A novel toxin homologous to large clostridial cytotoxins found in culture supernatant of *Clostridium perfringens* type C

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An unknown cytotoxin was identified in the culture supernatant of *Clostridium perfringens* type C. The cytotoxin, named TpeL, which was purified using mAb-based affinity chromatography, had a lethal activity of 62 minimum lethal dose (MLD) mg^{-1} in mice and a cytotoxic activity of 6.2×10^5 cytotoxic units (CU) mg^{-1} in Vero cells. The nucleotide sequence of TpeL was determined. The entire ORF had a length of 4953 bases, and the same nucleotide sequence was not recorded in the GenBank/EMBL/DDBJ databases. The molecular mass calculated from the deduced amino acid sequence was 191 kDa, and a signal peptide region was not found within the ORF. The deduced amino acid sequence exhibited 30–39 % homology to *Clostridium difficile* toxins A (TcdA) and B (TcdB), *Clostridium sordellii* lethal toxin (TcsL) and *Clostridium novyi* alpha-toxin (TcnA). The amino acid sequence of TpeL is shorter than these toxins, and the homologous region was located at the N-terminal site. Eighteen strains of *C. perfringens* types A, B and C were surveyed for the presence of the *tpeL* gene by PCR. The *tpeL* gene was detected in all type B (one strain) and C strains (five strains), but not in any type A strains (12 strains). TpeL was detected in culture filtrates of the five type C strains by dot-blot analysis, but not in the type B strain. It was concluded that TpeL is a novel toxin similar to the known large clostridial cytotoxins. Furthermore, the data indicated that TpeL is produced by many *C. perfringens* type C strains.

INTRODUCTION

Infection with *Clostridium perfringens* type C results in necrotizing enterocolitis associated with diarrhoea and dysentery in infant animals (Tweten, 2001). In humans, the bacteria cause necrotic enteritis, which is termed pigbel (Lawrence & Walker, 1976). *C. perfringens* has been classified into five types, A to E, according to the toxinogenicity of major extracellular toxins designated alpha-, beta-, epsilon- and iota-toxins. The *C. perfringens* strains defined as type C show alpha- and beta-, but not epsilon- and iota-toxinogenicities (Yoo *et al.*, 1997). The other toxins produced by *C. perfringens* type C have already been identified as beta2-, delta-, kappa- and theta-toxins, and enterotoxin (Gibert *et al.*, 1997; Niilo, 1987). Beta-toxin is correlated with the pathogenicity of *C. perfringens* type C, and diseases associated with type C strains have been controlled via treatment with beta-toxoid (Springer & Selbitz, 1999). Beta2-toxin is a new toxin recently discovered in *C.*

perfringens type C isolated from piglets with necrotic enteritis, and speculated to be important because its gene has been detected in most *C. perfringens* type C strains recovered from animals with clinical disease (Manteca *et al.*, 2002; Waters *et al.*, 2003).

Most of the toxins produced by *C. perfringens* type C are toxic to particular cells or cell lines. Beta- and beta2-toxins are toxic to HL60 cells (Nagahama *et al.*, 2003) and to CHO and I407 cells (Gibert *et al.*, 1997), respectively, while delta-toxin is toxic to various rabbit immune cells, i.e. alveolar macrophages, peritoneal appendix cells, bone marrow cells, splenocytes and thymocytes (Jolivet-Reynaud *et al.*, 1982). Theta-toxin exhibits cytotoxic effects on macrophages to escape from phagosomes in consort with alpha-toxin (O'Brien & Melville, 2004). In addition, alpha-toxin slightly damages the membranes of human diploid embryonic lung fibroblasts (Thelestam & Möllby, 1975). On the other hand, enterotoxin, which has molecular masses of 35 kDa as a monomer (Duffy *et al.*, 1982) and 90–200 kDa as aggregate forms with eukaryotic proteins (Singh *et al.*, 2001), is known to be highly cytotoxic to Vero and Caco-2 cells (McDonel & McClane, 1981; Singh *et al.*, 2001). Among the *C. perfringens* type C toxins reported previously, only enterotoxin is toxic to Vero cells, and no toxin is known to have a molecular mass of around 200 kDa without eukaryotic proteins.

Abbreviations: CBB, Coomassie brilliant blue; CU, cytotoxic units; LCT, large clostridial cytotoxin; MLD, minimum lethal dose.

The GenBank/EMBL/DDBJ accession number for the *tpeL* sequence reported in this paper is AB262081.

Multiple sequence alignment data of this toxin and large clostridial cytotoxins are available as a supplementary figure with the online version of this paper.

When we fractionated the culture filtrate of *C. perfringens* type C strain MC18 by HPLC, we noticed an unknown toxin that was lethal to mice. The toxin was toxic to Vero cells, and its molecular mass was estimated to be about 180 kDa by SDS-PAGE analysis. These characteristics completely differed from the previously reported toxins. For research on the toxin (named TpeL: toxin *C. perfringens* large cytotoxin), it was purified with an affinity column coupled with mAb. The gene (*tpeL*) encoding the toxin was then sequenced, and various *C. perfringens* strains were surveyed for the presence of the *tpeL* gene by PCR using primers based on the sequence data. In this paper, we report the details of the discovery and some of the characteristics of TpeL produced by *C. perfringens*.

METHODS

Bacterial strains, phages and phagemid. *C. perfringens* strains used in this study are listed in Table 1. Toxinotypes of *C. perfringens* strains were determined by PCR (Yoo *et al.*, 1997). MC18, which is beta2-toxin gene positive and enterotoxin gene negative by PCR analysis (Herholz *et al.*, 1999), was used for the preparation of purified TpeL, immunogen for production of the mAb, and sequencing of the *tpeL* gene. A lambda phage Zap Express vector digested with *Bam*HI (Stratagene) was used to prepare a DNA library, and the ExAssist helper phage (Stratagene) was employed for excision of the pBK-CMV phagemid vector from the ZAP Express vector with the inserted the *tpeL* gene. *Escherichia coli* XL-1 Blue MRF' and XL0LR were used for host cells of the Zap Express vector and pBK-CMV phagemid vector, respectively.

Discovery of TpeL by HPLC. *C. perfringens* MC18 was pre-cultured with cooked meat medium (Becton Dickinson) at 37 °C for 16 h. The growing cells were transferred to 150 ml TYG broth [30 g Trypticase Peptone (Becton Dickinson) l⁻¹ and 10 g Yeast Extract

(Becton Dickinson) l⁻¹ supplemented with 5 g glucose l⁻¹ and 0.5 g L-cysteine hydrochloride l⁻¹ (pH 7.4)] and incubated at 37 °C for 6 h (until the early stationary phase). The bacterial cells were removed by centrifugation and filtration, and the filtrate was concentrated to 1/50 of the original volume by ultrafiltration using a membrane with a 10 kDa cutoff. The sample was applied to a G6000PW gel filtration column (21.5 mm × 60 cm; Tosoh), which was equilibrated with 50 mM Tris/HCl buffer (pH 8.0). The column was washed with the same buffer at a flow rate of 5 ml min⁻¹. A 0.5 ml sample of each fraction was intravenously injected into a mouse, and the fractions lethal to the mice were pooled. The active fraction was then loaded onto a DEAE-5PW anion-exchange column (7.5 mm × 7.5 cm; Tosoh), and proteins were fractionated with a linear gradient of 0–0.5 M NaCl at a flow rate of 1 ml min⁻¹. An aliquot of 0.5 ml of each fraction was inoculated into a mouse. A fraction showing lethality was used for SDS-PAGE and N-terminal amino acid sequencing.

mAb. A toxoid for immunization was prepared as follows. MC18 cultured in TYG broth was inactivated by adding formalin at a final concentration of 0.2 % (v/v) at 37 °C for 1 day. The bacterial cells in the formalin-treated culture fluid were removed by centrifugation, and the supernatant was concentrated to approximately 1/25 of the original volume by ultrafiltration (10 kDa cutoff). An 8-week-old BALB/c mouse was immunized intramuscularly twice at 4 week intervals with 0.2 ml of the concentrated MC18 toxoid mixed with an equal volume of incomplete Freund's adjuvant (Sigma). Two weeks after the second immunization, spleen cells of the mouse were fused with SP2/0-Ag14 myeloma cells (Dainippon Sumitomo Pharma) using polyethylene glycol 1500 (Roche). The fused cells were dispensed onto 96-well microplates and incubated at 37 °C under 5 % CO₂. The hybridomas producing anti-TpeL antibody were screened via a neutralization test against the cytotoxicity of the MC18 culture filtrate as described previously (Amimoto *et al.*, 1998) except that the day of determination was day 3. They were cloned twice. The cloned hybridoma was grown with Iscove's Modified Dulbecco's Medium (Invitrogen) containing 20 % fetal calf serum,

Table 1. Bacterial strains

Toxin type	Strain	Reference and/or source
A	NCTC 8237	Titball <i>et al.</i> (1989)
	ATCC 3624	Weiss & Strong (1967)
	PB6K	Tsutsui <i>et al.</i> (1995)
	CP23	This study, isolated from bovine in Japan (1979)
	Ya8	This study, isolated from bovine in Japan (1989)
	Ya18	This study, isolated from bovine in Japan (1989)
	TYU	This study, isolated from bovine in Japan (2003)
	Tyu1	This study, isolated from bovine in Japan (2002)
	MC2	This study, isolated from swine in Japan (2001)
	MC14	This study, isolated from swine in Japan (2000)
	MC22	This study, isolated from swine in Japan (1999)
B	Shi1	This study, isolated from bovine in Japan (2000)
	ATCC 3626	Mollby & Holme (1976)
C	MC18	This study, isolated from swine in Japan (1999)
	CP46	This study, isolated from swine in Japan (1980)
	Shi2	This study, isolated from swine in Japan (2000)
	Chi8	This study, isolated from swine in Japan (2000)
	3511-2	This study, isolated from swine in Japan (2001)

and the hybridomas were injected intraperitoneally into BALB/c mice primed with pristane. Ascitic fluid containing mAb obtained from the mice was purified by ammonium sulfate precipitation and affinity-column chromatography with a protein G column (GE Healthcare).

Purification of TpeL. TpeL was purified from the culture filtrate by salt precipitation with ammonium sulfate and affinity column chromatography with the mAb. The affinity column was prepared by binding 8 mg mAb to a Hitrap NHS-activated HP column (GE Healthcare) according to the instruction manual. The culture filtrate was prepared from 4000 ml MC18 cultured in TYG broth at 37 °C for 6 h. The filtrate was concentrated by ultrafiltration, and TpeL in the concentrate was salted out with ammonium sulfate (50% saturation). The solution was centrifuged at 10 000 g for 30 min, and the precipitate was resuspended with 20 mM sodium phosphate buffer (pH 7.0). The suspension was dialysed with the same buffer for one night, and then applied to the affinity column. After the column was washed with the same buffer, TpeL bound to the column was eluted with 100 mM glycine/HCl buffer (pH 2.7) and neutralized to pH 7.0 with 1 M Tris/HCl buffer (pH 9.0) immediately. The protein concentration of the purified TpeL was estimated by the Lowry method.

Gel electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970). Samples were mixed with an equal volume of 2× sample buffer containing 10% (v/v) β-mercaptoethanol. The mixtures were heated in boiling water for 3 min and then analysed by SDS-PAGE with a 4% stacking gel and 6% running gel. The proteins resolved in the gel were stained with CBB R-250.

N-terminal amino acid sequencing. TpeL detected at the molecular mass position of about 180 kDa in the gel was transferred to a PVDF membrane (Millipore) by the method of Towbin *et al.* (1979) and sequenced 8 or 10 amino acid residues from the N-terminus by Edman degradation on a Procise cLC494 protein sequencer (Applied Biosystems).

Determination of lethal activity. The lethal activity assay was performed by twofold serially diluted purified TpeL (range 32 to 4 µg ml⁻¹) intravenously injected into four ddY female mice (mean: 20.2 g) at 0.5 ml for each dilution. Mice were observed for the occurrence of death, and the LD₅₀ determined by the method of Reed & Muench (1938) as well as the minimum lethal dose (MLD) per mouse were calculated.

Assay of cytotoxic activity. The cytotoxic activity was assayed using Vero cells. Eagle's minimum essential medium (Nissui) supplemented with 5% (v/v) fetal calf serum was used for the culture of Vero cells and dilution of samples. Vero cells were suspended at 2 × 10⁵ cells ml⁻¹ with the medium and 0.1 ml of this suspension was dispensed onto a 96-well microplate, and it was incubated at 37 °C for 24 h under 5% CO₂. A 0.1 ml aliquot of each twofold serial dilution of the sample was inoculated onto seeding Vero cells on a 96-well microplate. Morphological changes of Vero cells were observed with a microscope for 3 days. At post-inoculation day 3, the surviving cells were enumerated by measuring the dehydrogenase activity within the cells with a cell proliferation kit II (XTT) (Roche) (Roehm *et al.*, 1991). The reciprocal of the highest dilution of TpeL at which less than 50% of cells survived relative to the control well without TpeL was expressed in cytotoxic units (CU). The cytotoxicity of the purified TpeL was identified by the above-described neutralization test with the mAb.

Construction and screening of the DNA library. DNA of *C. perfringens* type C was extracted from MC18 using an Easy-DNA kit (Invitrogen). Twenty micrograms of DNA was partially digested with 0.0625 U of *Sau3AI* (BioLabs) at 37 °C for 1 h and 0.4 µg of the resulting product was ligated into the Zap Express vector

digested with *Bam*HI. The ligation product was packaged with Gigapack III gold (Stratagene). The library was introduced into *E. coli* XL-1 Blue MRF' to express the proteins for screening. The expressed proteins were transferred onto nitrocellulose membranes, and subsequently detected with 4 µg ml⁻¹ of the mAb against TpeL and an appropriate dilution of peroxidase-labelled anti-mouse IgG (KPL). Expression-positive plaques were purified twice. The inserted DNA was recovered into a pBK-CMV phagemid using the ExAssist helper phage with *E. coli* XL0LR.

Nucleotide sequencing of the *tpeL* gene. The *tpeL* gene in the pBK-CMV was sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Automated DNA sequencing of the sample DNA was performed by a 3730x1 DNA Analyser (Applied Biosystems). However, part of the 5' end of the *tpeL* gene could not be located in the pBK-CMV. Therefore, PCR using the DNA library as a template was carried out to determine the missing nucleotide sequence. The primers used in PCRs were PCT-5' primer (5'-ATATCCATCACCTAAATATCCC-3') based on the result of the partial sequencing and either T3 primer (5'-ATTAACCTCAACTA-AAGGGAA-3') or T7 primer (5'-GTAATACGACTCACTATAGG-GC-3') in pBK-CMV. PCRs were performed in a total reaction volume of 50 µl containing: 1× PCR buffer (Mg²⁺ free); 2.5 mM MgCl₂; 0.2 mM dNTP mixture; 2.5 units LA *Taq* DNA polymerase (TaKaRa); 50 pM of each primer; and 5 µl of the DNA library. The PCR cycle conditions were: denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min; and extension at 72 °C for 6 min; with a final extension step at 72 °C for 7 min. The PCR product was purified using a MinElute Gel Extraction kit (Qiagen) and sequenced directly.

Amino acid sequence analysis. An amino acid sequence was deduced from the nucleotide sequence. Prediction of a signal peptide was performed using the SignalP v 3.0 program (Bendtsen *et al.*, 2004). Sequences homologous to the deduced amino acid sequence were searched using the gapped BLAST program (Altschul *et al.*, 1997). The results were used in a phylogenetic analysis. A multiple sequence alignment was executed using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The reliability of the tree topology was analysed with the bootstrap method (Felsenstein, 1985).

Detection of the *tpeL* gene in various strains. The presence of the *tpeL* gene in 18 strains of *C. perfringens* was investigated by PCR. The strains incubated in 1.0 ml TYG broth were centrifuged at 10 000 g for 5 min and the precipitates were resuspended with 0.1 ml distilled water. The samples were boiled for 10 min and then centrifuged again at 10 000 g for 10 min. The supernatants were collected and used as template DNAs in PCR. PCR was performed in a total of 50 µl reaction mixture containing: 1× PCR buffer (Mg²⁺ free); 2.5 mM MgCl₂; 0.2 mM dNTP mixture; 2.5 units of LA *Taq* DNA polymerase (TaKaRa); 50 pM of primers (forward; 5'-ATATA-GAGTCAAGCAGTGGAG-3', reverse; 5'-GGAATACCACTTGATA-TACCTG-3'); and 5 µl template solution. The following conditions were used in the PCR: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min; and extension at 72 °C for 1 min; with the final extension step at 72 °C for 7 min. PCR products which exhibited a length of 466 bp were analysed by electrophoresis on 1.5% agarose gels.

Detection of TpeL from various strains. The production of TpeL by the various strains was confirmed by dot-blot analysis and cytotoxic assay. The proteins in the culture filtrates (0.4 ml) of the various strains were attached to a PVDF membrane. TpeL on the membrane was visualized with the method used for screening the DNA library. The cytotoxic activity of the culture filtrates was measured with Vero cells. The activity was determined by the

above-described XTT assay against Vero cells, which showed characteristic morphological changes in response to TpeL (cell rounding or enlargement). The culture filtrates showing cytotoxicity like TpeL were diluted to 8 CU, and identified with 1 µg mAb ml⁻¹ by the neutralization test.

RESULTS

Discovery and purification of TpeL

The growth of MC18 reached stationary phase at 6 h. Three millilitres of material for HPLC was prepared from 150 ml of the culture with a sterilization filter and an ultrafiltration membrane. In the first HPLC step, the substance lethal to mice was detected in fractions collected 30–32 min after the loading in a G6000PW gel filtration column. In the subsequent step with the lethal fractions, a toxin was eluted with approximately 0.14 M NaCl in anion-exchange column chromatography. The SDS-PAGE profile of the fraction obtained by HPLC is shown in Fig. 1(a). A major band of about 180 kDa was evident and minor bands of about 220 and 185 kDa were detected. TpeL was then further purified by affinity chromatography using a mAb that was shown to neutralize the toxicity of TpeL in cytotoxic assay. As a result, 150 µg TpeL was purified from 4000 ml of the MC18 culture medium. As shown in Fig. 1(b), TpeL was highly purified and its molecular mass was estimated at about 180 kDa by SDS-PAGE.

Toxicity of TpeL

TpeL was lethal to mice and toxic to Vero cells. The lethal activity of the purified TpeL in mice was estimated as 62

MLD mg⁻¹ (one MLD was 16 µg) and 91 LD₅₀ mg⁻¹ (one LD₅₀ was 11 µg) by intravenous injection.

The purified TpeL showed obvious cytotoxicity in Vero cells, and the specific activity was 6.2 × 10⁵ CU mg⁻¹ (one CU was 1.6 ng). Morphological changes induced by TpeL in Vero cells are shown in Fig. 2. The cytopathic effect induced by a low dose of TpeL was characterized by the enlargement of cells (Fig. 2b) and appearance of rounded cells (Fig. 2c). Vero cells exposed to a high dose of TpeL initially manifested similar changes to those inoculated with the low dose, then formed aggregates, and eventually detached from the well surface (Fig. 2d). The cytotoxicity induced by 4 CU of the purified TpeL was completely neutralized by 0.5 µg mAb ml⁻¹.

N-terminal amino acid sequencing

The N-terminal amino acid sequence of the major band obtained from the culture filtrate by HPLC was G-L-M-S-K-E-Q-L- without methionine as the first amino acid. The sequence of the purified TpeL was G-L-M-S-K-E-Q-L-I-I-, the same as the previous major band.

Sequencing of the *tpeL* gene

The gene encoding TpeL revealed the presence an ORF of 4953 bases. The sequence downstream from nucleotide position 1262 in the ORF was determined from the sequencing of the DNA library cloned by immunoscreening, and the other sequence was determined from sequencing of the PCR product amplified from the DNA library with PCT-5' and T7 primers.

The *tpeL* gene encoded 1651 amino acid residues, and the deduced N-terminal amino acid sequence was M-G-L-M-S-K-E-Q-L-I-I-, identical to that of the purified TpeL except that the initial amino acid was methionine. The molecular mass of TpeL calculated from the deduced amino acid sequence was 191 kDa. A signal peptide region was not found within the ORF using the SignalP program.

The deduced amino acid sequence shared homology with *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB), *Clostridium sordellii* lethal toxin (TcsL) and *Clostridium novyi* alpha-toxin (TcnA), called large clostridial cytotoxins (LCTs). The homology scores were 39 % to TcdA, 38 % to TcdB, 39 % to TcsL and 30 % to TcnA. The amino acid sequence of TpeL is shorter than that of any of these toxins, and the homologous region was located at the N-terminal site of the LCTs. A multiple sequence alignment of these toxins is available as Supplementary Fig. S1 with the online version of this paper, and a schematic representation of the alignment is shown in Fig. 3. A DXD motif in LCTs is essential for glycosyltransferase activity, and W102 in TcsL is an essential amino acid residue for the enzyme activity (Busch *et al.*, 2000a). TpeL conserved the DXD motif and W102 of TcsL. However, the C-terminal carbohydrate-binding sites of LCTs (von Eichel-Streiber *et al.*, 1992) were

Fig. 1. SDS-PAGE profiles of TpeL. (a) A fraction having lethality in mice purified by gel filtration and anion-exchange column chromatographies. (b) TpeL purified by affinity column chromatography with mAb. One microgram of the purified TpeL was loaded in a polyacrylamide gel. The gels were stained with CBB R-250.

Fig. 2. Morphological changes induced by TpeL in Vero cells. (a) Control Vero Cells. (b, c and d) Changes in Vero cells with 1.6, 3.2 and 6.4 ng TpeL, respectively. Vero cells grown on a 96-well microplate were inoculated with diluted TpeL. After 3 days, Vero cells were observed with a microscope. Scale bars, 100 μm.

not conserved. The phylogenetic tree obtained with the neighbour-joining method is shown in Fig. 4. TcdB and TcsL were phylogenetically very closely related, but the other toxins were separated.

Fig. 3. Schematic representation of the multiple sequence alignment. Dark grey boxes and black boxes show the glycosyl-transferase activity region and receptor-binding region, respectively (Busch *et al.*, 2000a, b; Faust *et al.*, 1998; Hofmann *et al.*, 1997; von Eichel-Streiber *et al.*, 1992). The predicted activity region of TpeL is indicated by light grey boxes.

Detection of the *tpeL* gene and its product in various strains

Eighteen strains of *C. perfringens* were analysed for the presence of the *tpeL* gene by PCR. All of the type B (only one strain tested) and type C (five strains tested) strains

Fig. 4. Phylogenetic tree of TpeL, TcdA, TcdB, TcsL and TcnA. The tree was constructed by the neighbour-joining method. Bootstrap values are shown on branches. The scale bar (0.05) represents substitutions per site.

contained the *tpeL* gene of the expected size, while 12 strains of type A carried no *tpeL* gene (Fig. 5a).

TpeL was detected in the culture filtrates of MC18, CP46, Shi2, Chi8 and 3511-2 by dot-blot analysis, and the intensity of the reaction differed among the strains (Fig. 5b). The culture filtrates of MC18 and Chi8 showed strong signals, and the signals of Shi2 and 3511-2 were slightly weaker than these. The culture filtrate of CP46 reacted very weakly with mAb, and TpeL in the culture filtrate of ATCC 3626 was not detected. The culture filtrates of MC18, Shi2, Chi8 and 3511-2 showed 320, 80, 320 and 80 CU of activity, respectively (Fig. 5c). In each culture filtrate, 8 CU of cytotoxic activity was completely neutralized by 1 μg mAb ml^{-1} . The activities of the culture filtrates of the other strains were all <10 CU, and resulted in cell disruption unlike the morphological change caused by TpeL at a dilution of 1:5.

DISCUSSION

The toxinotypes of *C. perfringens* are classified into A to E based on the production of alpha-, beta-, epsilon- and iota-toxins (Yoo *et al.*, 1997). In addition, certain strains of *C. perfringens* type C produce beta2-, delta-, kappa- and theta-toxins, and enterotoxin (Gibert *et al.*, 1997; Niilo, 1987). *C. perfringens* MC18 used in this study was analysed for the production of alpha-, beta- and beta2-toxins, whose molecular masses are about 43, 35 and 28 kDa, respectively (Gibert *et al.*, 1997; Tsutsui *et al.*, 1995). However, the

lethalities of the toxins were not detected in fractions from the G6000PW gel filtration column. The gel filtration column first used is appropriate for the separation of large molecular mass proteins and is not recommended to obtain proteins of relatively small molecular mass. So, we speculated that the toxins were lost during the gel filtration process. With the fractionation by anion-exchange column chromatography, the fraction detected as the major 180 kDa band in the SDS-PAGE profile corresponded to the lethal fraction in mice, and was confirmed to be toxic to Vero cells. To our knowledge, there is no previous report on a toxin that exhibits these characteristics. We considered that the protein may be an unknown toxin.

We obtained a highly purified TpeL using affinity chromatography with mAb. Among the toxins produced by *C. perfringens* type C, beta-toxin is known to be most lethal in mice: one MLD is 30 ng (Nagahama *et al.*, 1999) and that of beta2-toxin has been reported to be 3 μg (Gibert *et al.*, 1997). Although TpeL was toxic to mice, its lethal activity was about 1/500 and 1/5 that of beta- and beta2-toxins, respectively. These findings should be considered when the pathological and immunological roles of each toxin are investigated. At present, the roles of TpeL are not known exactly. The pathogenicity of TpeL might be hidden by the lethality of beta-toxin in animals.

The purified TpeL was prepared from the culture of MC18 at the early stationary phase. The bacteria would not likely undergo much autolysis and/or sporulation at this time. The

Fig. 5. Detection of the *tpeL* gene by PCR analysis and TpeL in culture filtrates by dot-blot analysis and cytotoxic assay. (a) PCR analysis was performed using the nucleotide sequences 2810–2829 (forward) and 3275–3254 (reverse) as primers. (b) Dot-blot analysis detected TpeL of culture filtrates. Proteins in the culture filtrate of each strain were attached to a PVDF membrane, and TpeL of culture filtrates was visualized with anti-TpeL mAb and anti-mouse IgG. (c) Cytotoxic activity was measured using Vero cells and expressed in CU. A dash indicates <10 CU. Lanes: 1, DNA size marker (100 bp ladder); 2, MC18; 3, CP46; 4, Shi2; 5, Chi8; 6, 3511-2; 7, NCTC 8237; 8, ATCC 3624; 9, ATCC 3626; 10, CP23; 11, PB6K; 12, Ya8; 13, Ya18; 14, TYU; 15, Tyu1; 16, MC2; 17, MC14; 18, MC22; 19, Shi1.

cytotoxicity of the culture filtrate increased along with growth of the bacteria (data not shown). It seems that TpeL was not released from bacteria following autolysis and/or sporulation. On the other hand, the N-terminal amino acid sequence of the purified TpeL lacked only methionine from the deduced amino acid sequence. This indicates that there is no signal peptide region in TpeL. Also, a signal peptide cleavage site in the sequence was not predicted by the SignalP program.

Among toxins produced by the *Clostridium* spp., TcdA, TcdB, TcsL and TcnA are known to be of LCT (Rupnik *et al.*, 2005). Comparisons of the characteristics of TpeL and LCTs are shown in Table 2. LCTs exhibit lethality in mice and cytotoxicity in various cell lines including Vero cells (Ball *et al.*, 1993). The lethal activity of TpeL was very weak when compared with that of LCTs in mice. Meanwhile, LCTs are highly cytotoxic as TpeL. TcdB and TcsL induce morphological changes in Vero cells similarly to TpeL (Ball *et al.*, 1993; Kato *et al.*, 1998; Nakamura *et al.*, 1984; Popoff, 1987). Anti-TcsL serum neutralizes TcdB activity but not TcdA activity (Popoff, 1987). This finding shows that TcsL is closely related to TcdB. Also in our phylogenetic analysis, TcsL and TcdB showed a high degree of relatedness, but the other toxins were divided into different groups. We tried a cross-neutralization test with TpeL and TcsL. An anti-TcsL

polyclonal antibody (Amimoto *et al.*, 2001) did not neutralize 4 CU TpeL in Vero cells, and 4 CU purified TcsL (Amimoto *et al.*, 2001) was not neutralized by 1.3 mg anti-TpeL mAb ml⁻¹ (data not shown). As for other characteristics of LCTs, no region encoding a signal peptide is included in the ORFs of these toxins, and the N-terminal amino acid of LCTs is methionine (Ball *et al.*, 1993), whereas that of TpeL is glutamine.

Glycosyltransferase active regions of TcdB, TcsL and TcnA are located in the first 551, 551 and 546 amino acid residues (Busch *et al.*, 2000a, b; Hofmann *et al.*, 1997), respectively. Moreover, that of TcdA is located within the first 659 N-terminal amino acids (Faust *et al.*, 1998). TpeL has the highly conserved N-terminal amino acid sequence of LCTs, containing a DXD motif. In TcsL, W102, which is involved in hydrophobic interaction with UDP-glucose, is essential for the enzyme activity (Busch *et al.*, 2000a). TpeL and the other LCTs conserved tryptophan corresponding to W102 in TcsL. We suspected that TpeL might have glycosyltransferase activity given the analyses. The C-terminal region of LCTs includes initial attachment sites for carbohydrates, and mediates non-specific interaction (von Eichel-Streiber *et al.*, 1992). However, TpeL appears to lack this region from the sequence. It is interesting that TpeL was toxic to Vero cells even without the binding sites. It is not known how TpeL binds to its receptor.

The *tpeL* gene was detected not only in type C strains isolated in recent years but also ATCC 3626, a type B strain preserved for many years. We therefore suggest that the *tpeL* gene has been conserved in *C. perfringens* DNA for a long time. Interestingly, beta-toxin-gene-positive strains completely coincided with *tpeL*-positive strains among the 18 strains examined in this study. Complete chromosomal and plasmid sequences of *C. perfringens* type A strain 13 are available (Shimizu *et al.*, 2002). Also, there is no *tpeL* gene sequence within the data. It has been pointed out that beta- and epsilon-toxin genes carried by plasmids are sometimes lost during the passage of the strains (Gibert *et al.*, 1997; Katayama *et al.*, 1996). So, when the strain loses the plasmids, it changes to type A. We confirmed toxinotypes of three high-passage-number strains (two strains of type C and one strain of type D). These strains carried only the alpha-toxin gene among the four major toxin genes, and the toxinotype of these strains had changed to type A. We tested these strains for the *tpeL* gene. However, *tpeL* was not detected in three strains for unknown reasons (data not shown). In our genetic study, total DNAs were used in the cloning and detection of the *tpeL* gene. So, it is not clear whether the *tpeL* gene is carried on a plasmid or on the chromosome.

Although the dot-blot analysis was more sensitive than the cytotoxic assay, the intensity of the signal in the dot-blot analysis corresponded to the cytotoxic activity. TpeL was not detected by Western blotting following SDS-PAGE with the mAb (data not shown). It is thought that the conformation of the epitope changed by SDS and

Table 2. Comparison of LCTs

Property	TpeL	TcdA	TcdB	TcsL	TcnA
Amino acid residues*	1651	2710	2366	2364	2178
Molecular mass (kDa)†	191	308	269	270	250
Signal peptide	none	none	none	none	none
Cytotoxic activity (1 CU)‡	1.6 ng	10 ng	1 pg	16 ng	100 pg
Lethal activity (1 MLD)§	16 µg	50 ng	50 ng	3 ng	5–10 ng
Glycosyltransferase activity	implication	yes	yes	yes	yes

*The sequence accession numbers of the toxins are CAA63564, CAA63562, CAA57959 and Q46149.

†Calculated from the deduced amino acid sequence.

‡The levels of cytotoxic activity in Vero cells of TcdA, TcdB and TcnA are from Ball *et al.* (1993). The titres are shown as the reciprocal of the highest dilution that caused rounding in more than 90 % of the cells. The activity of TcsL is from Popoff (1987). It is shown as the reciprocal of the highest dilution yielding a 50 % cytopathic effect.

§The lethal activities of TcdA, TcdB and TcnA are results obtained by intraperitoneal injection by Ball *et al.* (1993). The activity of TcsL is a result obtained by intraperitoneal injection by Popoff (1987).

||References for TcdA, TcdB, TcsL and TcnA are Busch *et al.* (2000a, b); Ciesla & Bobak (1998); Faust *et al.* (1998); and Hofmann *et al.* (1997).

2-mercaptoethanol did not fully recover. So, we employed a dot-blot assay for the detection of TpeL in the culture filtrate.

ATCC 3626 carried the *tpeL* gene, but TpeL in the culture filtrate was detected by neither a dot-blot analysis nor a cytotoxic assay in Vero cells. It was considered that ATCC 3626 was unable to produce a detectable level of TpeL or did not produce it at all. *C. perfringens* alpha-toxin is produced by strains of all types. Type A strains produce markedly more alpha-toxin than the other four types due to a mutation of a regulatory gene (Tsutsui *et al.*, 1995). The expression of TpeL of each strain might be different for similar reasons, because the level of expression was clearly different among the strains carrying the *tpeL* gene.

In conclusion, we have identified TpeL as a novel toxin of *C. perfringens* based on the following observations: (1) there is no reported toxin possessing the characteristics corresponding with those of TpeL; (2) there is no recorded sequence with significant homology to the *tpeL* gene. Moreover, TpeL might belong in the LCT family and has glucosyltransferase activity.

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