	−	−
294	Neonatal calves, hemorrhagic enteritis (50)	−	−
572	Neonatal calves, hemorrhagic enteritis (50)	−	−
576	Neonatal calves, hemorrhagic enteritis (50)	−	−
853	Neonatal calves, hemorrhagic enteritis (50)	−	−
1987	Neonatal calves, hemorrhagic enteritis (50)	−	−
B2085	Neonatal calves, hemorrhagic enteritis (50)	−	−

[a] The strain was originally from the collection of Russell Wilkinson.

anti-rabbit IgG-HRP (Sigma-Aldrich) for 1 h at room temperature. After three more washes, the blots were treated with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to X-ray film (Life Science Products) to detect the immunoreactive protein bands.

To quantify TpeL levels after different treatments or under different culture conditions, those Western blots were scanned and analyzed with NIH Image J software.

***C. difficile* toxin B Western blot analyses.** To analyze the time course of toxin B production and release by *C. difficile*, cooked meat medium stock cultures of *C. difficile* strains VPI 10463 (Robert Carman, TechLab) and 630 (ATCC) were inoculated into FTG, and those cultures were grown overnight at 37°C. A 0.2-ml aliquot of each starter culture then was passed into 10 ml of fresh TH medium (containing 0.1% sodium thioglycolate), and those cultures were grown at 37°C for 6, 24, or 48 h. Supernatants from each time point were collected and subjected to Western blot analyses using a rabbit polyclonal antiserum against the C-terminal domain of toxin B that was kindly supplied by Jimmy Ballard. The Western blots then were processed as described earlier for TpeL Western blots.

***C. perfringens* growth rate measurements.** To measure the culture growth rate of *C. perfringens* in this study, 0.2-ml aliquots of FTG overnight cultures were inoculated into 10 ml of TH, TY, or TGY and cultured at 37°C. At time intervals of 0, 2, 4, 6, 8, and 16 h, the culture was collected and mixed by gentle vortexing. The optical density at 600 nm (OD_{600}) then was recorded using a Bio-Rad Smartspec.

Preparation of samples for cytotoxicity experiments. Overnight (16-h) TH cultures of wild-type CN3685, mutants, or reversed mutants were centrifuged, and supernatants were filter sterilized using a 0.45-μm filter (Millipore). Those sterile supernatants then were concentrated 10-fold at 4°C using an Amicon Ultra centrifugal filter unit with an Ultracel-30 membrane (EMD Millipore), with buffer exchange to PBS (pH 7.4). Those concentrated supernatants then were diluted 1:10 using Hanks' balanced salt solution (HBSS; Corning) for cytotoxicity testing.

Trypsin treatment assay. To test whether TpeL is trypsin sensitive, processed TH culture supernatants of BMC107 containing TpeL were prepared as described above and then either treated at 37°C for 1 h with trypsin (Sigma-Aldrich) at the indicated concentrations (0, 0.04, 0.4, 4.0, 40, or 400 μg/ml) or treated with trypsin at a concentration of 12.5 μg/ml at 37°C for different times (0, 10, 20, or 60 min). After incubation, trypsin then was inactivated by adding an equal volume of trypsin inhibitor (3 to 6 mg of inhibitor ligand per ml gel; MP Biochemicals) at room temperature for 30 min. After centrifugation, the resultant supernatants were diluted 1:10 with HBSS and added to Vero cells to measure cytotoxicity (as described below) or for Western blot analyses (as described earlier).

***C. perfringens* supernatant cytotoxicity assay.** Vero cells were used for the *C. perfringens* supernatant toxin cytotoxicity assay based upon previous studies (6). Vero cells were maintained routinely in M199 (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS; Life Technologies), streptomycin (100 U/ml), and penicillin (100 μg/ml). Vero cells normally were harvested with 0.25% trypsin and 2.21 mM EDTA

(Gibco), resuspended in cell culture medium, and maintained at 37°C in a 5% CO_2 humidified atmosphere.

For assaying *C. perfringens* supernatant-induced cytotoxicity (morphological damage and quantitative cell death measurement), Vero cells were seeded into 12-well plates and incubated until confluent. For cell cytotoxicity experiments, the culture supernatant samples prepared as described earlier were added to the Vero cells for up to 5 h at 37°C.

The development of morphological changes, indicating cytotoxicity, was checked every hour using a Zeiss Axiovert 25 inverted microscope. After a 5-h treatment, the cytopathic effects caused by the various *C. perfringens* supernatants were photographed using a Canon Powershot G5 fitted to the Zeiss Axiovert 25 microscope. Images then were processed using Adobe Photoshop CS5.

Lactate dehydrogenase (LDH) release was measured to determine cell death, as described below.

Cytotoxicity neutralization assay. To help confirm whether the observed damage to Vero cells was caused specifically by the TpeL toxin present at natural concentrations in culture supernatants of CN3685 or derivatives, a serum neutralization approach was applied. Briefly, 50 μl of a neutralizing antiserum (described above) against TpeL toxin or preimmune serum was added to *C. perfringens* supernatant samples, prepared as described earlier, and those mixtures were incubated at room temperature for 15 min. The mixtures then were applied to Vero cells for 5 h at 37°C.

LDH release assay. To compare and quantitatively measure Vero cell cytotoxicity caused by different samples, the LDH release assay kit for mammalian cell death (Roche) was used. Vero cell monolayers were treated with different *C. perfringens* culture supernatant samples, prepared as described earlier, for 5 h at 37°C in a 5% CO_2 humidified atmosphere. Supernatants from the treated cell cultures were collected and used for measuring host cell LDH release. The absorbance of each sample then was measured at 490 nm with an iMark microplate reader (Bio-Rad). As described in the kit instructions, Vero cells treated with 1% Triton X-100 were used to determine maximal LDH release. The results are expressed as the percentage of maximal LDH release.

Statistical analysis. All values are expressed as means ± standard errors of the means (SEM). For statistical evaluations, one-way analysis of variance (ANOVA) with Tukey's *post hoc* test was performed, and P values of <0.05 were considered significant.

RESULTS

Evaluation of *tpeL* gene carriage in *C. perfringens*. Previous studies (24, 26, 27, 37) had detected the presence of the *tpeL* gene in some *C. perfringens* type A, B, and C isolates. To better understand the scope of *tpeL* gene carriage among *C. perfringens* isolates, the current study surveyed the presence of the *tpeL* gene in additional wild-type strains, including representatives of all *C. perfringens* toxigenic types and major pathogenic subtypes of type A.

PCR assays amplified products of the expected size, indicating

carriage of the *tpeL* gene, using DNA from only two of 22 surveyed type A strains with varied origins (Table 1). Both of those *tpeL*-positive type A strains were associated with avian necrotic enteritis. All four surveyed type A strains isolated from healthy North Americans, all four plasmid *cpe*-positive type A strains from non-food-borne human gastrointestinal diseases, and all nine surveyed chromosomal *cpe*-positive type A food poisoning strains tested *tpeL* negative. In contrast, eight of 10 surveyed type B or type C strains were *tpeL* positive (Table 1). Consistent with these PCR results, Southern blot experiments using a *tpeL*-specific probe confirmed the PCR results for *tpeL* gene carriage among the surveyed type A, B, and C strains of *C. perfringens* (Table 1). No *tpeL*-positive strain was detected by either PCR or Southern blot analyses among the surveyed *C. perfringens* type D and E strains (Table 1).

Western blot analyses of TpeL production and release from *C. perfringens* grown in different culture media. To compare culture medium effects on TpeL production and release into culture supernatants, *C. perfringens tpeL*-positive type A to C strains were grown in TH, TY, or TGY broth for 16 h. The supernatants of each culture then were collected and subjected to Western blot analyses using TpeL polyclonal antiserum. Results shown in Fig. 1A demonstrate that TH is consistently the best medium for supporting TpeL production and extracellular release, while TpeL production and release is consistently lowest in TGY. In order to determine whether the varied TpeL production and release observed in different culture media was due simply to effects on bacterial growth, growth curve experiments were performed. Measurement of culture OD$_{600}$ revealed similar growth rates for the strains when cultured in different media (Fig. 1B).

TpeL production begins during early-log-phase growth and peaks during early-stationary-stage growth in TH medium. Most toxins produced by *C. perfringens* are maximally expressed within ~10 h, i.e., during late-log or early-stationary growth phases (1, 2, 4, 38–40). However, other LCTs (such as *C. difficile* toxin A and toxin B) usually are maximally produced and released extracellularly after 24 h, i.e., at the late stationary stage of growth (11, 41, 42). Figure 2A confirms those previous conclusions for the timing of toxin B production and extracellular release by two *C. difficile* strains. These *C. difficile* cultures entered stationary phase after ~8 h (data not shown).

To begin evaluating the kinetics of TpeL production and extracellular release, several *tpeL*-positive *C. perfringens* strains were grown in TH medium, and supernatants from those cultures then were collected at 6 to 48 h for TpeL Western blot analyses. As shown in Fig. 2B, high supernatant levels of TpeL were detected when these *tpeL*-positive strains were grown in TH culture medium for 6 to 12 h, which corresponds to late-log-phase or early-stationary-phase growth (Fig. 1B).

To further determine the time course of TpeL production and release, CN3685 was grown in TH medium, and culture supernatants were collected at even earlier time points for Western blot analyses. As shown in Fig. 2C, TpeL was not detectable in 2-h TH culture supernatants but became readily apparent in 4-h TH culture supernatants. For CN3685, peak TpeL levels in culture supernatants were reached within ~8 h. Note that only limited degradation of extracellular TpeL was detectable in Fig. 2C, as well as in other experiments performed in this study (not shown).

To specifically examine the onset of TpeL production, washed CN3685 cells from TH cultures were lysed and subjected to TpeL
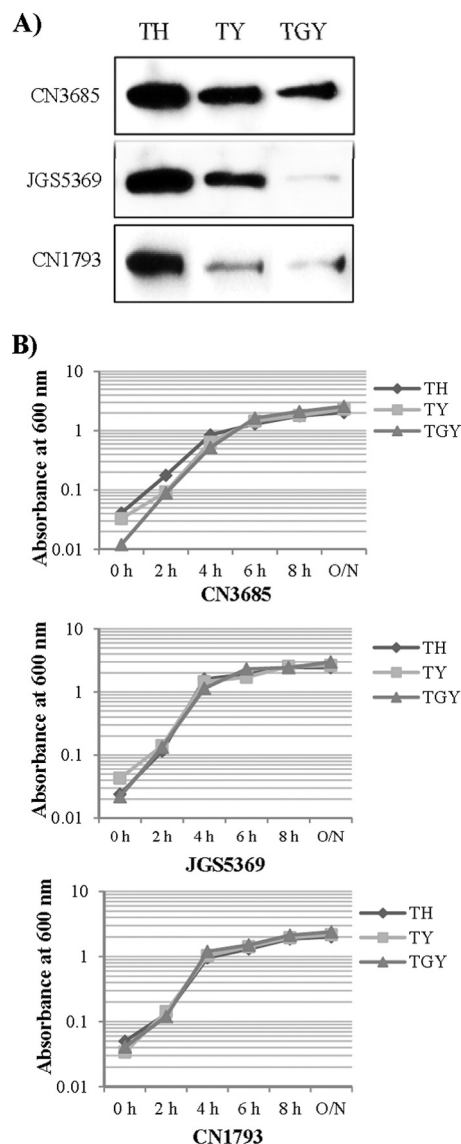

FIG 1 Comparison of TpeL production and extracellular release by *C. perfringens* growing in different culture media. (A) Western blot analyses of TpeL in culture supernatants from cultures of *C. perfringens* type A strain JGS5369, type B strain CN1793, and type C strain CN3685 grown for 16 h in TH, TY, or TGY. The immunoreactive protein shown is ~200 kDa, consistent with the expected mass of TpeL. Results shown are typical of three repetitions. (B) Comparison of growth characteristics of wild-type strains JGS5369, CN1793, and CN3685 at 37°C. The optical density (OD$_{600}$) of each strain growing in TH, TY, or TGY culture medium was measured using a Bio-Rad Smartspec spectrophotometer. A typical result of three repetitions is shown.

Western blotting. This analysis detected a TpeL signal within 2 h (Fig. 2C). Also notable on these blots was the substantial degradation of intracellular TpeL, with this degradation increasing with longer culture time. Considered collectively, the results shown in Fig. 2C indicated that, after production in the cytoplasm of *C. perfringens*, there is a delayed release of TpeL from the bacterium by a still-unknown mechanism.

As a final evaluation of the time course of TpeL production and extracellular release by *C. perfringens*, late-stationary-phase (72 h) culture supernatants of three *tpeL*-positive strains (type A strain
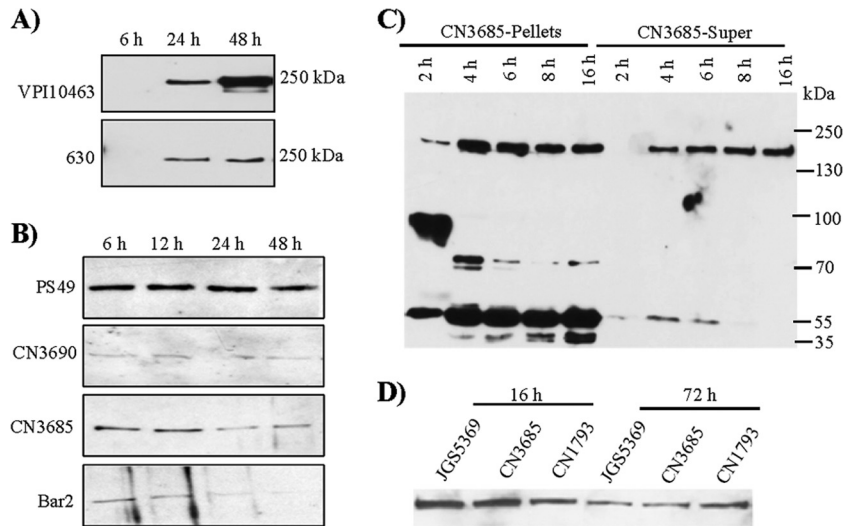
FIG 2 Western blot time course analyses of TpeL production and extracellular release. (A) Western blot analyses of toxin B levels in supernatants from cultures of *C. difficile* strains VPI 10463 and 630 grown at 37°C for 6, 24, or 48 h in TH. (B) Western blot analyses of TpeL levels in supernatants from cultures of *C. perfringens* strains PS49, CN3690, CN3685, and Bar2 grown for 6, 12, 24, or 48 h in TH. (C) Western blot analyses of TpeL levels in supernatants (Super) or washed and lysed cells (Pellets) of CN3685 cultures grown for 2, 4, 6, 8, or 16 h in TH. (D) Comparison of TpeL levels in 16-h or 72-h supernatants from TH cultures of JGS5369, CN3685, and CN1793. The immunoreactive protein shown is ~200 kDa, consistent with the expected mass of TpeL. Typical results from three repetitions are shown.

JGS5369, type B strain CN1793, and type C strain CN3685) also were tested by TpeL Western blot analyses. Those analyses showed that TpeL levels present in supernatants of 72-h TH cultures were less than those present in 16-h TH culture supernatants (Fig. 2D). Together, the results shown in Fig. 2 indicated that supernatant TpeL levels usually become maximal during the late log or early stationary stages of growth, which is similar to most other toxins produced by *C. perfringens*, rather than in late stationary phase, as for other LCTs.

Glucose and sucrose repress TpeL supernatant levels. Previous studies reported that production and release of LCTs often is repressed by glucose (43, 44). Another recent study demonstrated that sucrose, as well as glucose, inhibits the production of *C. perfringens* PLC (alpha-toxin) and PFO (theta-toxin) (45). Our Fig. 1A results detecting higher TpeL supernatant levels in TY (containing a basal sugar concentration of 0.25%) versus TGY (TY medium with an additional 2% glucose) broth are consistent with a recent report (46) that glucose also represses TpeL production and release by *C. perfringens* strain JIR12688, despite that strain maximally producing and releasing TpeL during late stationary phase, in contrast to our surveyed strains, where TpeL production starts early in log phase (Fig. 2).

To definitively evaluate if glucose also depresses TpeL supernatant levels for strains that maximally express this toxin during late-log or early-stationary-phase growth, *C. perfringens* was cultured in TY supplemented with different concentrations of glucose (0, 5, 10, 20, and 40 mg/ml). As shown in Fig. 3A, TpeL supernatant levels in an 8-h culture were significantly decreased by the addition of supplemental glucose. A similar reduction in TpeL supernatant levels also was observed using supplemental sucrose (Fig. 3B).

When quantified from three independent Western blot analyses, the data obtained (Fig. 3C) detected statistically significant different TpeL supernatant levels in the presence or absence of

supplemental glucose and sucrose. Measurement of changes in culture OD_{600} values over time revealed no differences in growth between cultures supplemented with ≤40 mg/ml concentrations of glucose or sucrose for these *C. perfringens* strains, indicating that the decreased TpeL supernatant levels shown in Fig. 3A and B were not from growth inhibition due to increasing sugar concentrations in the TY culture medium. However, higher supplemental sugar concentrations, i.e., those ranging from 60 to 80 mg/ml, did retard *C. perfringens* growth (data not shown).

Construction and characterization of a *tpeL* single null mutant and a *cpa, cpb, pfoA, tpeL* quadruple null mutant in CN3685. Recent studies showed that arbitrarily chosen amounts of purified TpeL cause cytotoxicity (6). To specially evaluate if, at natural levels present in culture supernatants, TpeL also can contribute to strain-induced cytotoxicity, the *tpeL* gene in *C. perfringens* type C animal disease strain CN3685 was insertionally inactivated with the *Clostridium*-modified Targetron insertional mutagenesis system (35). Construction of the *tpeL* isogenic null mutant of CN3685 was verified by PCR analyses using primers specific to *tpeL* sequences located upstream and downstream of the intron insertion site. These internal *tpeL* PCR primers specifically amplified a PCR product of ~600 bp from wild-type CN3685 DNA. However, the same primer pair amplified an ~1,500-bp PCR product from the DNA of the putative *tpeL* null mutant due to the insertion of a 900-bp intron into the *tpeL* ORF (Fig. 4A). Southern blot analyses using an intron-specific probe demonstrated the presence of a single intron insertion in the CN3685::*tpeL* mutant (Fig. 4B). As expected, no intron signal was detected using wild-type DNA from CN3685 (Fig. 4B).

The *tpeL* gene proved difficult to clone into a shuttle plasmid for complementation (data not shown). Instead, TpeL production by the CN3685::*tpeL* mutant was partially restored via a commonly used, LtrA-mediated trans-splicing approach (18) that removes the inserted intron from some *tpeL* mRNA, resulting in a
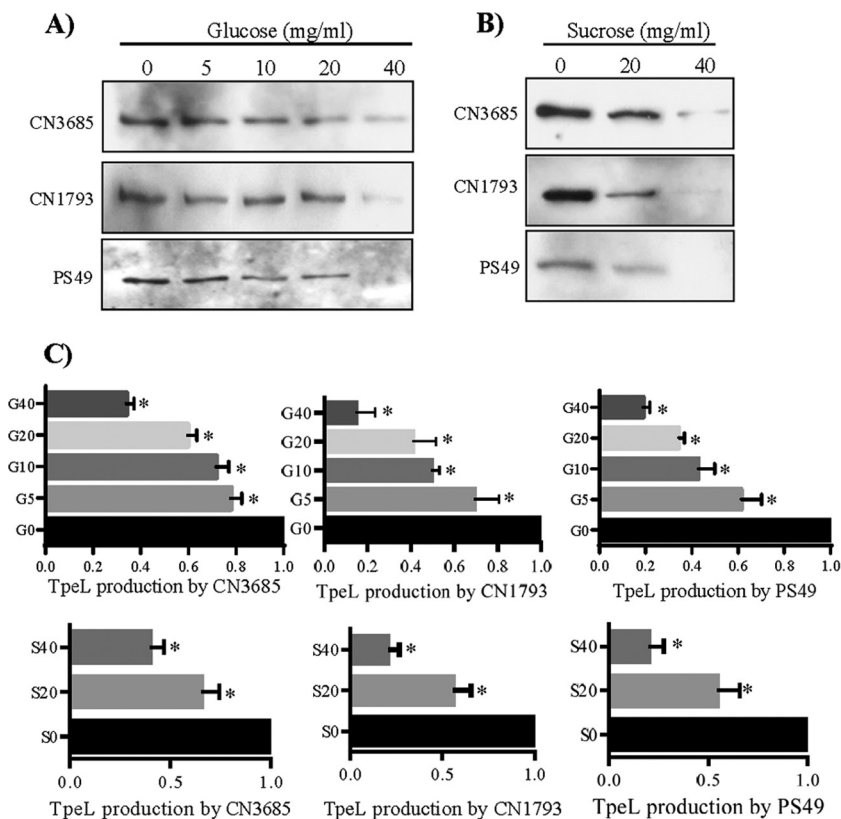
FIG 3 Comparison of TpeL supernatant levels for *C. perfringens* cultures grown in TY medium supplemented with different concentrations of glucose or sucrose. (A) Western blot analyses of TpeL levels in supernatants from 8-h TY cultures, which had been supplemented with glucose at the indicated concentrations of CN3685, CN1793, and PS49. Supernatants from matching TY cultures with no supplemental glucose were used as controls. (B) Western blot analyses of TpeL levels in supernatants from 8-h TY cultures, which had been supplemented with sucrose at the indicated concentrations of CN3685, CN1793, and PS49. Matching TY cultures with no supplemental sucrose were used as controls. The immunoreactive protein shown in panels A and B is ~200 kDa, consistent with the expected mass of TpeL. (C) Quantitative analysis of TpeL supernatant levels from 8-h TY cultures that had been supplemented with different concentrations of glucose (G) or sucrose (S). An asterisk indicates a statistically significant ($P < 0.05$) decrease of TpeL production in TY supplemented with glucose or sucrose compared to similar cultures without any carbohydrate supplementation. Shown are mean values ± standard errors of the means (SEM) based on three repetitions.

partial regain of TpeL translation. For this purpose, the plasmid pJIR750*tpeL*i-sense, which encodes a functional LtrA protein, was reintroduced into the CN3685::*tpeL* mutant. The resultant TpeL reversed mutant was grown at 30°C in the presence of antibiotics to maintain selective pressure for producing maximal LtrA protein, as required for splicing-induced intron removal. Western blot results showed that TpeL production was absent from the CN3685::*tpeL* mutant, while the reversed mutant exhibited partially restored TpeL production (Fig. 4C). This result also demonstrated that the anti-TpeL antibody prepared in this study specifically reacts with the TpeL protein.

C. perfringens type C strain CN3685 produces four known toxins, including CPA, CPB, PFO, and TpeL (18 and this study). To evaluate TpeL-induced cytotoxicity in the absence of any cytotoxic influence from other toxins, a strain producing only TpeL was constructed by using the Targetron procedure described above. For this purpose, the *tpeL* gene was insertionally inactivated in BMC107, which is a previously constructed CN3685 triple null mutant that does not produce CPA, CPB, or PFO (19). The genotype of this quadruple mutant, named the CN3685::αβθ*tpeL* mutant, was confirmed by PCR (data not shown). The *tpeL* gene in CN3685 is located 3 kb downstream of its *cpb* gene (Fig. 5A), which explains why Southern blots only detected three

intron signals using DNA from the CN3685::αβθ*tpeL* mutant (Fig. 5B). Long-range PCR confirmed the *cpb-tpeL* linkage in CN3685 using one primer to internal *tpeL* ORF sequences and a second primer to internal *cpb* ORF sequences (Fig. 5C). An overlapping PCR further demonstrated (Fig. 5D) this genetic linkage using a five-pair set of primers described in our previous studies (26, 27). Western blot analyses confirmed that the CN3685::αβθ*tpeL* mutant does not produce PFO, CPA, CPB, or TpeL.

Vero cell cytotoxicity caused by wild-type CN3685 and CN3685::*tpeL* mutant supernatants. To evaluate if, at natural production levels, TpeL can contribute to the cytotoxicity of *C. perfringens* supernatants, the CN3685 *tpeL* null mutant was tested for its cytotoxic effects on Vero cells. For this purpose, the TH culture supernatants of wild-type CN3685 and CN3685::*tpeL* and reversed mutants were collected, filter sterilized, concentrated 10-fold with buffer exchange, and then diluted 1:10 into HBSS. To further identify whether the cytotoxic consequences of CN3685 supernatants involved TpeL, an anti-TpeL neutralizing antiserum was added to some supernatant samples prior to the Vero cell cytotoxicity assay.

When the processed culture supernatants from wild-type CN3685, the CN3685 *tpeL* null mutant, or the reversed mutant were applied to Vero cells for 5 h, the culture supernatant samples
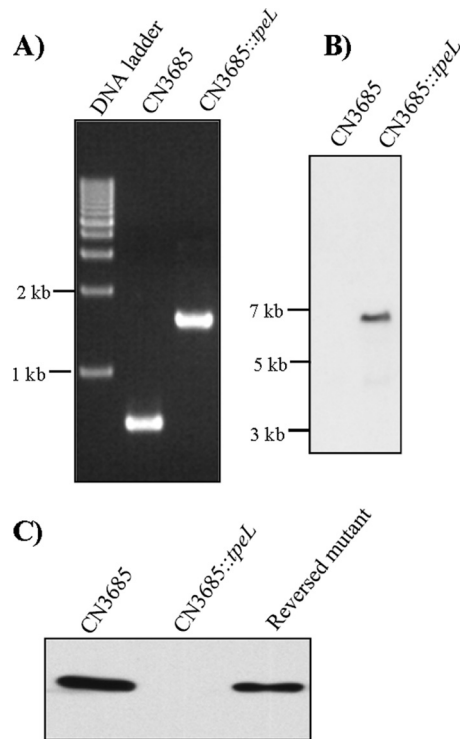
FIG 4 Construction of a CN3685 *tpeL* null mutant by intron-based insertional mutagenesis. (A) PCR analyses using DNA from wild-type CN3685 or the isogenic *tpeL*-null mutant (CN3685::*tpeL*). (B) Southern blot hybridization of wild-type CN3685 and the CN3685::*tpeL* mutant. DNA from each strain was digested with EcoRI, electrophoresed on a 0.8% agarose gel, and transferred onto a nylon membrane. The separated DNA then was hybridized with a DIG-labeled intron-specific probe. Sizes of DNA fragments in kilobases (kb) are shown on the left. (C) Western blot analyses for TpeL production by wild-type CN3685, the CN3685::*tpeL* mutant, or reversed mutant strains. The immunoreactive protein shown is ~200 kDa, consistent with the expected mass of TpeL.

from wild-type CN3685 caused significant Vero cell damage, including cell rounding, cell aggregation, and, eventually, cell detachment from the confluent monolayers (Fig. 6A). In contrast, morphological cell damage to Vero cells was visibly weaker, and cell detachment was absent, using similarly prepared culture supernatant from the CN3685 *tpeL* null mutant strain. Restoring some TpeL production to the *tpeL* null mutant by partially reversing the intron insertion in *tpeL* mRNA caused an increase in supernatant cytotoxic activity for Vero cells, which supported the attenuated cell damage caused by supernatants from the *tpeL* null mutant as being attributable to inactivation of *tpeL* gene expression (Fig. 6A). Furthermore, preincubating wild-type CN3685 culture supernatants with the TpeL-neutralizing antiserum further confirmed that much of the observed cytotoxicity activity was caused by TpeL toxin. Specifically, preincubation of processed CN3685 supernatant with TpeL-neutralizing antibody attenuated and delayed the development of morphological damage, while a similar preincubation with preimmune serum had little or no protective effects (Fig. 6A).

Lactate dehydrogenase (LDH) release from Vero cells then was measured to quantify the cytotoxicity of these processed culture supernatants (Fig. 6B). Cell death caused by wild-type CN3685 or the reversed mutant strains was significantly higher than that

caused by the *tpeL* null mutant. The lower cell death caused by the reversed mutant versus the wild-type strains is consistent with results shown in Fig. 4C, indicating that reversal of the *tpeL* mutation was partial and did not completely restore TpeL production, as is typical for this approach (18, 47). When the processed culture supernatants were preincubated with the TpeL-neutralizing antibody, cytotoxicity caused by the wild-type supernatant samples decreased significantly; in contrast, preincubation with preimmune serum did not affect cytotoxicity of this sample, confirming a substantial role for TpeL in the cytotoxic activity of these culture supernatants (Fig. 6B).

Glucose decreases the cytotoxic activity of BMC107 culture supernatants. As shown in Fig. 3, TpeL production and release by several *C. perfringens* strains, including CN3685, is repressed by either glucose or sucrose. Therefore, we assessed whether these sugars also modulate the cytotoxic effects of processed culture supernatants obtained from the TY cultures of TpeL-only-producing strain BMC107. After Vero cells were treated for 5 h with culture supernatant samples containing different concentration of glucose, LDH release from dead or damaged cells was measured. As shown in Fig. 7, this analysis detected less cytotoxicity with increasing glucose concentrations in these processed TY culture supernatants. This result further confirmed that glucose and sucrose not only represses TpeL levels in culture supernatants but also can have consequences for cytotoxicity.

TpeL is a trypsin-sensitive toxin. TpeL-producing type B and C strains, such as CN3685, cause diseases originating in the intestines (1), where secreted TpeL could contact proteases like trypsin. Therefore, the TpeL protein sequence was entered into the Expasy software program (http://web.expasy.org/peptide_cutter/) to predict potential TpeL cleavage sites by proteases. Approximately 200 potential trypsin cut sites were identified by this software in the full-length TpeL protein. To test definitively whether TpeL is a trypsin-sensitive toxin, processed culture supernatants of BMC107 were treated for 1 h at 37°C with different concentrations of purified trypsin. As indicated by Western blotting results shown in Fig. 8A, these trypsin-treated TpeL-containing supernatants contained a decreased amount of TpeL upon Western blot analysis. When similar TpeL-containing supernatants were treated with a single dose of trypsin (12.5 μg/ml) at 37°C for different times, the TpeL Western blot signal intensity decreased with longer trypsin treatment times (data not shown).

To evaluate the cytotoxic consequences of TpeL cleavage by trypsin, processed culture supernatant from BMC107 was treated with different concentrations of trypsin. After a 1-h treatment, remaining trypsin in these samples was inactivated by the addition of trypsin inhibitor. When these trypsin-treated supernatants were applied to Vero cells, morphological cell damage was much weaker and no cell detachment was observed compared to results for non-trypsin-treated supernatant samples (data not shown). As a control, processed culture supernatants of the CN3685::αβθ*tpeL* quadruple null mutant caused only very weak cell damage in this experiment, even without trypsin treatment (data not shown).

LDH release was quantitatively measured to evaluate the cytotoxic activity of processed supernatants that were or were not trypsin treated (12.5 μg/ml of trypsin). Cell death was much lower in Vero cells challenged with trypsin-treated supernatant. These results confirmed that the reduced cell death caused by the trypsin-treated supernatant samples is attributable to TpeL activity being destroyed by trypsin treatment (Fig. 8B). As a control for this
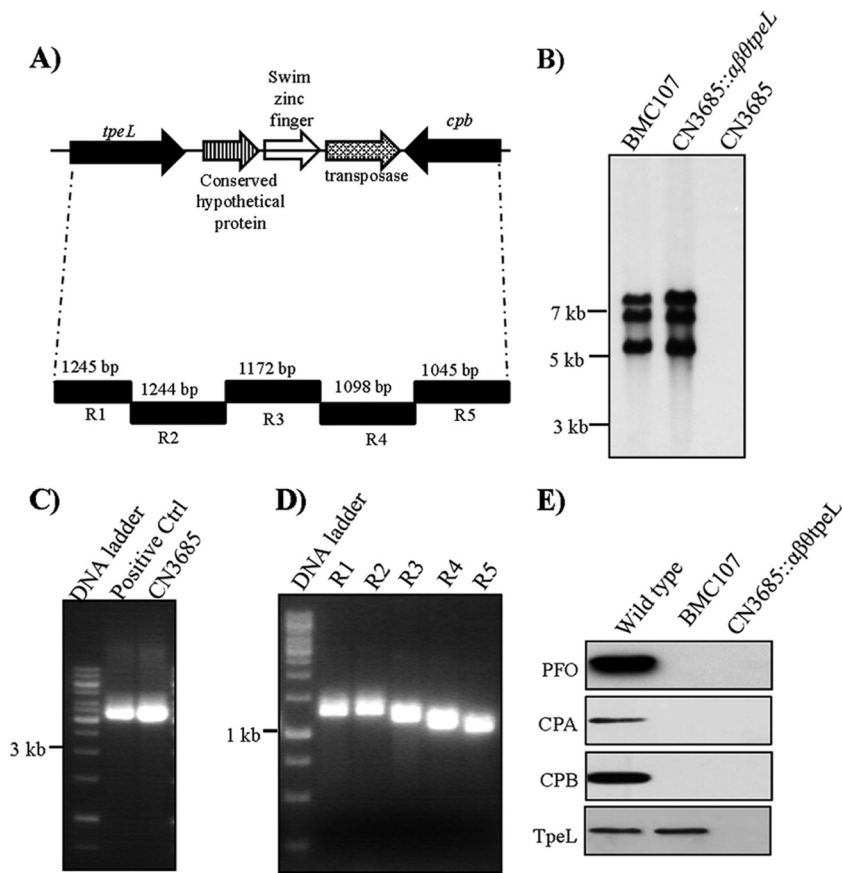
FIG 5 Construction of the quadruple *cpa*, *cpb*, *pfoA*, *tpeL* null CN3685::αβθ*tpeL* mutant by intron-based insertional mutagenesis. (A) Arrangement of the *cpb* and *tpeL* gene locus in *C. perfringens* type C strain CN3685 based upon previous studies (26, 27). (B) Southern blot hybridization of wild-type CN3685, the TpeL-producing triple toxin mutant BMC107, and the CN3685::αβθ*tpeL* quadruple null mutant. Sizes of DNA fragments in kilobases are shown to the left. (C) Long-range PCR analyses linking the *tpeL* and *cpb* genes in CN3685. (D) Overlapping PCR analyses of the region extending from *cpb* to *tpeL* in CN3685 using primers R1 to R5. (E) Western blot analyses of CPA, CPB, PFO, and TpeL levels in 16-h TH culture supernatants of wild-type CN3685, the TpeL-producing triple toxin mutant BMC107, and the CN3685::αβθ*tpeL* quadruple null mutant. The immunoreactive proteins in panel E matched the expected masses, as relevant, of PFO (~55-kDa), CPA (~47 kDa), CPB (~35 kDa), and TpeL (~200 kDa).

experiment, little cell damage was measured in Vero cells challenged with the processed supernatant from CN3685::αβθ*tpeL* mutant cultures; this result also supports conclusions from Fig. 7 that TpeL contributes to natural CN3685 cytotoxicity.

Lastly, to evaluate the cytotoxic effects of the trypsin-pretreated samples shown in Fig. 8A, various concentrations of the trypsin-treated supernatant samples were applied to Vero cells after removal of residual trypsin activity by the addition of trypsin inhibitor. The percentage of Vero cell death caused by the non-trypsin-treated sample was more than 65%, while Vero cell death resulting from challenge with different concentrations of trypsin-treated supernatants decreased from 49.6% to 12.8% as the trypsin concentration used for supernatant treatment increased (Fig. 8C).

DISCUSSION

This study provides several important insights into *tpeL* carriage, as well as the production and release, cytotoxic contributions, and trypsin sensitivity of the TpeL protein. With respect to *tpeL* carriage, the current results are consistent with previous reports indicating that many type C strains, and nearly all type B strains, carry this toxin gene, which is often (but not always) located in

these strains near the *cpb* gene on plasmids of ~90 kb or ~60 to 65 kb (26, 27). The current results also detected the *tpeL* gene in two type A avian necrotic enteritis strains, both *netB* positive (data not shown); this finding is consistent with previous reports suggesting that TpeL-positive strains often are associated with avian necrotic enteritis, although NetB toxin is considered to play the major role in pathogenesis (24, 48). However, the *tpeL* gene was not detected in other type A strains, including enteropathogenic type A strains carrying either a chromosomal or plasmid-borne *cpe* gene. To date, the copresence of both *tpeL* and *cpe* genes has never been detected in any single *C. perfringens* strain. One potential explanation for this situation involves incompatibility between the plasmids carrying *tpeL* and those carrying the *cpe* gene. If this is correct, it would still remain unclear why type A chromosomal *cpe* strains also rarely, if ever, carry the *tpeL* gene.

When the current study similarly evaluated carriage of the *tpeL* gene among type D and E strains of *C. perfringens*, none of the surveyed isolates carried this toxin gene. Again, this pattern could be due to incompatibility issues between *tpeL*-carrying plasmids and the plasmids carrying *etx* genes in type D strains or iota toxin genes (*itxA* and *itxB*) in type E strains. If, as proposed in the preceding paragraph, *tpeL* plasmids generally are incompatible with
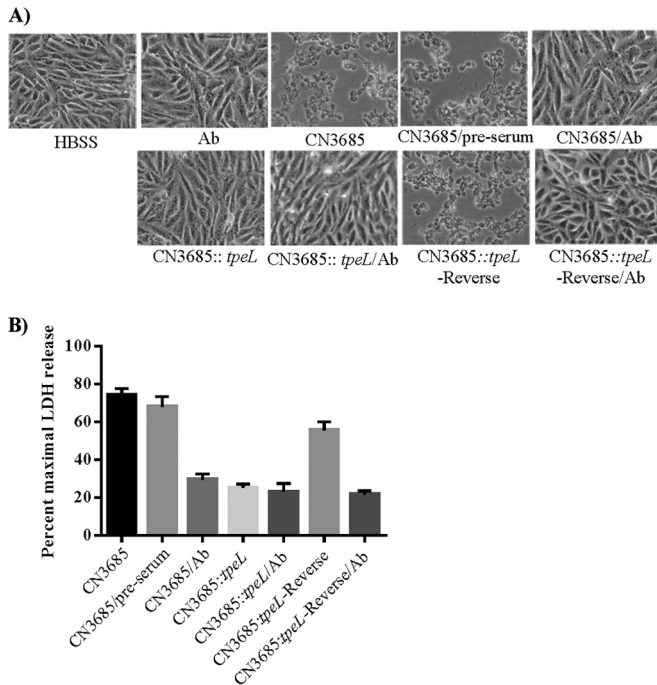
A)

B)

FIG 6 Vero cell cytotoxicity caused by processed supernatants for cultures of wild-type CN3685 and the CN3685::tpeL mutant. (A) Morphological damage to Vero cells. Processed (see Materials and Methods) 16-h TH culture supernatants from wild-type CN3685, CN3685::tpeL, or the reversed mutant were tested for their cytotoxic effects after either preincubation with preimmune serum (preserum) or TpeL neutralizing serum (Ab), as indicated. HBSS shows the effect of buffer alone. (B) Lactate dehydrogenase (LDH) release by Vero cells after treatment with different samples described in panel A. Cytotoxicity caused by processed culture supernatant from the CN3685::tpeL mutant shows a statistically significant ($P < 0.05$) decrease compared to that of Vero cells treated with similarly processed samples, including wild-type CN3685 culture supernatant, wild-type CN3685 culture supernatant preincubated with anti-TpeL serum, or culture supernatant of the CN3685::tpeL reverse complement strain. The experiment was repeated three times, and results shown are the mean values. Error bars represent SEM.
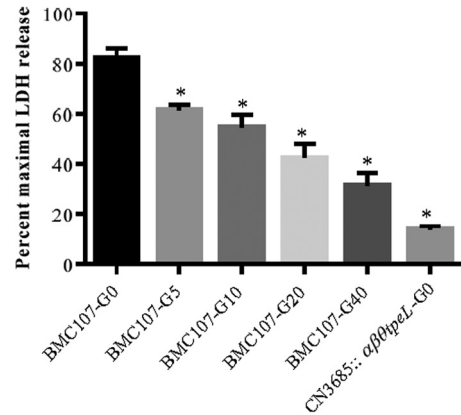

FIG 7 Glucose affects the cytotoxicity caused by supernatants from BMC107 cultures. Lactate dehydrogenase (LDH) release from Vero cells after treatment with processed (see Materials and Methods) supernatants from 8-h cultures of BMC107 grown in TY supplemented with different glucose concentrations. The experiment was repeated three times, and results shown are the mean values. Error bars represent SEM. An asterisk shows a statistically significant difference ($P < 0.05$) between a sample without glucose supplementation (BMC107-G0) and samples supplemented with different concentrations of glucose. G0, G5, G10, G20, and G40 represent different concentrations of glucose supplementation in TY culture.

cpe plasmids, this may help to explain the absence of tpeL plasmids from many type E strains, since iota toxin plasmids often are related to cpe plasmids, including their carriage of cpe genes or silent cpe sequences (49–51). While the presence of plasmids carrying tpeL (and cpb) genes in type B strains indicates that not all etx plasmids are incompatible with tpeL plasmids, it is noteworthy that only a single ~65-kb etx plasmid has been detected in type B strains (27, 52). In contrast, a diversity of etx plasmids exists among type D strains (28), and it has been proposed that many of those plasmids are incompatible with cpb plasmids, since they are never found in type B strains (27, 52). Therefore, it is possible that the tpeL and cpb plasmids found in type B strains are not compatible with the etx plasmids present in many type D strains.

A recent report (46) indicated that, as for other LCTs, glucose represses TpeL levels in culture supernatants of C. perfringens strain JIR12688, which produces and releases TpeL during late stationary phase. The current study now extends that finding by showing this relationship also holds true for those C. perfringens strains producing TpeL during the log or early stationary phase of growth (see the next paragraph). Furthermore, our results indicated that TpeL production and release by early TpeL-producing strains also is repressed in the presence of sucrose. Thus, tpeL

expression, as well as production of CPA and PFO (45), appears to be regulated by both glucose and sucrose catabolite repression in most or all TpeL-positive C. perfringens strains. While the mechanism of this repression in C. perfringens requires further study, it is notable that the C. difficile literature reports that, under nutrient-rich conditions, CodY represses TcdA and TcdB production (53). This may be relevant for understanding the regulation of TpeL production, since C. perfringens also produces a functional CodY protein (54).

There have been discrepancies in the literature regarding the timing of TpeL production by C. perfringens. A recent study reported that, like TcdA and TcdB production by C. difficile, C. perfringens strain JIR12688 produces TpeL during late stationary phase, with maximal production at 72 h (46). However, an earlier study detected tpeL mRNA expression in 6-h cultures of 5 different type C strains (26). In the current study, Western blot kinetic analyses clearly showed that TpeL production by the surveyed C. perfringens strains can begin within 2 h and becomes maximal during the late log or early stationary phase of growth. Therefore, there appears to be heterogeneity in the timing of TpeL production and release among C. perfringens strains, with many or most strains producing and releasing TpeL during the late log or early stationary growth phase (this study), although at least one strain (JIR12688) produces and releases this toxin primarily during late stationary phase (46). The explanation for these timing differences will require further study, but it is notable that JIR12688 appears to be an unusually slow-growing C. perfringens strain (46). It is possible that this growth defect reflects a metabolic difference that impacts the timing of TpeL production and release by this strain.

Previous studies have shown that TpeL-containing supernatants (46), or arbitrarily chosen amounts of purified TpeL (6), are cytotoxic for cultured mammalian cells. By using toxin mutants, the current study now specifically establishes that, at natural production levels, TpeL can contribute to the cytotoxic properties of
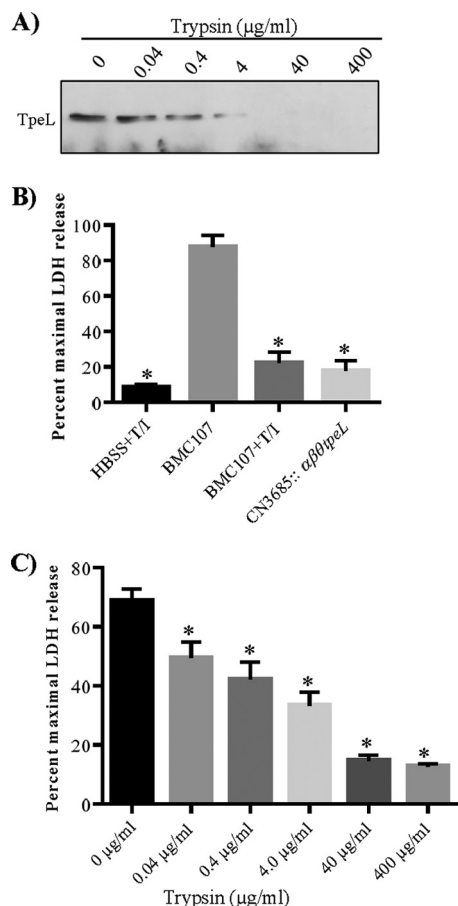
FIG 8 TpeL is sensitive to trypsin. (A) Processed (see Materials and Methods) TpeL-containing culture supernatant from BMC107 was treated with trypsin at the indicated concentration for 1 h at 37°C. Trypsin (T) activity then was removed from the samples by trypsin inhibitor (I) prior to TpeL Western blot analyses. The immunoreactive protein shown is ~200 kDa, consistent with the expected mass of TpeL. (B) Quantitative Vero cytotoxicity (measured by LDH release) of supernatants from BMC107 treated with trypsin and trypsin inhibitor (T/I). HBSS buffer similarly treated with T/I was used as a trypsin treatment control, and the processed supernatant from the CN3685::αβθtpeL mutant (no T/I treatment) was used as a TpeL-negative control. An asterisk shows a statistically significant ($P < 0.05$) decrease in LDH cytotoxicity caused by the samples treated with T/I compared to sample without this treatment. (C) Cytotoxic effects were evaluated after treatment of processed supernatants with different trypsin concentrations. LDH release was used to quantitatively evaluate cytotoxicity. An asterisk shows a statistically significant difference ($P < 0.05$) compared to the value for BMC107. The error bars represent SEM calculated from three independent experiments from panels B and C.

supernatants from cultures of TpeL-producing *C. perfringens*. This finding suggests potential TpeL contributions to virulence, but this hypothesis still must be tested in animal models. It is notable that some isogenic toxin mutants of the same CN3685 strain used for constructing mutants in the current study have been tested previously for virulence in animal models (18, 19). Those studies, conducted before the discovery of TpeL, showed that CPB production is required for this strain to cause hemorrhagic necrotic enteritis in rabbit small intestinal loops and is also a major contributor to enterotoxemic lethality in a mouse oral challenge model. In contrast, loss of either CPA or PFO production had no discernible effect on CN3685 pathogenicity in the small intestinal loop model and caused only a slight attenuation in

the lethality of this strain in the mouse enterotoxemia model (18, 19). While those previous studies established the importance of CPB for CN3685 virulence, they do not necessarily exclude TpeL contributions to the virulence of this strain. Of note, the earlier studies had challenged animals with TGY cultures of CN3685, which (as now shown in the current study and the study by Carter et al. [46]) is not the most favorable culture medium for TpeL production and release. Alternatively, it is possible that TpeL contributes additively or synergistically to CPB action, similar to the recent discovery of CPB and CPE synergism for the enteric pathogenicity of some TpeL-negative type C strains (47).

Finally, the current study examined whether TpeL is sensitive to trypsin, which is relevant since (i) during intestinal infections, this toxin should be produced by TpeL-positive type B and C strains and (ii) there is considerable variability in the effects of trypsin on *C. perfringens* toxins, with some (i.e., ETX, CPE, and ITX) toxins being activated by trypsin (41, 55–57), while others (i.e., CPB and beta2 toxin) are inactivated by trypsin (58, 59). Our findings clearly demonstrated that TpeL is trypsin sensitive, like *C. difficile* toxin B (60), which is another LCT. Demonstrating that TpeL is trypsin sensitive does not diminish the potential importance of this toxin for disease, since TpeL is produced mainly by type B and C strains. Those strains often cause disease in hosts with reduced intestinal trypsin levels due to age, nursing, diet, or other factors (1).

Collectively, these findings provide important new insights into TpeL, an unusual LCT. Future studies will investigate the activity of TpeL *in vivo* and the regulation of production of this toxin.

ACKNOWLEDGMENTS

REFERENCES

1. **McClane BA, Uzal FA, Miyakawa MF, Lyerly D, Wilkins TD.** 2006. The enterotoxic Clostridia, p 688–752. *In* Dworkin M, Falkow S, Rosenburg E, Schleifer H, Stackebrandt E (ed), The prokaryotes, 3rd ed. Springer Press, New York, NY.
2. **Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, Rood JI, McClane BA.** 2013. Toxin plasmids of *Clostridium perfringens*. Microbiol Mol Biol Rev **77:**208–233. http://dx.doi.org/10.1128/MMBR.00062-12.
3. **Petit L, Gibert M, Popoff MR.** 1999. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol **7:**104–110. http://dx.doi.org/10.1016/S0966-842X(98)01430-9.
4. **Chen J, Ma M, Uzal FA, McClane BA.** 2014. Host cell-induced signaling causes *Clostridium perfringens* to upregulate production of toxins important for intestinal infections. Gut Microbes **5:**96–107. http://dx.doi.org/10.4161/gmic.26419.
5. **McClane BA, Robertson SL, Li J.** 2013. *Clostridium perfringens*, p 465–489. *In* Doyle MP, Buchanan RL (ed), Food microbiology: fundamentals and frontiers, 4th ed. ASM Press, Washington, DC.
6. **Amimoto K, Noro T, Oishi E, Shimizu M.** 2007. A novel toxin homologous to large clostridial cytotoxins found in culture supernatant of *Clostridium perfringens* type C. Microbiology **153:**1198–1206. http://dx.doi.org/10.1099/mic.0.2006/002287-0.
7. **Popoff MR, Bouvet P.** 2009. Clostridial toxins. Future Microbiol **4:**1021–1064. http://dx.doi.org/10.2217/fmb.09.72.
8. **Schirmer J, Aktories K.** 2004. Large clostridial cytotoxins: cellular biology of Rho/Ras-glucosylating toxins. Biochim Biophys Acta **1673:**66–74. http://dx.doi.org/10.1016/j.bbagen.2004.03.014.
9. **Jank T, Aktories K.** 2008. Structure and mode of action of clostridial glucosylating toxins: the ABCD model. Trends Microbiol **16:**222–229. http://dx.doi.org/10.1016/j.tim.2008.01.011.

10. **Just I, Hofmann F, Aktories K.** 2000. Molecular mode of action of the large clostridial cytotoxins. Curr Top Microbiol Immunol **250:**55–83.

11. **Voth DE, Ballard JD.** 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev **18:**247–263. http://dx.doi.org/10.1128/CMR.18.2.247-263.2005.

12. **Guttenberg G, Hornei S, Jank T, Schwan C, Lu W, Einsle O, Papatheodorou P, Aktories K.** 2012. Molecular characteristics of *Clostridium perfringens* TpeL toxin and consequences of mono-O-GlcNAcylation of Ras in living cells. J Biol Chem **287:**24929–24940. http://dx.doi.org/10.1074/jbc.M112.347773.

13. **Nagahama M, Ohkubo A, Oda M, Kobayashi K, Amimoto K, Miyamoto K, Sakurai J.** 2011. *Clostridium perfringens* TpeL glycosylates the Rac and Ras subfamily proteins. Infect Immun **79:**905–910. http://dx.doi.org/10.1128/IAI.01019-10.

14. **Pauillac S, D'Allayer J, Lenormand P, Rousselle JC, Bouvet P, Popoff MR.** 2013. Characterization of the enzymatic activity of *Clostridium perfringens* TpeL. Toxicon **75:**136–143. http://dx.doi.org/10.1016/j.toxicon.2013.07.003.

15. **Schorch B, Song S, van Diemen FR, Bock HH, May P, Herz J, Brummelkamp TR, Papatheodorou P, Aktories K.** 2014. LRP1 is a receptor for *Clostridium perfringens* TpeL toxin indicating a two-receptor model of clostridial glycosylating toxins. Proc Natl Acad Sci U S A **111:**6431–6436. http://dx.doi.org/10.1073/pnas.1323790111.

16. **Sakurai J, Duncan CL.** 1978. Some properties of beta-toxin produced by *Clostridium perfringens* type C. Infect Immun **21:**678–680.

17. **Fernandez-Miyakawa ME, Fisher DJ, Poon R, Sayeed S, Adams V, Rood JI, McClane BA, Uzal FA.** 2007. Both epsilon-toxin and beta-toxin are important for the lethal properties of *Clostridium perfringens* type B isolates in the mouse intravenous injection model. Infect Immun **75:**1443–1452. http://dx.doi.org/10.1128/IAI.01672-06.

18. **Sayeed S, Uzal FA, Fisher DJ, Saputo J, Vidal JE, Chen Y, Gupta P, Rood JI, McClane BA.** 2008. Beta toxin is essential for the intestinal virulence of *Clostridium perfringens* type C disease isolate CN3685 in a rabbit ileal loop model. Mol Microbiol **67:**15–30. http://dx.doi.org/10.1111/j.1365-2958.2007.06007.x.

19. **Uzal FA, Saputo J, Sayeed S, Vidal JE, Fisher DJ, Poon R, Adams V, Fernandez-Miyakawa ME, Rood JI, McClane BA.** 2009. Development and application of new mouse models to study the pathogenesis of *Clostridium perfringens* type C enterotoxemias. Infect Immun **77:**5291–5299. http://dx.doi.org/10.1128/IAI.00825-09.

20. **Freedman JC, Li J, Uzal FA, McClane BA.** 2014. Proteolytic processing and activation of *Clostridium perfringens* epsilon toxin by caprine small intestinal contents. mBio **5:**e01994–14. http://dx.doi.org/10.1128/mBio.01994-14.

21. **Chen J, McClane BA.** 2012. Role of the Agr-like quorum-sensing system in regulating toxin production by *Clostridium perfringens* type B strains CN1793 and CN1795. Infect Immun **80:**3008–3017. http://dx.doi.org/10.1128/IAI.00438-12.

22. **Minami J, Katayama S, Matsushita O, Matsushita C, Okabe A.** 1997. Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiol Immunol **41:**527–535. http://dx.doi.org/10.1111/j.1348-0421.1997.tb01888.x.

23. **Chen J, Rood JI, McClane BA.** 2011. Epsilon-toxin production by *Clostridium perfringens* type D strain CN3718 is dependent upon the *agr* operon but not the VirS/VirR two-component regulatory system. mBio **2:**e00275–11. http://dx.doi.org/10.1128/mBio.00275-11..

24. **Coursodon CF, Glock RD, Moore KL, Cooper KK, Songer JG.** 2012. TpeL-producing strains of *Clostridium perfringens* type A are highly virulent for broiler chicks. Anaerobe **18:**117–121. http://dx.doi.org/10.1016/j.anaerobe.2011.10.001.

25. **Harkness JM, Li J, McClane BA.** 2012. Identification of a lambda toxin-negative *Clostridium perfringens* strain that processes and activates epsilon prototoxin intracellularly. Anaerobe **18:**546–552. http://dx.doi.org/10.1016/j.anaerobe.2012.09.001.

26. **Gurjar A, Li J, McClane BA.** 2010. Characterization of toxin plasmids in *Clostridium perfringens* type C isolates. Infect Immun **78:**4860–4869. http://dx.doi.org/10.1128/IAI.00715-10.

27. **Sayeed S, Li J, McClane BA.** 2010. Characterization of virulence plasmid diversity among *Clostridium perfringens* type B isolates. Infect Immun **78:**495–504. http://dx.doi.org/10.1128/IAI.00838-09.

28. **Sayeed S, Li J, McClane BA.** 2007. Virulence plasmid diversity in *Clostridium perfringens* type D isolates. Infect Immun **75:**2391–2398. http://dx.doi.org/10.1128/IAI.02014-06.

29. **Li J, McClane BA.** 2014. Contributions of NanI sialidase to Caco-2 Cell adherence by *Clostridium perfringens* type A and C strains causing human intestinal disease. Infect Immun **82:**4620–4630. http://dx.doi.org/10.1128/IAI.02322-14.

30. **Fisher DJ, Miyamoto K, Harrison B, Akimoto S, Sarker MR, McClane BA.** 2005. Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol Microbiol **56:**747–762. http://dx.doi.org/10.1111/j.1365-2958.2005.04573.x.

31. **Craven SE, Blankenship LC, McDonel JL.** 1981. Relationship of sporulation, enterotoxin formation, and spoilage during growth of *Clostridium perfringens* type A in cooked chicken. Appl Environ Microbiol **41:**1184–1191.

32. **Hunter SE, Clarke IN, Kelly DC, Titball RW.** 1992. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. Infect Immun **60:**102–110.

33. **Sawires YS, Songer JG.** 2005. Multiple-locus variable-number tandem repeat analysis for strain typing of *Clostridium perfringens*. Anaerobe **11:**262–272. http://dx.doi.org/10.1016/j.anaerobe.2005.03.004.

34. **Robertson SL, Li J, Uzal FA, McClane BA.** 2011. Evidence for a prepore stage in the action of *Clostridium perfringens* epsilon toxin. PLoS One **6:**e22053. http://dx.doi.org/10.1371/journal.pone.0022053.

35. **Chen Y, McClane BA, Fisher DJ, Rood JI, Gupta P.** 2005. Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. Appl Environ Microbiol **71:**7542–7547. http://dx.doi.org/10.1128/AEM.71.11.7542-7547.2005.

36. **Yao J, Zhong J, Fang Y, Geisinger E, Novick RP, Lambowitz AM.** 2006. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of Ll.LtrB group II intron splicing. RNA **12:**1271–1281. http://dx.doi.org/10.1261/rna.68706.

37. **Nowell VJ, Poppe C, Parreira VR, Jiang YF, Reid-Smith R, Prescott JF.** 2010. *Clostridium perfringens* in retail chicken. Anaerobe **16:**314–315. http://dx.doi.org/10.1016/j.anaerobe.2009.11.004.

38. **Rood JI.** 1998. Virulence genes of *Clostridium perfringens*. Annu Rev Microbiol **52:**333–360. http://dx.doi.org/10.1146/annurev.micro.52.1.333.

39. **Fisher DJ, Fernandez-Miyakawa ME, Sayeed S, Poon R, Adams V, Rood JI, Uzal FA, McClane BA.** 2006. Dissecting the contributions of *Clostridium perfringens* type C toxins to lethality in the mouse intravenous injection model. Infect Immun **74:**5200–5210. http://dx.doi.org/10.1128/IAI.00534-06.

40. **Sayeed S, Fernandez-Miyakawa ME, Fisher DJ, Adams V, Poon R, Rood JI, Uzal FA, McClane BA.** 2005. Epsilon-toxin is required for most *Clostridium perfringens* type D vegetative culture supernatants to cause lethality in the mouse intravenous injection model. Infect Immun **73:**7413–7421. http://dx.doi.org/10.1128/IAI.73.11.7413-7421.2005.

41. **Geric B, Carman RJ, Rupnik M, Genheimer CW, Sambol SP, Lyerly DM, Gerding DN, Johnson S.** 2006. Binary toxin-producing, large clostridial toxin-negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters. J Infect Dis **193:**1143–1150. http://dx.doi.org/10.1086/501368.

42. **Carter GP, Rood JI, Lyras D.** 2012. The role of toxin A and toxin B in the virulence of *Clostridium difficile*. Trends Microbiol **20:**21–29. http://dx.doi.org/10.1016/j.tim.2011.11.003.

43. **Antunes A, Martin-Verstraete I, Dupuy B.** 2011. CcpA-mediated repression of *Clostridium difficile* toxin gene expression. Mol Microbiol **79:**882–899. http://dx.doi.org/10.1111/j.1365-2958.2010.07495.x.

44. **Dupuy B, Sonenshein AL.** 1998. Regulated transcription of *Clostridium difficile* toxin genes. Mol Microbiol **27:**107–120. http://dx.doi.org/10.1046/j.1365-2958.1998.00663.x.

45. **Mendez MB, Goni A, Ramirez W, Grau RR.** 2012. Sugar inhibits the production of the toxins that trigger clostridial gas gangrene. Microb Pathog **52:**85–91. http://dx.doi.org/10.1016/j.micpath.2011.10.008.

46. **Carter GP, Larcombe S, Li L, Jayawardena D, Awad MM, Songer JG, Lyras D.** 2014. Expression of the large clostridial toxins is controlled by conserved regulatory mechanisms. Int J Med Microbiol **304:**1147–1159. http://dx.doi.org/10.1016/j.ijmm.2014.08.008.

47. **Ma M, Gurjar A, Theoret JR, Garcia JP, Beingesser J, Freedman JC, Fisher DJ, McClane BA, Uzal FA.** 2014. Synergistic effects of *Clostridium perfringens* enterotoxin and beta toxin in rabbit small intestinal loops. Infect Immun **82:**2958–2970. http://dx.doi.org/10.1128/IAI.01848-14.

48. **Keyburn AL, Bannam TL, Moore RJ, Rood JI.** 2010. NetB, a pore-forming toxin from necrotic enteritis strains of *Clostridium perfringens*. Toxins **2:**1913–1927. http://dx.doi.org/10.3390/toxins2071913.

49. **Miyamoto K, Yumine N, Mimura K, Nagahama M, Li J, McClane BA, Akimoto S.** 2011. Identification of novel *Clostridium perfringens* type E strains that carry an iota toxin plasmid with a functional enterotoxin gene. PLoS One **6:**e20376. http://dx.doi.org/10.1371/journal.pone.0020376.

50. **Li J, Miyamoto K, McClane BA.** 2007. Comparison of virulence plasmids among *Clostridium perfringens* type E isolates. Infect Immun **75:**1811–1819. http://dx.doi.org/10.1128/IAI.01981-06.

51. **Billington SJ, Wieckowski EU, Sarker MR, Bueschel D, Songer JG, McClane BA.** 1998. *Clostridium perfringens* type E animal enteritis isolates with highly conserved, silent enterotoxin gene sequences. Infect Immun **66:**4531–4536.

52. **Miyamoto K, Li J, Sayeed S, Akimoto S, McClane BA.** 2008. Sequencing and diversity analyses reveal extensive similarities between some epsilon-toxin-encoding plasmids and the pCPF5603 *Clostridium perfringens* enterotoxin plasmid. J Bacteriol **190:**7178–7188. http://dx.doi.org/10.1128/JB.00939-08.

53. **Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL.** 2007. Repression of *Clostridium difficile* toxin gene expression by CodY. Mol Microbiol **66:**206–219. http://dx.doi.org/10.1111/j.1365-2958.2007.05906.x.

54. **Li J, Ma M, Sarker MR, McClane BA.** 2013. CodY is a global regulator of virulence-associated properties for *Clostridium perfringens* type D strain CN3718. mBio **4:**e00770–13. http://dx.doi.org/10.1128/mBio.00770-13.

55. **Gibert M, Petit L, Raffestin S, Okabe A, Popoff MR.** 2000. *Clostridium perfringens* iota-toxin requires activation of both binding and enzymatic components for cytopathic activity. Infect Immun **68:**3848–3853. http://dx.doi.org/10.1128/IAI.68.7.3848-3853.2000.

56. **Richardson M, Granum PE.** 1983. Sequence of the amino-terminal part of enterotoxin from *Clostridium perfringens* type A: identification of points of trypsin activation. Infect Immun **40:**943–949.

57. **Bokori-Brown M, Savva CG, Fernandes da Costa SP, Naylor CE, Basak AK, Titball RW.** 2011. Molecular basis of toxicity of *Clostridium perfringens* epsilon toxin. FEBS J **278:**4589–4601. http://dx.doi.org/10.1111/j.1742-4658.2011.08140.x.

58. **Vidal JE, McClane BA, Saputo J, Parker J, Uzal FA.** 2008. Effects of *Clostridium perfringens* beta-toxin on the rabbit small intestine and colon. Infect Immun **76:**4396–4404. http://dx.doi.org/10.1128/IAI.00547-08.

59. **Gibert M, Jolivet-Reynaud C, Popoff MR.** 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene **203:**65–73. http://dx.doi.org/10.1016/S0378-1119(97)00493-9.

60. **Katoh T, Honda T, Miwatani T.** 1988. Purification and some properties of cytotoxin produced by *Clostridium difficile*. Microbiol Immunol **32:**551–564. http://dx.doi.org/10.1111/j.1348-0421.1988.tb01417.x.

61. **Sawires YS, Songer JG.** 2006. *Clostridium perfringens*: insight into virulence evolution and population structure. Anaerobe **12:**23–43. http://dx.doi.org/10.1016/j.anaerobe.2005.10.002.