Clostridium perfringens BETA2 TOXIN: A POTENTIAL ACCESSORY TOXIN IN GASTROINTESTINAL DISEASES OF HUMANS AND DOMESTIC ANIMALS

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Clostridium perfringens is a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium that causes histotoxic infections and enterotoxaemias in humans and domestic animals. This bacterium owes its pathogenicity to the production of a large arsenal of toxins, including the *C. perfringens* enterotoxin (CPE) and beta2 toxin (CPB2). Type A, *cpe*-chromosomal isolates cause human food-poisoning (FP), whereas type A, *cpe*-plasmid isolates cause human antibiotic associated diarrhea (AAD). Symptoms of AAD are more severe and longer in duration than symptoms of FP. We hypothesized that AAD isolates may produce an accessory toxin, which would contribute to pathogenesis, as an explanation for these symptomatic differences. Consequently, the goal of this dissertation was to determine; 1) whether the recently discovered, plasmid-encoded, CPB2 toxin was produced by AAD isolates, 2) how this toxin functions *in vitro*, and 3) its role *in vivo* (in the context of human and animal disease). PCR analysis, pulsed-field gel electrophoresis/Southern blotting, and DNA sequencing determined that the *cpb2* gene is preferentially associated with AAD isolates (but not FP isolates), in part, due to its presence on some *cpe*-encoding plasmids. Sequencing of *cpe* and *cpe*/*cpb2* plasmids found a large region of plasmid DNA, encoding a conjugal-gene cluster, which appears conserved amongst most *C. perfringens* virulence plasmids. Subsequent sequencing of a number of *cpb2* genes identified two CPB2 variants, both of which are produced (detected using Western blot). Neutral red cytotoxicity assays using purified CPB2 demonstrated that CPB2h1 is more cytotoxic for Caco-2 cells than CPB2h2 and this activity is heat labile. Further *in vitro* work (using ^{86}Rubidium release assays and osmotic stabilizers) suggested that CPB2 acts by disrupting membrane permeability. *In vivo* experiments confirmed that our CPB2 preparations also were lethal using a mouse intravenous injection model. However, efforts to develop a more realistic model of pathogenesis (which initiates in the intestines) should be continued to further

analyze the role of CPB2 in pathogenesis. Ultimately, the production of CPB2 by most AAD isolates, as opposed to FP isolates, could provide an explanation for the more severe symptoms associated with AAD cases and suggests that CPB2 may be involved in human gastrointestinal disease.

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1.0 GENERAL INTRODUCTION TO *Clostridium perfringens* AND FOUNDATION OF HYPOTHESIS

Clostridium perfringens, a Gram-positive, anaerobic, spore-forming bacillus is well known for its prolific toxin production (114, 131). Research to elucidate the mechanism of action of these toxins and to discern their roles during infections caused by multitoxigenic isolates is the primary focus of the *C. perfringens* research community (107). Subsequently, the overall aim of this dissertation was to further understand the role of the *Clostridium perfringens* beta2 toxin (CPB2) in the pathogenesis of gastrointestinal (GI) infections in humans and domestic animals. At the outset of my graduate research in 2002, CPB2 was a relatively new toxin to the *C. perfringens* field and only eleven CPB2-related publications were available on Pubmed. Most of these articles involved epidemiological studies linking *cpb2* to GI diseases of domestic animals and there was much debate within the field as to the importance of CPB2 in GI disease. To clarify the role of CPB2 in GI disease, basic questions needed to be answered in regard to CPB2 including; 1) determining the scope and genetic diversity of *cpb2* across different *C. perfringens* toxin types, 2) assessing the CPB2 production capabilities of these isolates versus their ability to produce other potent toxins, 3) identifying the mechanism of action and potency of CPB2 *in vitro* and, 4) evaluating whether CPB2 could cause damage in a defined animal GI disease model. The majority of this dissertation will detail the research undertaken to tackle these questions and the results of those efforts. To provide a framework for understanding why CPB2 was the focus of this dissertation, *C. perfringens* will be introduced in this chapter and information regarding the great pathogenic potential of this organism will be discussed. Special emphasis will be placed on the *C. perfringens* enterotoxin, the impetus for our early interest in CPB2 and the seed for our hypothesis, as well as information derived from fledgling studies regarding CPB2.

1.1 *Clostridium perfringens*

1.1.1 Organism (phylogeny and bacteriology)

The organism *Clostridium perfringens* was first identified in 1892 by Welch and Nuttall and has been known by a number of names including *Bacillus perfringens, Bacillus welchii*, and *Clostridium welchii* (30). *C. perfringens* was initially discovered due to its role in gas gangrene (it is now known to be the most common causative agent of clostridial myonecrosis), a scourge amongst soldiers since the inception of war (prior to the discovery of antibiotics). In fact, the name *perfringens*, meaning "breaking through", is in reference to the propensity of these bacteria to destroy or "break through" muscle tissue during clostridial myonecrosis. *C. perfringens* belongs to the class clostridia within the phylum firmicutes, where it clusters with other pathogenic classes, including bacillales, lactobacillales, and mollicutes. Within the class clostridia, *C. perfringens* is located in the order clostridiales, family clostridiacaea. The genus *Clostridium* includes many other biomedically-important bacterial pathogens including *C. difficile, C. septicum*, *C. botulinum*, and *C. tetani*. Not surprisingly, these related organisms cause diseases mediated primarily through the action of potent, proteinacious toxins.

C. perfringens is a Gram-positive, low GC content organism (a highly variable feature of the clostridia – 24-54% GC) (30). Genome sequencing of three *C. perfringens* strains has revealed an average GC content of 28%, similar to the GC content of other sequenced pathogenic clostridial genomes (102, 125). Clostridial genomes show considerable variation in size, ranging from 2 to 6.5 Mbp, possible reflecting their wide environmental-dispersal (30). Specifically, *C. perfringens* genome sizes range from 3 to 4 Mbp (based upon sequencing and pulsed-field gel electrophoresis estimates) (38, 102, 125). This large variability in genome size is not type dependent (see Table 1.1 and section 1.1.2 for further discussion on the *C. perfringens* typing scheme) and is thought to be due, in part, to the insertion of mobile genetic elements including phage-like sequences (102). Presently, it is not clear if these genome size differences reflect pathogen host specificity, allow for survival in particular environmental niches, or are "non-functional" DNA elements.

C. perfringens is a ubiquitous organism that has the widest environmental distribution of any pathogenic microorganism and can be isolated from soil, sewage, and the mammalian digestive tract (30). Distinguishing characteristics of *C. perfringens* include; non-motility, a

catalase/oxidase negative phenotype, anaerobic growth, an optimal growth temperature of 42°C, a lecithinase-positive phenotype (apparent on egg-yolk agar plates), a double-zone of hemolysis on blood agar plates (BAP), and the reduction of sulphite (seen as black colonies on Shahidi-Ferguson *perfringens* agar (SFP) plates) (4, 30). However, it should be noted that only 90% of isolates produce black colonies on SFP and that most, but not all isolates (notably, an important cluster of *C. perfringens* isolates associated with food-poisoning) produce double hemolytic zones on BAP. *C. perfringens* also produces toxins that act in synergy with toxins from Group B *streptococcus* resulting in an increased zone of beta hemolysis on BAP than isolates grown separately, which serves as the basis for the Reverse CAMP test (30).

Microscopic examination of *C. perfringens* grown in media composed of peptone, yeast extract, and glucose reveals it to be a Gram-positive, straight rod with blunt ends that vary in size from 0.6-2.4 μm by 1.3-19 μm and can be present singly or in pairs. Colonies resulting from overnight growth on blood agar plates are typically 2.5 mm in diameter, circular, entire, dome-shaped, yellowish-gray, and translucent with a glossy surface. Roughly 75% of *C. perfringens* produce a capsule composed of varying polysaccharides, possibly accounting for the glossy appearance of the colonies of many strains. These capsules have been used as the basis for serotyping *C. perfringens*; however, this method remains more popular in Japan than in other areas of the world (65, 142).

C. perfringens, while more aerotolerant than other clostridia, still requires an environment lacking O_2 for growth (69). Common methods for overcoming this growth limitation include the use of anaerobic jars or anaerobic chambers. Not surprisingly, based upon its anaerobic lifestyle, *C. perfringens* lacks most components of the TCA cycle and does not possess genes required for ATP generation via respiration (125). These bacteria derive their energy from anaerobic fermentation, producing acetic, butyric, and lactic acid as fermentation end products. These acidic end-products contribute to the low pH of cultures grown *in vitro* (which can approach a pH of 5) and are thought to produce a reducing environment during *in vivo* growth and infection (particularly in myonecrosis) that is favorable for its anaerobic life-style (141). The growth of *C. perfringens* also results in production of large amounts of H_2, highly visible at the surface of broth cultures, which is responsible for the gas associated with myonecrotic infections (gas gangrene).

A distinguishing characteristic of the firmicutes is their ability to form endospores, though many do so only poorly under laboratory conditions (30). Sporulation is typically initiated under conditions of nutrient depletion and helps to preserve genetic content until better conditions are present. Due to the importance of sporulation for the production of the

Clostridium perfringens enterotoxin (see section 1.2 for the role of CPE in disease), the known processes of sporulation as they pertain to enterotoxin (CPE) production will be briefly discussed. Sporulation can be divided into VII stages with stage 0 consisting of initiation signals resulting in a cascade of exquisitely timed events that ultimately result in autolysis and release of the mature spore (stage VII) (110). For most *cpe*-positive FP isolates, sporulation can be initiated *in vitro* by growth in Duncan-Strong media which contains starch, phosphate, and no glucose (81). These components are thought to be important signals in the initiation of sporulation (or in repressing sporulation, in the case of glucose).

Upon entry into sporulation a cascade of events occur, many of which are regulated by sporulation-specific sigma factors including $\sigma^{E,F,G,}$ and ^{K} (110). CPE production, which is restricted to the mother cell (and therefore presumably under the control of mother cell-associated σ^{E} and/or ^{K}), is thought to begin during stages I and II, since mutants blocked in stage 0 are unable to produce CPE (95, 156). Furthermore, sporulation mutants blocked in stages III, IV, and V continue to produce CPE, suggesting that the factors required for CPE production are produced during stages I and II (95). Regulation of CPE appears to be tightly governed by sporulation related processes as no *cpe* mRNA or protein can be detected during vegetative growth (42). Release of CPE occurs during autolysis of the mother cell (stage VII), i.e. CPE is not a secreted protein.

The process of sporulation in *C. perfringens* and other clostridia, while similar to the well-studied pathways identified in *Bacillus subtilis*, must contain unique mechanisms to achieve final spore formation due to the absence of early genes (such as the phospho-relay genes and regulators that activate the sporulation master switch, SpoOA) known to be required for initiation of spore formation in *Bacillus* (124). However, despite the obvious importance of spore formation in the long-term survival of *C. perfringens* as well as the role of the sporulation process and/or the spores themselves in the pathogenesis of tetanus, *Clostridium difficile* associated diarrhea (CDAD), and *C. perfringens* food-poisoning and antibiotic associated diarrhea, sporulation remains a much understudied topic in clostridia.

1.1.2 *Clostridium perfringens* typing scheme

The large arsenal of toxins (>14) produced by *C. perfringens* has been used to subdivide *C. perfringens* into five toxinotypes, A-E, based upon the production of four toxins, $\alpha, \beta, \varepsilon,$ and

ι toxins, which are classically referred to as the four major *C. perfringens* lethal toxins (131). The first four types, A-D, were established by Wilsdon in 1931 with the fifth type, E, introduced in 1943 by Bosworth (30). The α toxin is produced by all toxinotypes, whereas differential production of β, ε, and ι toxin serves as the basis for separating *C. perfringens* into types B-E (Table 1.1). Not surprisingly, due to its presence in all toxinotypes, the *plc* gene (encoding α toxin) is located on the chromosome, while the other typing toxin genes are located on large virulence plasmids (107).

Classically, isolates were typed using a guinea pig skin test or a mouse lethality assay (30). For the first assay, supernatants from isolates of unknown types were pre-incubated with or without type-specific neutralizing rabbit antiserum and then injected intradermally into the back skin of guinea pigs. Prevention of visible pathology by pre-treatment with a specific antiserum compared to an untreated sample would identify the isolate type. For example, supernatant from a type E isolate would not cause pathology at the injection site when the sample was pre-treated with type E specific antiserum, but would cause pathology if untreated or pre-treated with anti-type A, C, or D antiserum. Using a similar experimental design, isolates also were typed by assessing the lethality of untreated or pre-treated (with type specific antiserum) supernatants from unknown isolates in a mouse intravenous injection (via the tail vein) model. It is important to note that the type-specific neutralizing antiserum used for these assays was often developed by injecting rabbits with crude toxoids derived from supernatants. Hence, type-specific antiserum may have been neutralizing other toxigenic factors plus the typing toxins.

Currently, a molecular biology-based typing approach has mostly replaced the animal-based typing assays. This new assay, known as the multiplex PCR toxin genotyping assay, uses primers that amplify internal fragments of the genes encoding the four typing toxins as well as the genes of two other lethal and biomedically-relevant toxins, *cpe* and the *C. perfringens* beta2 toxin (*cpb2*) (Figure 1.1) (51). While the multiplex PCR assay 1) provides faster typing results, 2) gives information about the presence of non-typing toxins (*cpe* and *cpb2*), 3) is more economical, and 4) reduces animal use compared to the guinea pig skin test and mouse lethality assay, it does not provide information regarding the actual production of toxins, but merely the presence of a portion of the toxin gene. This is an important distinction in light of recent reports that have identified a number of isolates that fail to produce detectable levels of their typing toxin *in vitro* (48, 121), thus indicating that toxin genotype and toxin phenotype do not always correlate.

Table 1.1 *C. perfringens* toxinotyping scheme, toxin genotypes, and type/disease associations.

	Typing Toxins[a]					Diseases[e]	
Toxinotype	α[b]	β[c]	ε[c]	ι[c]	Toxin genotypes[d]	Human	Domestic Animals
A	+	-	-	-	*plc*	Gangrene	Diarrhea (dogs, pigs, etc.)
					plc, *cpe*[f/b/c]	GI disease (food poisoning,	Necrotic enteritis (fowl)
					plc, *cpb2*[c/f]	antibiotic associated diarrhea,	
					plc, *cpb2, cpe*	sporadic diarrhea)	
B	+	+	+	-	*plc*, *cpb*[b], *etx*[b]		Dysentery (lambs)
					plc, *cpb*, *etx*, *cpb2*		Enterotoxemia (sheep)
C	+	+	-	-	*plc*, *cpb*	Enteritis necroticans (pigbel)	Necrotic enteritis (piglets,
					plc, *cpb*, *cpb2*		foals, etc.)
					plc, *cpb*, *cpb2, cpe*		Acute Enterotoxemia (adult sheep)
					plc, *cpb*, *cpe*		
D	+	-	+	-	*plc*, *etx*		Enterotoxemia (goats, sheep,
					plc, *etx*, *cpb2*		etc.)
					plc, *etx*, *cpb2, cpe*		
					plc, *etx*, *cpe*		
E	+	-	-	+	*plc*, *iap/ibp*, *cpe*[g]		Enterotoxemia (calves and rabbits)
					plc, *iap/ibp*, *cpe*[g], *cpb2*		

[a]Genes encoding toxins are *plc* (α), *cpb* (β), *etx* (ε), *iap/ibp* (ι)

[b]Toxin gene is located on the chromosome

[c]Toxin gene is located on a large virulence plasmid (50-150 kb)

[d]Possible toxin genotypes determined using multiplex PCR typing method

[e]Diseases are listed for each toxinotype and are not organized to correlate with the toxin genotypes

[f]*C. perfringens* enterotoxin (CPE) and *C. perfringens* beta2 toxin (CPB2)

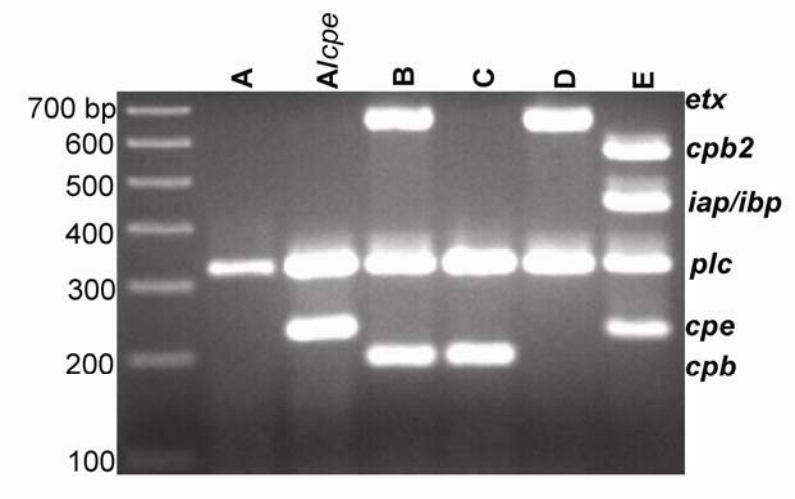

Figure 1.1 The *C. perfringens* multiplex PCR genotyping assay. PCR was performed on colony lysates and products were run on a 2% agarose gel and stained with ethidium bromide (modified from Figure 1 from (50)).

1.1.3 *Clostridium perfringens*-associated diseases

C. perfringens holds the distinction of being the most widely dispersed pathogenic bacteria, and arguably, also produces the most toxins of any bacterial species (134). However, as mentioned in 1.1.2, an individual isolate does not produce every toxin, allowing *C. perfringens* to be separated into five toxinotypes. Not surprisingly, owing to the production of different toxins with differing modes of actions, *C. perfringens* types are associated with distinct diseases (Table 1.1). Most infamously, *C. perfringens* type A isolates are known to be the primary agent responsible for clostridial myonecrosis (also referred to as gas gangrene) (113). Clostridial myonecrosis is a very destructive infection of muscle tissue that requires aggressive treatment, most commonly gross amputation, to prevent death of the infected animal or patient (23). The association between type A isolates and gas gangrene is thought to be due to 1) the overwhelming preponderance of type A isolates in the environment and mammalian digestive tract compared to types B-E (type A isolates represent >95% of *C. perfringens* isolates) (27)

and 2) an increased production of α toxin by *C. perfringens* bacteria isolated from gas gangrene cases (113). In an elegant study that has served as a model for determining the role of individual toxins in clostridial disease, single and double isogenic α toxin and perfringolysin O (*pfoA*) toxin knockout mutants were used to demonstrate that α toxin is necessary and sufficient to elicit most of the pathology associated with experimental gas gangrene (PFO was found to play an accessory role in disease) (7). Interestingly, α toxin was the first toxin for which enzymatic activity was ascribed (86). A summary of basic physicochemical properties, mechanism of actions, and LD_{50} values of toxins important for this dissertation are listed in Table 1.2.

Table 1.2 Physical and biological properties of select *C. perfringens* toxins.

	Typing toxins			Other toxins		
Properties	**α**	**β**	**ε**	**CPE**	**CPB2**	**PFO**
size, kDa	43	35	33	35	28	54
delivery method	secreted	secreted	secreted	intracellular[a]	secreted	secreted
receptor	phospholipids/sphingomyelin	unknown	unknown	claudins	unknown	cholesterol
mode of action	phospholipase C[c]	pore formation	pore formation	pore formation	?	pore formation
LD_{50}, i.v.[b]	3 μg	30 ng	10 ng	1-2 μg	3-30 μg	13-16 μg

[a]released upon mother cell lysis at the end of sporulation, [b]LD_{50} reported for mice, [c]also initiates cell signaling cascades

In opposition to gas gangrene, which is a very aggressive and invasive infection, disease caused by type B-E isolates and CPE-producing type A isolates (to be discussed in more detail in section 1.2) are non-invasive enterotoxaemias (Table 1.1) (134). Type B, D, and E isolates are primarily associated with diseases of domestic animals that initiate in the intestine. Animals suffering from diseases caused by type B-D isolates often show signs of systemic toxicity including neurological distress and edema in some organs and body cavities, believed to result from the action of ε and/or β toxins circulating systemically after crossing the intestinal epithelium. The mechanism by which these toxins breach the intestinal barrier is not known, and it has been hypothesized that accessory toxins (all *C. perfringens* isolates produce more than one lethal toxin) may facilitate this process. Once systemic, ε and β toxins act as potent neurotoxins (ε and β toxins are the third and fourth most potent clostridial toxins, respectably,

after botulinum and tetanus toxins); the associated neurotoxicity and low LD_{50} of ε toxin has led to its inclusion as a class B select agent on the overlap CDC and USDA select agent lists (31).

Type C isolates hold the distinction of being the only non-type A isolates known to routinely cause enterotoxaemias in humans as well as animals (82). Type C isolates cause necrotic or hemorrhagic enteritis in domestic animals (most commonly in young animals such as piglets and foals) while in adult sheep, type C isolates cause a rapidly fatal enterotoxaemia known more colorfully as struck (the animals are reported to die so fast they appear to have been "struck" by lightning) (134). For unknown reasons, struck has only been reported in the United Kingdom. Type C isolates also cause disease in humans known as enteritis necroticans (EN). EN was first associated with type C isolates in the late 1940s in Germany where it was called Darmbrand (82) and later identified in large outbreaks of EN in Papua, New Guinea (where it is known as pigbel (83)) and in Khmer Rouge camps in Cambodia (70). The incidence of EN in Papua, New Guinea (where it was once the leading cause of death in children over 1 year in age (2)) was greatly reduced following a vaccine campaign using a type C supernatant toxoid. While rare, EN cases do appear in post-industrial countries, including the United States, typically in persons with pre-existing medical conditions (such as pancreatic disease) that affect normal function of the gastrointestinal tract (56, 84, 108, 122, 134, 144). These cases are typically quite severe, often requiring surgical re-sectioning of the affected portion of the intestine and can result in death.

Since type B-D isolates produce multiple toxins (their typing toxins plus non-typing toxins), it is not known which toxin(s) contribute to the subsequent disease pathology. Vaccines against type B-D infections (and type C infections in pigbel endemic areas) have been effective in reducing the incidence of disease in domestic animals (which in some species cause herd morbidity rates of 15-50% and mortality rates approaching 100% (134)). However, these vaccines require repeated administration due to waning immunity (which last 6-12 months (134)), vaccine efficacy varies between companies, and vaccine composition varies from supernatant toxoids alone to toxoids with or without bacterin (121, 138, 145, 150). The multi-component nature of these vaccines makes it difficult to determine which factor is actually eliciting the protective immune response and an open debate remains as to the importance of the antitoxin vs. the antibacterial immune response (134). Ideally, vaccines should provide long-term protection with a minimal injection routine. Toxin phenotype analysis and the construction and testing of isogenic toxin knockout mutants in experimental disease models to determine which toxin(s) are necessary and/or sufficient for disease would allow companies to target

vaccines to specific antigens, possibly increasing the efficacy and time period of protection afforded by vaccination.

1.2 INTRODUCTION TO CPE AND FOODBORNE VERSUS NON-FOODBORNE GASTROINTESTINAL DISEASE

1.2.1 *Clostridium perfringens* gastrointestinal disease and CPE

1.2.1.1 Discovery of CPE. The role of *C. perfringens* in food poisoning was first suggested by McClung in 1945 (93) and by Hobbs *et al.* (62, 63) who identified large growths of heat resistant, *C. perfringens* type A isolates in food samples associated with food-poisoning outbreaks. Subsequent human experimental trials demonstrated that a select group of *C. perfringens* type A isolates could cause food-poisoning like symptoms in volunteers when $5x10^8$ viable bacteria where introduced orally (4). Further studies of these food-poisoning associated bacteria identified a protein factor in the supernatants of sporulating cultures that caused fluid accumulation in rabbit ileal loops (a feature consistent with diarrhea) (139, 140). This protein factor was called the *C. perfringens* enterotoxin (CPE).

Two studies have since solidified the role of CPE in human GI disease. Firstly, purified CPE, ingested by human volunteers, is sufficient to cause the symptoms of food poisoning including diarrhea and cramping (130). Secondly, a molecular biology based approach, in which the *cpe* genes from two *cpe*-positive human GI disease isolates were inactivated, further established the requirement for the production of CPE to elicit GI damage in an experimental animal disease model (119). In this second study, which satisfies molecular Koch's postulates, Δ*cpe*-mutants were unable to cause pathology in a rabbit ileal loop model whereas the parent isolates and Δc*pe*-mutants complemented with a *cpe*-expressing vector caused damage in rabbit ileal loops including flattening and desquamation of intestinal villi and fluid accumulation. These morphological and physiological changes in rabbit ileal loops are consistent with changes induced by treatment of human ileal tissue with CPE in an Ussing chamber (47).

Currently, CPE-producing *C. perfringens* type A isolates are the third most common cause of food-poisoning in the United States (and the second leading cause of food-poisoning in the United Kingdom) (3, 106). Symptoms of CPE food-poisoning (FP) typically begin ~12 hrs after ingestion of viable bacteria from a contaminated food source (typically meat products improperly cooked or stored) and include diarrhea and cramping, sometimes accompanied by nausea (127). Symptoms typically resolve themselves within 24 hrs of onset. The onset of disease symptoms correlates with the time required for *C. perfringens* to complete sporulation (as discussed in section 1.1.1, CPE is only produced during sporulation). Vegetative *C. perfringens* typically sporulate within the gastrointestinal tract in response to unknown signals present in the stomach and/or intestines, though a recent study has suggested that inorganic phosphate levels may stimulate sporulation (109). Once end stage sporulation has been completed the mother cell will lyse and release CPE into the lumen of the intestine. Methods for identifying CPE-producing *C. perfringens* as the causative agent of foodborne disease include; 1) a count of 10^5 or greater *C. perfringens* CFU per g of suspected food, 2) 10^6 or greater CFU per g of patient stool, 3) isolation of CPE-producing bacteria from a patient's stool, and 4) direct detection of CPE using serological methods (4).

Studies in the 1980s began to suggest that CPE-producing *C. perfringens* isolates also were associated with sporadic diarrhea (SD) in the community as well as nosocomial infections in patients undergoing antibiotic therapy (antibiotic associated diarrhea, or AAD) with penicillin, cephalosporins, trimethoprim, or cotrimoxazole (6, 16-19, 21, 68, 87, 100, 101). Recently, CPE-producing *C. perfringens* isolates also have been associated with nosocomial infections in patients not undergoing antibiotic therapy (5). AAD patients were typically elderly and were not colonized by *Clostridium difficile* (another common cause of nosocomial diarrhea). In contrast to food poisoning symptoms, these patients had diarrheic episodes with a median duration of seven days and some patients had relapses of diarrhea (18, 100). It is estimated that CPE-producing *C. perfringens* may account for 5-15% of nosocomial infections in the United States and the United Kingdom (100). Interestingly, AAD/SD infections are thought to be initiated by a smaller inoculum of bacteria than food poisoning infections (29).

It is now known that the *cpe* gene is also carried by a number of type C and D isolates, and that these isolates can produce CPE *in vitro* (48, 121). The *cpe* gene has not been reported in type B isolates and type E isolates carry a silent copy of the *cpe* gene on a plasmid upstream of *iab*/*ibp* (15). The role of CPE in type C and D diseases of domestic animals and humans (in the case of type C isolates) is not known. Interestingly, a recent study determined that a large

number of type C isolates from human EN cases are positive for *cpe* and that these isolates are capable of producing the toxin *in vitro* (48).

1.2.1.2 Mechanism of action of CPE. Purification of CPE in the 1970s (140), identification of CPE-sensitive cells enabling *in vitro* studies (91), and the subsequent cloning of the *cpe* gene (43), have allowed the mechanism of action of CPE as well as structure/function analysis to be intensely studied. The collective findings of these studies will be briefly discussed in this subsection.

CPE is produced as a 319 amino acid single polypeptide of ~35 kDa that is released into the environment at the end stage of sporulation upon lysis of the mother cell. Once free from the bacteria, CPE recognizes and binds to its receptor on CPE-sensitive cells via a binding domain localized within the 30 C-terminal amino acids. CPE is known to bind to certain claudins, a family of >20 small, transmembrane proteins found in tight junctions. This interaction manifests itself as a SDS-sensitive, non-cytotoxic small complex of approximately 90 kDa. After the initial binding step, CPE is believed to interact with other CPE molecules via an N-terminal latch domain resulting in the formation of a larger (155 kDa), SDS-resistant complex. Formation of this 155 kDa large complex is concomitant with disruption of membrane permeability (induced via a mechanism consistent with pore formation) and results in cell death via apoptosis or oncosis. The resultant cell death pathway is dependent upon the dose of toxin used *in vitro* and is directly related to the amount of calcium entering the cell through the CPE-induced cell lesion. Following extended incubation of CPE with certain CPE-sensitive cells, a second SDS-resistant large complex (200 kDa) will form, resulting from interactions between CPE and occludin (another tight junction associated protein). However, this 200 kDa large complex is not required for cytotoxicity. These CPE-mediated processes also cause disruption of epithelial tight junctions. For a more detailed review on these *in vitro* experimental findings see (131).

The *in vivo* effects of CPE have been elucidated using a rabbit ileal loop disease model and purified CPE (124). Soon after treatment with 75 μg/ml of CPE, rabbit ileal loops stop absorbing fluid and eventually fluid transport is reversed, which results in a net secretion of fluid into the lumen of the intestine. This is consistent with the resultant diarrhea seen in human patients, and recent studies with human ileal tissue treated with CPE in Ussing chambers showed an increase of fluid in the apical side of the Ussing chamber (47). The dosage of CPE used in the rabbit ileal loop studies is within the range of CPE measured in the stool samples from patients suffering from CPE-associated GI disease, which varies from 1 μg/ml to 100 μg/ml

(32). Micro histopathological examination of CPE-treated ileal loops found evidence of villous blunting and desquamation (particularly at the tips of villi).

1.2.2 The genetics of *cpe* in relation to disease

1.2.2.1 Genetics. As discussed in section 1.1.1, CPE production is regulated by sporulation specific factors. This regulatory control mechanism results in expression of *cpe* mRNA and production of CPE only during sporulation, beginning during stage I of sporulation. Experiments in which the *cpe* gene was introduced into *cpe*-negative *C. perfringens* isolates demonstrated that the sporulation-specific regulation of CPE production is maintained in naturally *cpe*-negative type A-D isolates (42). Recent studies also have shown that naturally *cpe*-positive type C and D isolates only produce CPE during sporulation (48, 121).

Information regarding the location of the *cpe* gene within the *C. perfringens* genome and organization of the *cpe* locus was first published by Cornillot *et al.* who determined that *cpe* could be located on either a plasmid (veterinary GI disease isolates) or on the chromosome (FP isolates) (41). When encoded by a plasmid, the *cpe* gene was flanked by an IS*1151* element, which also has been shown to flank a number of other plasmid-encoded *C. perfringens* toxin genes including *etx* and *iab/ibp*. IS*1151*-mediated mobility has been suggested as a potential mechanism for transfer of these toxin genes between segments of DNA.

Further supporting the dual location of *cpe*, work by Collie and McClane demonstrated that isolates associated with human AAD and SD cases carry a plasmid *cpe* gene (~100 kb), while FP isolates typically carry a chromosomal *cpe* gene (40). Subsequent research has demonstrated that chromosomal *cpe* isolates are much more resistant to environmental stresses, such as heat and cold, than are plasmid *cpe* isolates (85, 120). This resistance to environmental stress may explain why chromosomal *cpe* isolates act as agents of food-poisoning. Neither the *cpe* gene, CPE, nor the *cpe* plasmid are responsible for the environmental sensitivity differences between chromosomal and plasmid *cpe*-positive disease isolates (111). Furthermore, a survey of foods for *cpe*-positive *C. perfringens* isolates only identified chromosomal *cpe* isolates, suggesting that these isolates may reside within food reservoirs making them more likely to be associated with foodborne illness (154).

Restriction fragment length polymorphism (RFLP) analysis using either *Eco*RI or *Nru*I followed by Southern blot analysis to detect the *cpe* gene determined that *cpe* is present on

either 10 kb or 5 kb fragments, respectively, in FP isolates, but is typically encoded on >20 kb fragments in AAD/SD isolates and most *C. perfringens* type A veterinary GI disease isolates (9 of 10 isolates) (137). Later, limited sequencing of the plasmid (97) and chromosomal (26) *cpe* loci from AAD and FP isolates identified various insertion elements flanking the *cpe* gene (Figure 1.2). While all *cpe* loci contain an IS*1469* sequence upstream of *cpe*, the loci then diverge resulting in two different plasmid *cpe* loci and one chromosomal *cpe* locus. A multiplex PCR assay designed to specifically amplify fragments of differing length from the variant *cpe* loci have not identified a fourth locus, i.e. no unexpected fragments were amplified using this PCR approach to survey a collection of FP, AAD, and SD isolates (99).

The IS elements near the *cpe* gene (Figure 1.2) have been hypothesized to mobilize *cpe*, possible explaining its plasmid or chromosomal location. Supporting this hypothesis, Brynestad *et al.* were able to demonstrate, via PCR, the presence of circular DNA elements carrying different combinations of *cpe* and its flanking IS elements (24). Unfortunately, while showing that those insertions elements could mobilize DNA from the chromosome, reinsertion of the elements back into the chromosome could not be detected.

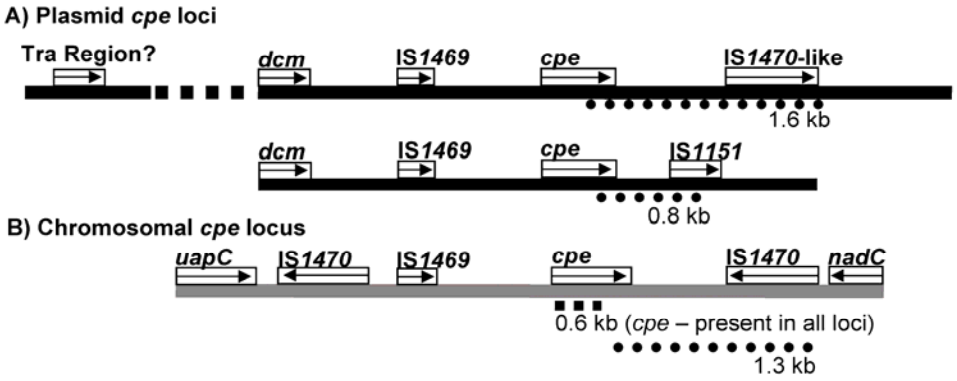

Figure 1.2 Organization of the *cpe* loci. Dotted lines represent the products of locus specific PCR reactions (99).

1.2.2.2 *cpe* location and disease relationship. Interestingly, FP cases and AAD/SD cases are not only caused by isolates with different *cpe* loci and different environmental stress resistance characteristics, but patients presenting with FP versus AAD/SD also have different clinical disease symptoms. FP cases have symptoms that are less severe and shorter in duration, with diarrhea and cramping lasting ~24 hours after symptoms first begin with patients producing loose stool without blood (127). After symptoms resolve (typically without antibiotic treatment), GI disease does not normally reoccur in the absence of a second case of FP, i.e. new FP isolates must be introduced to initiate a second diarrheic episode. In contrast to FP, patients with AAD or SD typically have episodes of diarrhea lasting, on average, one week and these symptoms include cramping and diarrhea accompanied with blood and mucous (18, 100).

A number of hypotheses to explain the differing symptomatic etiologies of AAD versus FP have been explored. FP and AAD isolates produce, on average, the same amount of CPE and the production of CPE is restricted to sporulating conditions for both FP and AAD isolates (39). The CPE amino acid sequences also are identical between FP and AAD isolates and, subsequently, their CPE protein is equally cytotoxic for CPE-sensitive cell lines (39). An experiment demonstrating the ability of the *cpe* plasmid carrying the IS*1470*-like gene to transfer between naturally *cpe* negative *C. perfringens* isolates via conjugation (Figure 1.2 A) does offer a possible explanation for the protracted symptoms of AAD cases (25). Transfer of the *cpe* plasmid to resident *C. perfringens* flora that are adapted for colonization of the intestinal tract would allow for repeated episodes of diarrhea as spores remaining in the GI tract could recolonize the intestinal epithelium upon germination. However, this scenario does not completely explain why AAD symptoms are more severe than the symptoms resulting from FP.

1.3 THE *Clostridium perfringens* BETA2 TOXIN

1.3.1 Discovery of the *cpb2* gene

1.3.1.1 Identification of *cpb2*. The *C. perfringens* beta2 toxin (CPB2) was first identified in 1997 making CPB2 the newest toxin for the *C. perfringens* field (52). The discovery of CPB2 is due, in

part, to work by Hunter *et al.* that identified the DNA sequence of *cpb* (66). Based on the predicted *cpb* ORF, β toxin was expected to have a mass after proteolytic removal of the secretion signal (identified using *in silico* analysis and experimentally verified via N-term sequencing) of 34,861 Da. Previously, the molecular weight of β toxin purified from type C isolates had been reported as ranging from 20-42 kDa based on SDS-PAGE and gel filtration chromatography (116-118). This discrepancy in mass has been attributed, in part, to the proteolytic sensitivity of β toxin. The predicted ~35 kDa weight of β toxin conflicted specifically with a previous study that identified a 28 kDa protein purified from a type C isolate that was initially thought to be β toxin (71). This 28 kDa protein caused hemorrhagic necrosis of the intestinal mucosa without fluid accumulation (at high doses) in a guinea pig ileal loop model and was cytotoxic for Chinese Hamster Ovary (CHO) cells. However, in light of the definitive determination of the molecular weight of β toxin, researchers that took part in the Jolivet-Reynaud *et al.* (71) study sought to determine if the 28 kDa protein was an unidentified *C. perfringens* toxin. This follow up study established that the 28 kDa protein was in fact an unidentified toxin, which they named the *C. perfringens* beta2 toxin (CPB2) (52).

Using the type C strain CWC245, Gibert *et al.* (52) identified the *cpb2* gene using degenerate PCR primers based on the sequencing of N-terminal and internal amino acid fragments of CPB2 purified from CWC245. Sequencing of a *cpb2*-positive 1.3 kb DNA fragment, determined that CPB2 is encoded by an ORF of 798 nucleotides (including the termination codon) coding for 265 amino acid with a predicted molecular weight of 30962 Da. CPB2 has no similarity at the amino acid or nucleotide level with any genes of known or unknown function within the GenBank database, including β toxin (despite their similar names). Seven nucleotides upstream of the ATG initiation codon is a consensus ribosomal binding site (GGGGG) and an inverted repeat is located immediately after the termination codon. ΔG values (-20 kcal) for the inverted repeat indicated that a stable hairpin structure could form, possible resulting in rho-independent termination of transcription. More recent work (104) has identified the -35 and -10 boxes and the transcriptional start site, located 19-bp upstream from the initiation codon by performing promoter extension analysis using the *cpb2*-positive *C. perfringens* type A isolate, strain 13 (125). The *cpb2* ORF and 5' and 3' sequences of the *cpb2* genes from CWC245 (type C, original *cpb2* gene) and strain 13 are highly conserved at the nucleotide level (95% identical ORFs) (Figure 1.3).

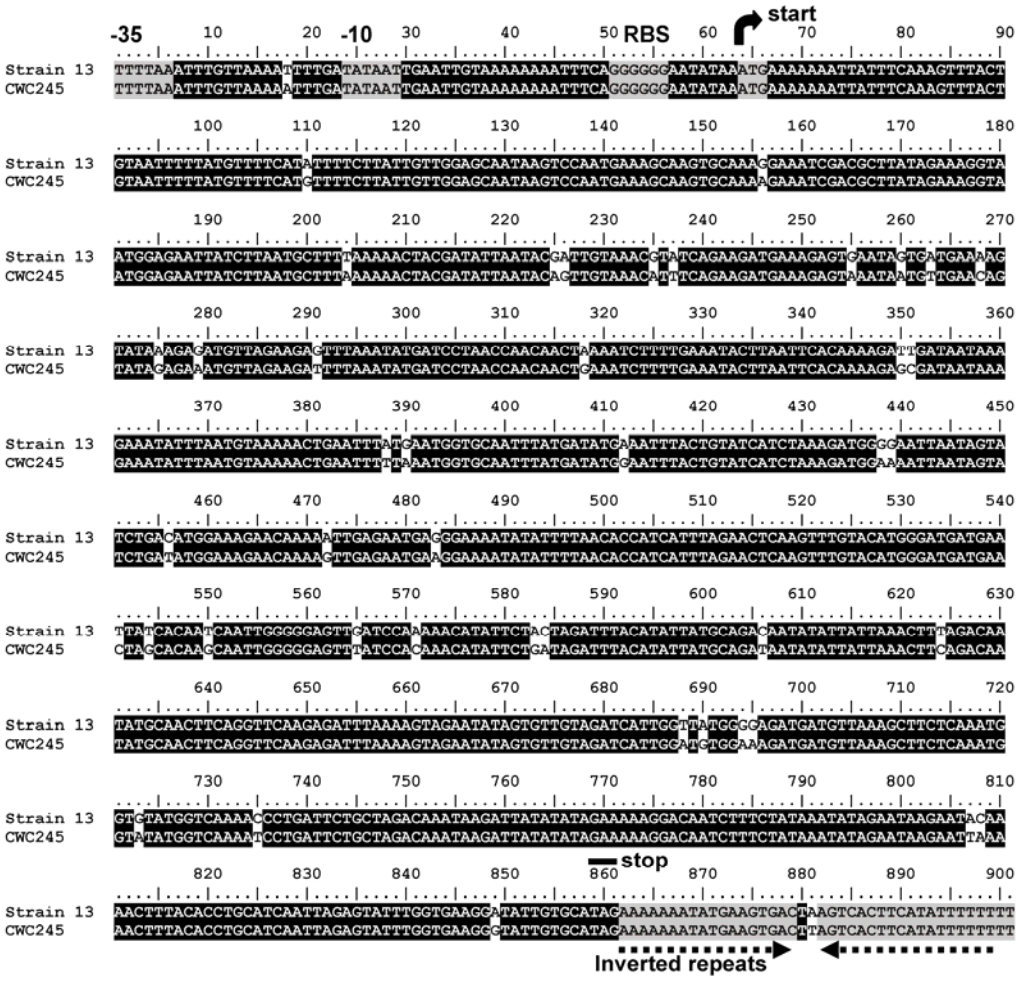

Figure 1.3 Nucleotide alignment of the *cpb2* gene from *C. perfringens* type A (strain 13) and type C (CWC245) strains. The -35, -10, ribosomal binding site (RBS), start codon, and inverted repeats are identified by gray shading.

1.3.1.2 Mapping of *cpb2*. Southern blotting analysis to determine the location of the *cpb2* gene in *cpb2*-positive, *cpe*-negative type A and C isolates localized *cpb2* to plasmids (120-140 kb, N = 11) (52). *Eco*RI restriction enzyme digestion of these plasmids followed by Southern blotting to detect *cpb2* identified 3 different sized fragments that hybridized with the *cpb2* probe. In this study, *cpb* and *cpb2* hybridized to different fragments in type C isolates suggesting that the toxin

genes are not close together on the “type C” plasmid, or alternatively, that they are located on separate plasmids.

More recent Southern blot analysis of *cpb2*-positive type A and type C porcine GI disease isolates (N = 18) (153) and *cpb2*-positive type A equine GI disease isolates (N = 7) (152) determined that *cpb2* is located on ~50 kb plasmids (N = 18). RFLP/Southern blotting analysis of those porcine and equine disease isolates with *Ecor*RV and *Hpa*I identified at least 4 different sized *cpb2* fragments using *Hpa*I (5 kb to >20 kb) and either 4 or 2 different sized fragments using *Eco*RV (6 kb to <20 kb) for the porcine or equine disease isolates, respectively.

In strain 13, which has a sequenced genome, *cpb2* is present on a 54310 bp plasmid (pCP13) (125). Digestion of this plasmid with either *Eco*RV or *Hpa*I should generate *cpb2*-bearing fragments of ~5300 kb or ~29600 kb, respectively (based on *in silico* analysis). These sizes are consistent with the results for 21 of the 23 equine disease isolates, but only one of the 29 *cpb2*-positive porcine disease isolates analyzed via RFLP/Southern blotting. These studies suggest that *cpb2* can be located on different sized plasmids and that the *cpb2* locus varies.

The location of *cpb2* on a large plasmid is similar to a number of other *C. perfringens* toxin genes, including the *cpe, etx, cpb,* and *iab/ibp* toxin genes, which have been localized to plasmids ranging in size from ~90-140 kb (40, 46, 77). However, with the exception of the *cpe* loci, little work has been done to determine whether the loci of other toxin genes from *C. perfringens* virulence plasmids are as variable as the *cpb2* locus (based on RFLP/Southern blotting results).

1.3.1.3 Production of CPB2. Western blot detection of CPB2 in culture supernatants grown under vegetative conditions indicates that CPB2 can be secreted by *C. perfringens* (27, 52). The size of the mature peptide from CWC245 is predicted to be 26670 Da, very close to the experimentally determined mass of 28 kDa via Western blot analysis. The predicted pI of CPB2 is 5.01 (with an experimental pI of 5.4-5.5) (52). The relative similarity between the size (28 vs. 35 kDa) and isoelectric points of CPB2 and β toxin (experimental pI of 5.6) coupled with the possible presence of CPB2 in type C supernatants (~50% of type C isolates are *cpb2*-positive) makes it plausible that the different sizes reported for purified β toxin in early studies (pre-1988) may have be due to purification of CPB2 instead of β toxin.

In silico analysis (http://www.bioinformatics.leeds.ac.uk/prot_analysis/Signal.html) of the translated amino acid sequence of the *cpb2* gene predicts a typical Gram-positive secretion signal comprising the first 30 amino acids with cleavage between the 30th and 31st amino acids

(27 KASA-K 31). The predicted signal peptide cleavage site was confirmed via N-terminal amino acid sequencing of CPB2 purified from CWC245 supernatants (52). Amino acid sequence alignments between the CPB2 ORFs from strain 13 and CWC245 reveal that these proteins are 92% identical (Figure 1.4). The variation in CPB2 amino acid sequence between these isolates is unique among *C. perfringens* toxins compared to CPE, PFO, β, ι, ε, or α toxin which are typically at least 98% identical (with the exception of a single α toxin variant that shows 80% identity to the normal α toxin sequence (76)).

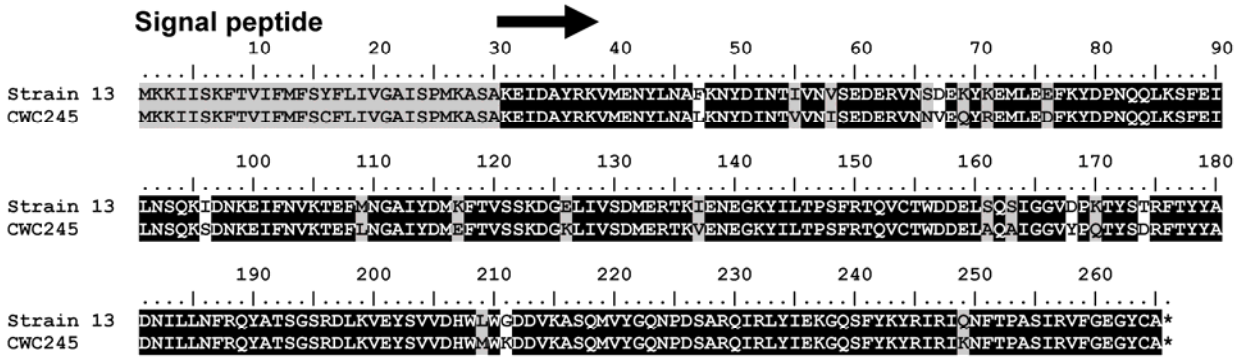

Figure 1.4 Amino acid alignment of CPB2 from *C. perfringens* type A strain 13 and type C strain CWC245. The signal peptide is shown in gray and the first amino acid present in the mature peptide (position 31) is indicated by the black arrow at the top of the alignment.

1.3.1.4 Regulation of *cpb2*. In the *C. perfringens* type A isolate, strain 13, expression of *cpb2* is known to be controlled by a two-component bacterial regulator, VirR and VirS (104). VirS is a predicted transmembrane protein that functions as a sensor histidine kinase (8). Upon binding of an unknown signal (presumably a small peptide) VirS undergoes an autophosphorylation process that adds a phosphate group to a C-terminal histidine residue. This phosphate group is transferred to an aspartic acid group on VirR, the response regulator. Activated VirR then binds to conserved regions in DNA known as VirR boxes and either promotes or represses

transcription. In the best understood VirS/VirR pathway, VirR binds to two adjacent VirR boxes in the *pfoA* promoter resulting in expression of *pfoA* (35). Activated VirR also can indirectly regulate genes which do not have VirR boxes present in their promoter region through the action of a regulatory RNA molecule named VR-RNA (VirR regulated RNA) (126). However, the mechanism of VR-RNA regulation is not understood.

VirR/VirS have been shown to directly and indirectly regulate a number of *C. perfringens* proteins in a positive or negative manner, including at least two other *C. perfringens* toxins, PFO (directly, see above) and *plc* (indirectly) (126). Similar to *plc*, *cpb2* also is positively regulated in an indirect manner by VirR/VirS via VR-RNA (104). This was determined by measuring production of *cpb2* mRNA transcript in strain 13 isolates harboring isogenic VirR or VR-RNA knockouts. Both knockout constructs resulted in reduced expression of the 1.2 kb *cpb2* mRNA transcript, which could be restored by complementing with vectors producing VirR or VR-RNA. The *cpb2* gene is the first plasmid-encoded toxin gene in *C. perfringens* shown to be regulated by a chromosomally-encoded bacterial two-component regulator. This study also found that expression of *cpb2* mRNA peaks during late-log phase under vegetative growth conditions and the size of the mRNA transcript (1.2 kb) suggest that *cpb2* is not present in an operon. Unfortunately, experiments to determine whether *cpb2* expression is similarly regulated by VirR/VirS in different isolates or *C. perfringens* types were not performed. It also should be noted that this study did not attempt to detect the production of CPB2 in culture supernatants.

Interestingly, at least two other regulatory mechanisms to control CPB2 production have been postulated, both involving *cpb2*-positive type A equine disease isolates. It has been reported that a number of *cpb2*-positive type A equine disease isolates fail to produce CPB2 *in vitro* under culture conditions in which most type A and C porcine disease isolates produce CPB2 (148, 152). This was surprising since two studies have demonstrated, via immunohistochemistry, the presence of CPB2 at intestinal lesion sites in horses suffering from GI disease (but not in healthy, control horses) suggesting that CPB2 was produced *in vivo* (9, 148). In one of these studies (148), DNA sequencing analyses of the *cpb2* gene from a number of equine disease isolates revealed the loss of a single nucleotide within the poly-A tract located immediately after the *cpb2* initiation codon (Figure 1.3), which is similar to the “slippery codon sequence” present in a number of transposases and viruses (33, 149, 151). This deletion would result in a frame shift mutation and a truncated protein (after 9 amino acids). The animal sources of these isolates carrying the abnormal *cpb2* genes had shown increased signs of disease and died after gentamicin treatment. Growth of these abnormal *cpb2*-positive equine isolates in the presence of gentamicin resulted in the production of detectable levels of CPB2,

possibly due to interaction between gentamicin and the 50s ribosomal subunit causing the ribosome to misread the shortened poly-A tract, resulting in production of CPB2. This control of CPB2 production by translational slippage is the first demonstration of ribosomal frame shifting controlling the production of a toxin (148). Similar induction of CPB2 production was seen by treating cultures with streptomycin, which also interferes with ribosomal activity. This mechanism of regulation could allow *C. perfringens* to respond to environmental stress by the production of toxins.

In contrast to this study, a recent study of *cpb2*-positive type A equine disease isolates failing to produce CPB2 found that the *cpb2* ORFs were intact (152). However, these isolates produced low levels of *cpb2* mRNA compared to the *cpb2* mRNA levels of *cpb2*-positive porcine disease isolates that produced detectable levels of CPB2. The level of mRNA, and subsequently the production of CPB2, could be induced by artificially increasing the number of *cpb2* gene copies through the introduction of *cpb2* on a multicopy *C. perfringens* shuttle vector. The low levels of *cpb2* mRNA in these isolates under *in vitro* conditions could not be explained by differences in transcript length, differences in sequences upstream or downstream of the *cpb2* ORF, or differences within the ORF. It is possible that mRNA levels are increased during *in vivo* growth compared to *in vitro* culture conditions. Oddly, the *cpb2*-positive equine disease isolates used in this study were all collected from diseased horses located in similar parts of Europe as the previous translational slippage study (148), yet these isolates show heterogeneity in gene regulation and amino acid sequence. The variation in *cpb2* plasmid size, loci, nucleotide and amino acid sequence, and regulation sets it apart from other *C. perfringens* toxins.

1.3.2 Epidemiological studies linking CPB2 to gastrointestinal disease in domestic animals

Early studies to evaluate a possible link between CPB2 and gastrointestinal diseases of domestic animals were primarily epidemiological in nature. The criterion used by those studies to establish that CPB2 might be involved in disease was to determine whether the *cpb2* gene had a higher or exclusive association with diseased animals compared to healthy animals. Essentially, these studies used PCR or Southern blotting hybridization assays to detect the *cpb2* gene in *C. perfringens* isolated from the feces and intestinal contents of diseased and healthy domestic animals. Obviously, an epidemiology study can only determine whether or not CPB2 is linked with disease, but cannot elucidate how the factor contributes to disease.

Furthermore, it remains possible that CPB2 might be associated with disease only as a marker of disease and may not be directly responsible for the pathology observed with the particular disease. Nonetheless, the presence of a correlation between *cpb2* and gastrointestinal disease would indicate that it at least has the potential to cause disease.

A summary of the more conclusive epidemiological studies (those studies that included healthy animal controls) is shown in Table 1.3. The strongest epidemiological link between *cpb2* and gastrointestinal disease is found amongst swine, and particularly amongst piglets (135). Healthy swine very rarely carry *cpb2*-positive *C. perfringens* isolates and almost all *cpb2*-positive porcine disease isolates are able to produce CPB2 *in vitro*. A reasonable case also could be made for *cpb2*-positive isolates playing a role in equine typhlocolitis (9, 27, 60, 148). More recent work has demonstrated that a number of *cpb2*-positive equine disease isolates fail to produce CPB2 *in vitro* unless grown in the presence of sub-inhibitory concentrations of gentamicin (as discussed in section 1.3.1.3) suggesting that these isolates may play a direct role in antibiotic-associated diarrhea in horses (148). However, in a more recent study a number of equine disease isolates failed to produce detectable levels of CPB2 *in vitro* due to low levels of *cpb2* mRNA (152). While it is certainly possible that those isolates produce detectable levels of CPB2 *in vivo*, these conflicting reports make it difficult to determine if CPB2 is important in equine gastrointestinal disease.

Table 1.3 Summary of epidemiological studies linking *cpb2* to gastrointestinal diseases of domestic animals.

Type	Animal	Disease	% cpb2$^+$	N	% CPB2+	N	Reference
?	porcine	enteritis, diarrhea	86	381			Bueschel *et al.* 2003
A	porcine	not reported[c]	57	103			Jost *et al.* 2006
A	piglet	enteritis, diarrhea	91	220	97	32	Bueschel *et al.* 2003
A	porcine	dairrheic	100	21			Waters *et al.* 2003
A	piglets	entertitis	100	12			Gibert *et al.* 1997
A	piglet	not reported[c]	79	29			Garmory *et al.* 2000
A	piglet	enteritis	74	87			Klaasen *et al.* 1999
A	porcine	enteritis	91	11			Johansson *et al.* 2006
C	porcine	not reported[c]	100	16			Jost *et al.* 2006
C	porcine	diarrheic	100	8			Waters *et al.* 2003
C	piglet	enteritis, diarrhea	97	36	100	6	Bueschel *et al.* 2003
C	piglet	entertitis	86	14			Gibert *et al.* 1997
C	piglet	not reported[c]	100	4			Garmory *et al.* 2000
C	piglet	enteritis	73	82			Klaasen et al. 1999
A	porcine	healthy	11	9			Bueschel *et al.* 2003
A	porcine	healthy	0	5			Waters *et al.* 2003
A	piglet	healthy	0	7			Garmory *et al.* 2000
C	porcine	healthy	0	1			Waters *et al.* 2003
?	equine	not reported[c]	21	86	100	2	Bueschel *et al.* 2003
A	equine	typhlocolitis	52	25			Herholz *et al.* 1999
A	equine	non-typhlocolitis	38	16			Herholz *et al.* 1999
A	equine	not reported	91	11			Jost *et al.* 2006
A	equine	colitis	100	15			Gibert *et al.* 1997
A	equine[a]	enteritis	69	58			Bacciarini *et al*. 2003
A	equine[a]	non-enteritis	9	11			Bacciarini *et al*. 2003
A	equine	diarrhoea	75	8			Johansson *et al.* 2006
A	equine	healthy	0	58			Herholz *et al.* 1999
A	equine	healthy	0	2			Johansson *et al.* 2006
A	foals	not reported[c]	50	24			Garmory *et al.* 2000
A	foals	healthy	50	10			Garmory *et al.* 2000
C	bovine	not reported[c]	33	6			Jost *et al.* 2006
E	bovine	not reported[c]	100	12			Jost *et al.* 2006
A	bovine	hemorrhagic bowel syndrome	41	143	25	36	Dennison *et al.* 2005
A	bovine	left-displace abomasum	24	147	0	36	Dennison *et al.* 2005
A	bovine calves	not reported[c]	18	11			Garmory *et al.* 2000
A	bovine calves	not reported[c]	12	51	10	50	Bueschel *et al.* 2003
C	bovine calves	not reported[c]	40	5			Bueschel *et al.* 2003
E	bovine calves	not reported[c]	97	37	0	7	Bueschel *et al.* 2003
A	bovine calves[b]	enterotoxaemia	62	83			Manteca *et al*. 2002
?	bovine	enteritis, sudden death	21	336			Bueschel *et al.* 2003
A	bovine calves[b]	healthy	59	51			Manteca *et al*. 2002
A	avian	all conditions	33.8	133	40	5	Bueschel *et al.* 2003
A	avian	healthy	96	48			Siragusa *et al.* 2006
?	canine	not reported[c]	37.9	140	0	2	Bueschel *et al.* 2003
A	lamb	not reported[c]	29	24			Garmory *et al.* 2000
A	lamb	healthy	60	10			Garmory *et al.* 2000
A	environmental		3.6	28			Bueschel *et al.* 2003

[a]The *cpb2* gene was detected using a colony hybridization approach instead of a *cpb2* specific PCR.

[b]Percent *cpb2*-positive and N values are reported as the number of calves carrying *cpb2*-positive isolates rather than the total number of isolates (multiple isolates were collected per animal whereas other studies used one isolate per animal).

[c]Not reported indicates that the specific type of gastrointestinal disease was not mentioned. However, these isolates are from diseased animals.

1.3.3 Early studies on the biological activity of CPB2

The first study describing the activity of CPB2 was performed by Jolivet-Reynaud *et al.* in 1986, although those investigators thought they were studying the effects of β toxin purified from a type C isolate (71). The purified protein of 28 kDa with a pI of 5.4-5.5 (later identified as CPB2 and not β toxin) was found to have a mouse LD_{50} of ~4 μg when injected intravenously (mice were observed for one day and developed signs of paralysis). This purified protein also was dermonecrotic in a guinea pig model (using 2 μg). In a guinea pig ligated intestinal loop model, incubation with CPB2 for 6 hrs with a low dose of toxin (4 μg) caused fluid accumulation (0.4 ml/cm) without hemorrhaging and this effect was abolished if the protein was pretreated with trypsin. At higher doses (40 or 100 μg) hemorrhagic effects (including destruction of the intestinal villi and mucosa) were observed without fluid accumulation after 6 hrs of CPB2 treatment. ^{3}H-thymidine and ^{14}C-leucine incorporation studies found that CHO cells were sensitive to toxin (dose response curves were performed with 0.1 μg to 10 μg of protein). Vero cells, mouse or human fibroblasts, Hep cells, and transformed intestinal cells (types were not specified) were found to not be sensitive to purified CPB(2).

As mentioned in section 1.3.1.1, Gibert *et al*. (52) later determined that the experiments performed by Jolivet-Reynaud *et al*. (71) used CPB2 and not β toxin. Gibert *et al*. (52) cloned the *cpb2* gene from a type C isolate (CWC245) behind the *iab/ibp* promoter to create pMRP268. This plasmid was transformed into the naturally *plc*-negative *C. perfringens* type A strain 667-76 and this transformed strain was used to produce recombinant CPB2 for antibody production and biological activity assays. Rabbit polyclonal anti-CPB2-antibodies reacted weakly with purified β toxin suggesting that despite the sequence differences, these proteins may be weakly immunologically related. However, polyclonal horse anti-β toxin-antibodies did not cross react with purified CPB2.

The purified CPB2 had a LD_{50} of 3 μg in a mouse i.v. injection model and was cytotoxic for CHO cells (52). However, a dose of 20 μg, instead of the previously reported dose range of 0.1 to 10 μg of CPB2, was required to inhibit incorporation of ^{3}H-thymidine or to cause cell rounding as determined by phase contrast microscopy in CHO/I407 cells. The difference in doses required to damage cells was attributed to the instability of CPB2. The authors report observing a loss of specific activity during purification even if steps were performed in the presence of protease inhibitors (such as benzamide, 2 μg/ml) or 50% glycerol. CPB2-sensitive

cell lines were reported to round-up after toxin treatment. However, unlike other *C. perfringens* toxins (such as ι toxin) that disrupt the actin cytoskeletal network, phalloidon staining did not reveal drastic rearrangement of the cell's cytoskeleton after CPB2 treatment. CPB2 also does not possess ADP-ribosylation (used by ι toxin) or UDP-glucosylation activity (used by large clostridial toxins).

More recently, two groups have briefly explored the role of CPB2 *in vivo*. To investigate the role of CPB2 in bovine enterotoxaemia, Manteca *et al*. (88) initially observed that diseased calves were significantly more likely to be colonized with *cpb2*-positive *C. perfringens* type A isolates than healthy, control calves and that diseased animals had a higher bacterial load of *C. perfringens* than healthy animals (10^6-10^{10} vs. 10^3-10^7, respectively). *C. perfringens cpb2*-positive and *cpb2*-negative isolates were then grown in brain heart infusion media and used to challenge a healthy calf using the ligated intestinal loop model. Lesion scores were higher for *cpb2*-positive isolates than for *cpb2*-negative isolates and lesions contained regions of necrosis and hemorrhaging. Polymorphonuclear infiltration was also observed. However, this study included a small number of isolates, did not contain replicates, and the *cpb2*-positive isolates also produce α toxin and PFO (and possible other unknown factors) that could have contributed to or caused the observed pathology.

A second study by Bacciarini *et al.* (9) using immunohistochemistry demonstrated the presence of CPB2 *in vivo* in horses that succumbed to gastrointestinal diseases (such as typhlocolitis). For detection of CPB2, polyclonal anti-CPB2-antibodies were raised in a rabbit using a recombinant CPB2 molecule containing a 6x his-tag that was purified from *E. coli*. CPB2 was only detected in intestinal tissue obtained from horses with gastrointestinal disease and not from horse dying from non-gastrointestinal disorders. Staining for CPB2 was primarily localized to areas with a high concentration of rod shaped bacteria (presumably *C. perfringens*). While this study demonstrated that CPB2 can be present under *in vivo* conditions, it did not determine what role CPB2 has in equine gastrointestinal disease.

2.0 STATEMENT OF THE PROBLEM

As discussed in section 1.2.2, *cpe*-positive isolates cause foodborne (chromosomal *cpe* isolates) and nonfoodborne (plasmid *cpe* isolates) human GI disease. Symptoms from cases of CPE-associated nonfoodborne human GI disease are longer in duration (averaging one week) and more severe (may involve bloody, mucoid stool) than those symptoms resulting from cases of CPE-associated foodborne human GI disease (average duration of 24 hours with loose, watery stool). The *cpe* gene itself is not responsible for these symptomatic differences since CPE production (levels and regulation) and cytotoxicity are the same regardless of the location of the *cpe* gene. While the location of the *cpe* gene on conjugative plasmids (which could transfer the *cpe* gene to resident *C. perfringens* flora that are adapted for intestinal colonization) has been proposed as one explanation for these symptomatic differences, conjugation does not provide a complete explanation as to why nonfoodborne GI disease symptoms are more severe than symptoms from foodborne human GI disease.

As a possible explanation for the symptomatic differences between foodborne and nonfoodborne human GI disease cases, we hypothesize that in opposition to foodborne GI disease isolates, nonfoodborne GI disease isolates carry and express *cpb2*. The recently discovered CPB2 toxin has been linked by epidemiological studies to GI disease in domestic animals (particularly piglets) and *in vivo* experiments have demonstrated its ability to damage the intestinal epithelium (in intestinal loop models) making it an attractive candidate for an accessory toxin in human GI disease. Secondly, we hypothesize that CPB2 contributes/causes GI disease in humans and domestic animals by disrupting the intestinal epithelium via its cytotoxic activity on individual cells. It is important to note that the strains used to create isogenic *cpe* knockout mutants (discussed in 1.2.1.1) are *cpb2*-negative, so intestinal loop experiments with those mutants were not assessing the role of CPB2 as an accessory toxin in disease.

The production of a second toxin, namely CPB2, with cytotoxic activity towards enterocytes in the presence of CPE (in the case of human GI disease isolates and a number of other toxins in the case of domestic animal disease), would be expected to cause increased pathology in the GI tract and subsequently, more severe symptoms in infected patients. Furthermore, the production of a vegetative toxin (CPB2) by nonfoodborne GI disease isolates would result in the presence of a possible GI disease-inducing factor in the absence of sporulation (required for CPE production), possibly explaining, in part, the increased duration of nonfoodborne GI disease versus foodborne GI disease. Lastly, the production of a toxin that disrupts intestinal permeability could result in increased absorption of other toxins, namely β toxin (implicated in animal and human GI disease) and ε toxin (animal GI disease only), resulting in a more severe and life-threatening disease.

To address these hypotheses, this dissertation will describe research performed to 1) determine the genotype and phenotype of human and non-human GI disease isolates in regard to the location of *cpb2* and the production of CPB2, 2) assess the *in vitro* sensitivity of enterocyte-like cells to CPB2 and the mechanism of action of CPB2 against sensitive cell lines and, 3) elucidate the role of CPB2 *in vivo* using the mouse intravenous injection model and the rabbit ileal loop model.

3.0 MATERIALS AND METHODS

The majority of material and methods can be found in attached manuscripts located in the Appendix. The primary exceptions to this statement are methods pertaining to Section 4.2 in which the mechanism of action of CPB2 was explored, and Section 4.3.1 involving *in vivo* experiments with rabbits. To serve as a quick reference for the large number of isolates used in this dissertation, *E. coli* and *C. perfringens* isolates (recombinant and wild type) used in this study are listed in this chapter.

3.1 BACTERIAL CULTURING CONDITIONS AND ISOLATE CATALOGUES

E. coli DH5α (used for all routine cloning and recombinant protein production procedures) was grown overnight in 3 ml Luria Bertoni (LB) broth at 37°C with shaking or on LB agar plates at 37°C under aerobic conditions. Appropriate antibiotics were added to LB and agar plates as follows; ampicillin (Sigma) – 100 μg/ml, erythromycin (Sigma) – 150 μg/ml, or chloramphenicol (Sigma) – 100 μg/ml. Growth conditions were altered for production of recombinant CPB2 as described in chapter 3.3.3. *E. coli* stocks were maintained in glycerol (60%) and stored at either -80°C or -20°C.

For routine growth, *C. perfringens* was cultured overnight in 10 ml fluid thioglycollate medium (FTG, [Difco]) at 37°C without shaking. After incubating overnight, FTG cultures were plated on TSC agar plates (Shahidi-Ferguson *perfringens* agar base [Difco]) and incubated for 20 hr at 37°C in anaerobic jars. An isolated colony was then inoculated into 10 ml FTG media, which was grown overnight prior to further experiments. Those FTG cultures were then passed

to different media depending upon the experiment to be performed. To assess culture purity for experiments using *C. perfringens,* cultures were routinely streaked after experiments onto brain heart infusion (BHI [Difco]) agar plates, which were then grown overnight at 37°C under aerobic conditions. For experiments involving recombinant *C. perfringens,* antibiotic concentrations were as follows; erythromycin – 50 μg/ml or chloramphenicol – 20 μ/ml.

Long-term *C. perfringens* stock cultures (lasting decades) were prepared by passing 0.1 ml of an overnight FTG culture to 10 ml of cooked meat media, which was then grown at 37°C for one week to allow for sporulation. Cooked meat stocks containing spores were stored at -20°C. Most *C. perfringens* type A isolates form heat stable spores in cooked meat cultures that can be signaled to germinate by a brief exposure to heat. However, most non-type A isolates either sporulate poorly in cooked meat media or do not form heat resistant spores and will not survive a brief heat exposure. To resuscitate all *C. perfringens* cooked meat stocks, 0.1 ml of cooked meat culture stocks were added to two separate tubes containing 10 ml of FTG. One tube of inoculated FTG was then incubated immediately at 37°C (for heat-sensitive isolates), while the second tube was incubated at 75°C for 10 minutes (to signal germination) before being placed at 37°C. A number of isolates used in the following studies were received as lyophilized cultures (all cultures with AM prefixes [Table 3.1]). To resuscitate those bacteria, the freeze dried *C. perfringens* were suspended in 1 ml of FTG media and transferred to 10 ml of FTG for overnight growth at 37°C.

Short-term *C. perfringens* stocks (lasting ~1 year) were made by spreading 100 μl of an overnight FTG culture onto a TSC agar plate which was then grown overnight at 37°C under anaerobic conditions to form a bacterial lawn. One ml of glycerol stock media (50% glycerol, 0.1% L-cystine, 50% BHI) was then added to the surface of the TSC agar plate and the bacterial lawn was scraped from the plate using a glass rod. The bacteria/glycerol was then transferred to 1.5 ml cryovials and stored at -20°C. To obtain vegetative cultures from these stocks, 100 μl of the glycerol stock was added to 10 ml of FTG and grown overnight at 37°C.

Since a large number of isolates (recombinant *E. coli* and recombinant or wildtype *C. perfringens*) are cited throughout this dissertation (and in the Appendix), a catalogue of strains is included. Table 3.1 contains the names, references, disease origin, toxin type, and relevant genetic information including the toxin genotype, location of genes (plasmid versus chromosomal), and IS elements if present.

Table 3.1 Recombinant and wildtype *E. coli* and *C. perfringens* isolates used in this study. Isolates located in the boxes are clonal.

Isolate name	(Alternative name)	Origin and/or reference	Description (disease, location/date of isolation)	Type (Toxin Genotype)	IS element[e]
			***E. coli* and *C. perfringens* recombinant constructs**		
DF(pMAL-c2vcpb2S)		This study	*E. coli* DH5α with pMAL-c2vcpb2S		
DF(pMAL-c2vcpb2NS)			*E. coli* DH5α with pMAL-c2vcpb2NS		
DF(pGEXvcpb2S)			*E. coli* DH5α with pGEX-2Tvcpb2S		
DF(pBADvcpb2S)			*E. coli* DH5α with pBADvcpb2S		
DF(pBADvcpb2NS)			*E. coli* DH5α with pBADvcpb2NS		
DF(pBAD/Thiovcpb2S)			*E. coli* DH5α with pBAD/Thiovcpb2S		
DF(pBAD/Thiovcpb2NS)			*E. coli* DH5α with pBAD/Thiovcpb2NS		
Songer(pTrcHisvcpb2S)		Bueschel *et al.* 2003	*E. coli* DH5α with pTrcHisBvcpb2S		
(pB2h1v751)3624		Fisher *et al*. 2005	ATCC 3624 with pB2(h1)v751	A (*cpb2h1*[a,b])	
(pB2h2v750)3624		This study	ATCC 3624 with pB2(h2)v750	A (*cpb2h2*[a])	
(pJIR751)3624			ATCC 3624 with pJIR751	A	
(pJIR750)3624			ATCC 3624 with pJIR750	A	
			***C. perfringens* isolates**		
Strain 13		Shimizu *et al.* 2002	Unknown (canine), Canada	A (*cpb2typ)*	
ATCC 3624		Kokai-Kun *et al.* 1994	Gas gangrene	A	
NCTC8239		Collie and	Food poisoning, Europe 1950s, clonal	A (*cpe*[d])	A
NCTC8798		McClane, 1998	Food poisoning, Europe 1950s	A (*cpe*[d])	A
NCTC10239			Food poisoning, Europe 1950s	A (*cpe*[d])	A
NCTC8235			Food poisoning, Europe 1950s	A (*cpe*[d])	A
NCTC8238			Food poisoning, Europe 1950s	A (*cpe*[d])	A
NCTC8359			Food poisoning, Europe 1950s	A (*cpe*[d])	A
NCTC8679			Food poisoning, Europe 1950s	A (*cpe*[d])	A
NCTC8799			Food poisoning, Europe 1950s	A (*cpe*[d])	A
191-10		Sparks *et al.* 2001	Food poisoning, Hawaii, 1980s	A (*cpe*[d], *cpb2h1*)	A
FD1041		Sparks *et al.* 2001	Food poisoning, North America, 1980s	A (*cpe*[d])	A
C-1851		Sparks *et al.* 2001	Food poisoning, Vermont 1980s	A (*cpe*[d], *cpb2h1*)	A
C-1869			Food poisoning, Vermont 1980s	A (*cpe*[d], *cpb2h1*)	A
C-1881			Food poisoning, Vermont 1980s	A (*cpe*[d], *cpb2h1*)	A
C-1887			Food poisoning, Vermont 1980s	A (*cpe*[d], *cpb2h1*)	A
C-1841			Food poisoning, Vermont 1980s	A (*cpe*[d], *cpb2h1*)	A
Ohio #23		Sparks *et al.* 2001	Food poisoning, North America, 1990s	A (*cpe*[d])	A
513			Food poisoning, North America, 1990s	A (*cpe*[d])	A
527			Food poisoning, North America, 1990s	A (*cpe*[d])	A
528			Food poisoning, North America, 1990s	A (*cpe*[d])	A
537-5			Food poisoning, North America, 1990s	A (*cpe*[d])	A
538-1			Food poisoning, North America, 1990s	A (*cpe*[d])	A
E13			Food poisoning, North America, 1990s	A (*cpe*[d])	A
R42			Food poisoning, North America, 1990s	A (*cpe*[d])	A
803 MA		Bos *et al.* 2005	Food poisoning, Oklahoma, 1999, clonal	A (*cpe*[d])	A
869 MH			Food poisoning, Oklahoma, 1999	A (*cpe*[d])	A
809 MH			Food poisoning, Oklahoma, 1999	A (*cpe*[d])	A
802 MA			Food poisoning, Oklahoma, 1999	A (*cpe*[d])	A
F101 810			Food poisoning, Oklahoma, 1999	A (*cpe*[d])	A
F4969		Collie and	Sporadic Diarrhea, Europe 1990s	A (*cpe*[c])	C
F4406		McClane 1998	Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2h1*)	B
F5603			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2h1*)	B
F4591			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c])	C
F4013			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2*)	B
F5537			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2*)	C
F5599			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2*)	C
F4393			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2*)	B
F4396			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2*)	C
F4859			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2h2*)	C
F4129			Sporadic Diarrhea, Europe 1990s	A (*cpe*[c], *cpb2*)	C
B11			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
B38			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
B40			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
B41			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
B2			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
B45			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
NB16			Antibiotic Associated Diarrhea, Europe, 1980s	A (*cpe*[c], *cpb2*)	B
F36081		Sparks *et al.* 2001	Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	B
F38660			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c])	C
H38094			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	B
M18069			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c])	C
M19874			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[b])	C
M22792			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[b])	C
M24326			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[b])	C
M26413			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[b])	C
M34401			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[b])	C
M35584			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
M39558			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
S43526			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
T29447			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c])	C

Table 3.1 (continued)

Isolate name	(Alternative name)	Origin and/or reference	Description (disease, location/date of isolation)	Type (Toxin Genotype)	IS element[e]
			C. perfringens		
T34058			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
T44123			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	B
T57603			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	B
W24820			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
W30554			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
W43181			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
F16865			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2h2*)	C
S10653			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	B
S10748			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
T285546			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
T32214			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
T39814			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
W2624			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
W30554			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2h2*)	C
W32500			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2*)	C
X5722			Antibiotic Associated Diarrhea, North America, 1990s	A (*cpe*[c], *cpb2h2*)	C
MR2-4		Miyamoto *et al.* 2001	Normal Human Isolate, Japan, 1990s	A (*cpe*[c], *cpb2*)	C
PS49		Ron Labbe		B (*cpb2atyp*)	
NCTC8533	NCIB8693	Rick Titball, UK	Lamb dysentary, UK, 1953	B (*cpb2atyp*)	
Bar 2		Francisco Uzal, UC Davis	Unavailable, Argentina	B (*cpb2atyp*)	
JGS1984		McClane lab culture	Unavailable, North America, 1990s	B (*cpb2atyp*)	
AM923	CN1794	BW via Haywood/Wilkinson	Vet lab WRL, UK, 1947	B (*cpb2*)	
AM925	CN1990		Lamb jejunum, Wales, 1947	B (*cpb2*)	
AM927	NCIB3110		Beckenham, 1930	B (*cpb2*)	
AM928				B (*cpb2*)	
AM 929	CN677		Acute lamb dysentery, Newcastle, 1942	B (*cpb2*)	
AM935	CN1793		Toxigenic, 1947	B (*cpb2*)	
AM936	CN1795		Toxigenic, 1947	B (*cpb2*)	
AM937	CN1886		Lamb stomach, Wales, 1947	B (*cpb2*)	
AM 939	CN2003		Lamb ileum, Wales, 1947	B (*cpb2*)	
AM940	CN2414		3 day old lamb, Wales, 1948	B (*cpb2*)	
AM941	CN2416		Stomach 5 day old lamb, Wales, 1948	B (*cpb2*)	
AM942	CN3425		Jejunum 5 day old lamb, Wales, 1948	B (*cpb2*)	
AM943	CN3434		Hanover, 1951	B (*cpb2*)	
AM945	CN3447		Lamb dysentery, UK, 1951	B (*cpb2*)	
AM1023	CN3922		MAFF, Weybridge, UK, 1955	B (*cpb2*)	
AM930	CN684	BW via Haywood/Wilkinson	Lamb dysentery, atypical strain, UK, 1943	C	
AM931	CN685		VLamb dysentery, atypical strain, UK, 1943	C	
AM933	CN689		First lamb dysentery strain "Wilsdon", UK, 1922	C	
CN5383		McClane Lab cultures	Pigbel, Papua, New Guinea, 1970s	C	
AM950	CN367	BW via Haywood/Wilkinson	Vet Lab WRL, Paludis, 1942	C	
AM951	CN882		Sheep with struck, 878, Wales, 1934	C	
AM952	CN885		Vet Lab WRL, Ashby, 1940	C	
AM953	CN886		Vet Lab WRL, Ashby, 1932	C	
AM954	CN887		Sheep with struck, 993, Wales, 1934	C	
AM955	CN1797		Toxigenic strain, Ashby, 1932	C	
AM956	CN2065		Peritoneal fluid, Struck ewe, 1947	C	
AM958	CN2109		NCTC 3227, Wye 3, 1948	C	
AM961	CN3685		Peritoneal fliud, Sheep with struck, Ashford, 1954	C	
AM965	CN3706		Hereford cattle, VL. 8056/1, Colorado, 1955	C	
AM966	CN3708		Hereford cattle, VL. 8056/5, Colorado, 1955	C	
AM969	CN3711		Hereford cattle, VL. 8056/10, Colorado, 1955	C	
AM989	CN3717		Hereford cattle, VL. 8056/18, Colorado, 1955	C	
AM990	CN3727		Hereford cattle, VL. 8056/2, Colorado, 1955	C	
AM991	CN3728		Hereford cattle, VL. 8056/3, Colorado, 1955	C	
AM999	CN3955		Ewe Peritoneal Fliud, CWC.5, MAFF, Weybridge, 1956	C	
Bar3		Francisco Uzal, UC Davis	Unavailable, Argentina	C (*cpb2atyp*[g])	
NCTC10719	CWC1	NCTC	Porcine necrotic enteritis, Coenhagen, 1970s	C (*cpb2*)	
JGS1495		Glenn Songer, Univ of Arizona	Porcine, unavailable (TIGR[i])	C (*cpb2typ*)	B
JGS1504			Porcine necrotic enteritis, North America, 1990s	C (*cpb2*)	
JGS1070			Porcine enteritis, North America, 1990s	C (*cpb2*)	
JGS1071			Porcine enteritis, North America, 1990s	C (*cpb2typ*)	
JGS1075			Porcine enteritis, North America, 1990s	C (*cpb2*)	
JGS1076			Porcine enteritis, North America, 1990s	C (*cpb2*)	
JGS1659			Porcine enteritis, North America, 1990s	C (*cpb2*)	
AM962	CN3686	BW via Haywood/Wilkinson	Piglets, 236/7972, Cambridge, 1954	C (*cpb2typ*)	
AM963	CN3687		Piglets, 20/7972, Cambridge, 1954	C (*cpb2*)	
AM964	CN3690		Piglets, 201/7972, Cambridge, 1954	C (*cpb2*)	
AM968	CN3710		Hereford cattle, VL. 8056/9, Colorado, 1955	C (*cpb2atyp*)	
AM988	CN3715		Hereford cattle, VL. 8056/16, Colorado, 1955	C (*cpb2atyp*)	
AM992	CN3729		Hereford cattle, VL. 8056/7, Colorado, 1955	C (*cpb2atyp*)	
AM993	CN3730		Hereford cattle, VL. 8056/8, Colorado, 1955	C (*cpb2atyp*)	
AM994	CN3732		Hereford cattle, VL. 8056/14, Colorado, 1955	C (*cpb2atyp*)	
AM995	CN3795		Pig intestine, MRCVS, 1955	C (*cpb2*)	
AM997	CN3947		Piglet acute enteritis, CWC.7, MAFF, Weybridge, 1956	C (*cpb2typ*)	
CN5388		McClane Lab cultures	Pigbel, Papua, New Guinea, 1970s	C (*cpe, cpb2atyp*[g])	

Table 3.1 (continued)

Isolate name	(Alternative name)	Origin and/or reference	Description (disease, location/date of isolation)	Type (Toxin Genotype)	IS element[e]
			C. perfringens		
AM1035	CN2076	BW via Haywood/Wilkinson	Darmbrand, Zeissler, Hamburg, 2, 1948	C (*cpe*)	
AM1039	CN3748		Darmbrand, Zeissler, Hamburg, 4P1, 1955	C (*cpe*)	
AM1043	CN3753		Darmbrand, Zeissler, Hamburg, 9, 1955	C (*cpe*)	
AM1046	CN3758		Darmbrand, Zeissler, Hamburg, 42a, 1955	C (*cpe*)	
AM1048	CN3763		Darmbrand, Zeissler, Hamburg, 64, 1955	C (*cpe*)	
NCTC8346	CN1635	NCTC	Sheep, Beckenham, 1951	D	
NCTC8503	CN366	NCTC	Beckenham, 1950	D	
PS52		Unknown		D	
Bar9		Francisco Uzal, UC Davis	Unavailable, Argentina	D	
Bar15			Unavailable, Argentina	D	
Bar17			Unavailable (cecum), Argentina	D	
JGS1182		Glenn Songer, Univ of Arizona	Ovine, sudden death, Canada, 1990s	D (*cpb2atyp*)	
JGS1240			Ovine, bronchopneumo USA, 1990s	D (*cpb2atyp*)	
JGS1558			Caprine, diarrhea, USA, 1990s	D	
JGS1705				D	
JGS1721			Ovine enteritis, USA, 1990s (TIGR[f])	D	B
JGS1945			Caprine, diarrhea, USA, 1990s	D	
JGS1948			Caprine, enterotoxemia, Canada, 1990s	D	
JGS4105			Ovine, sudden death, USA, 1990s	D (*cpe*)	
JGS4138			Caprine, sudden death, USA, 2000s	D (*cpe*, *cpb2atyp*)	
JGS4139			Caprine, sudden death, USA, 2000s	D (*cpe*, *cpb2*)	
AM944	CN3446	BW via Haywood/Wilkinson	Lamb dysentery, 1951	D	
AM1002	CN462		Goat, 1942	D (*cpe*)	
AM1003	CN1020		Canada, Deposited as C. haemolyticum, 1944	D	
AM1004	CN1183		Lamb, Wales, 1942	D (*cpe*, *cpb2atyp*)	
AM1005	CN1184		Ewe, Brecon. Starin Macauley, 1945	D	
AM1007	CN1634		Lamb with suspected dysentery, 1945	D	
AM1008	CN1675		Ewe's udder, Wales, 1946	D	
AM1009	CN1798		Toxigenic, 1947	D	
AM1011	CN2062		Goat fatal diarrhoea, bowel contents, 1948	D	
AM1013	CN2068		Lamb stomach, Newcastle, 1948	D	
AM1015	CN3693		Kid 6 months, ileum, 1954	D	
AM1016	CN3718		Guinea pig heart blood, Buenos Aires 1955	D	
AM1017	CN3793		Vaccine production strain, Kenya, 1955	D	
AM1020	CN3842		Ewe, small intestine, Leamington Spa, 1955	D (*cpe*)	
AM1024	CN3977		Lamb, carcase, 8549/4, Dartford, 1956	D	
AM1025	CN3978		Lamb, carcase, 8549/3, Dartford, 1956	D	
AM1026	CN3980		Lamb, carcase, 8549/3, Chelmsford, 1956	D (*cpe*)	
AM1027	CN4003		3 day old lamb, stormont, 1956	D (*cpe*, *cpb2atyp*)	
AM1030	CN3841		Ewe intestinal contents, Boss-on-Rye< Smooth, 1955	D	
AM1031	CN3948		Sheep abomasum, Teheran 1956	D (*cpe*)	
AM1032	CN4029		Sheep carcase, 8586 A/1, Edmunds, 1956	D	
AM1033	CN4031		Sheep carcase, 8586 B/4, Edmunds, 1956	D	
AM1034	CN4167		Montana State, Frank AM J Vet Res 117:492-494, 1956	D	

[a]ATCC3624 is naturally *cpb2*-negative, but is listed as positive for *cpb2* due to the presence of a *cpb2*-containing vector.

[b]The h1 (human variant 1), h2 (human variant 2), typ (typical), or atyp (atypical) suffixes on *cpb2* refer to the different *cpb2* sequence variants (based on the nucleotide sequences). The *cpb2* variants are only identified for those isolates which had either the entire *cpb2* gene or a portion of the *cpb2* gene sequenced. The *cpb2* genes without suffixes have not been sequenced.

[c]All isolates with *cpe*[c] listed for their genotype have been demonstrated (by PCR (99)) to carry a plasmid-encoded *cpe* gene.

[d] All isolates with *cpe*[d] listed for their genotype have been demonstrated (by PCR (99)) to carry a chromosomally-encoded *cpe* gene.

[e]The presence of different IS elements was determined using PCR (all *cpe*-positive type A isolates (99)) or through sequencing (type C and D isolates [TIGR, unpublished]). IS elements are designated by A (IS*1470*), B (IS*1151*) and C (IS*1470*-like).

[f]TIGR used these isolates to sequence the *cpb* plasmid (type C) and the *etx* plasmid (type D) [unpublished].

[g]The *cpb2atyp* genes carried by these isolates contains multiple deletions within the ORF and CPB2 can not be detected in culture supernatants.

3.2 MANIPULATION OF DNA AND CONSTRUCTION OF RECOMBINANT BACTERIA

3.2.1 Vector construction

A number of vectors were designed for the purposes of 1) expressing different recombinant *cpb2* constructs in *E. coli* or 2) expressing wildtype *cpb2* in naturally *cpb2*-negative *C. perfringens* (49). In general, cloning was performed using the pathway diagrammed in Figure 3.1. Specific details, including vector and insert sizes and primers used for cloning are included in Table 3.2 and Table 3.3, respectively. TaqComplete master mix (GeneChoice) was routinely used to amplify template DNA (derived from colony lysates). Unless stated otherwise, PCR amplification conditions were as follows: 95°C 5 min (initial denaturation) followed by 30 cycles at 95°C 1 min, 55°C 1 min, and 72° (1 min per 1 kb of DNA). A final 5 min extension was run at 72°C. PCR products were cloned directly into the cloning vector pCR2.1 TOPO (Invitrogen) using the manufacturer's directions.

For ligation reactions, digested DNA was first separated on agarose gels and visualized with ethidium bromide staining. After identification of the expected vector and insert bands, DNA was excised from the agarose using a clean razorblade and the DNA was extracted using the Freeze-N-Squeeze kit (BIO-RAD) as directed. DNA was then precipitated in eppendorf tubes by mixing the extracted solution with 2 volumes of 95% ethanol and 0.1 vol sodium acetate (3 M, [Sigma]). This solution was incubated at -20°C for >3 hr and then centrifuged at 13000 rpm for 30 min. Precipitated DNA was washed with 70% ethanol, dried at 50°C, and resuspended in 15 μl of sterile, molecular grade H_2O (Sigma). Insert and vector were then mixed at a 3:1 ratio with

ligase (Invitrogen) to give a final volume of 15 μl and ligated as suggested by the manufacturer. After ligation, the ligase was inactivated by heating the ligation reaction to 70°C for 10 min. Ligation products were then used to transform either TOP10 chemically competent or electroporatable *E. coli* DH5α as directed (from Invitrogen). Transformants were selected on LB agar plates with appropriate antibiotics and clones were then screened for inserts by performing colony PCR using the same primers used to generate the original TOPO clones. Clones positive by PCR were then screened for production of CPB2 and/or sequenced depending upon the vector constructed. When necessary, electroporation was used to construct *C. perfringens* transformants as described in Fisher *et al.* (49). Positive-transformants were selected via colony PCR as described above for selecting TOPO clones.

For creating recombinant *E. coli* carrying expression vectors (Table 3.2) encoding the *cpb2* gene in frame with vector-specific protein tags (for detection/purification) primers (Table 3.3) were designed to amplify the *cpb2* ORF either with or without the 5' signal peptide. For vectors containing an N-terminal amino acid tag (pGEX-2T and pMAL-c2), the *cpb2* 3' termination codon was included in the 3' cloning primer. When cloning into vectors encoding a C-terminal amino acid tag (pBAD and pBAD/THIO-TOPO) the *cpb2* 3' termination codon was not included in the 3' cloning primer to allow read through of the C-terminal tag during translation.

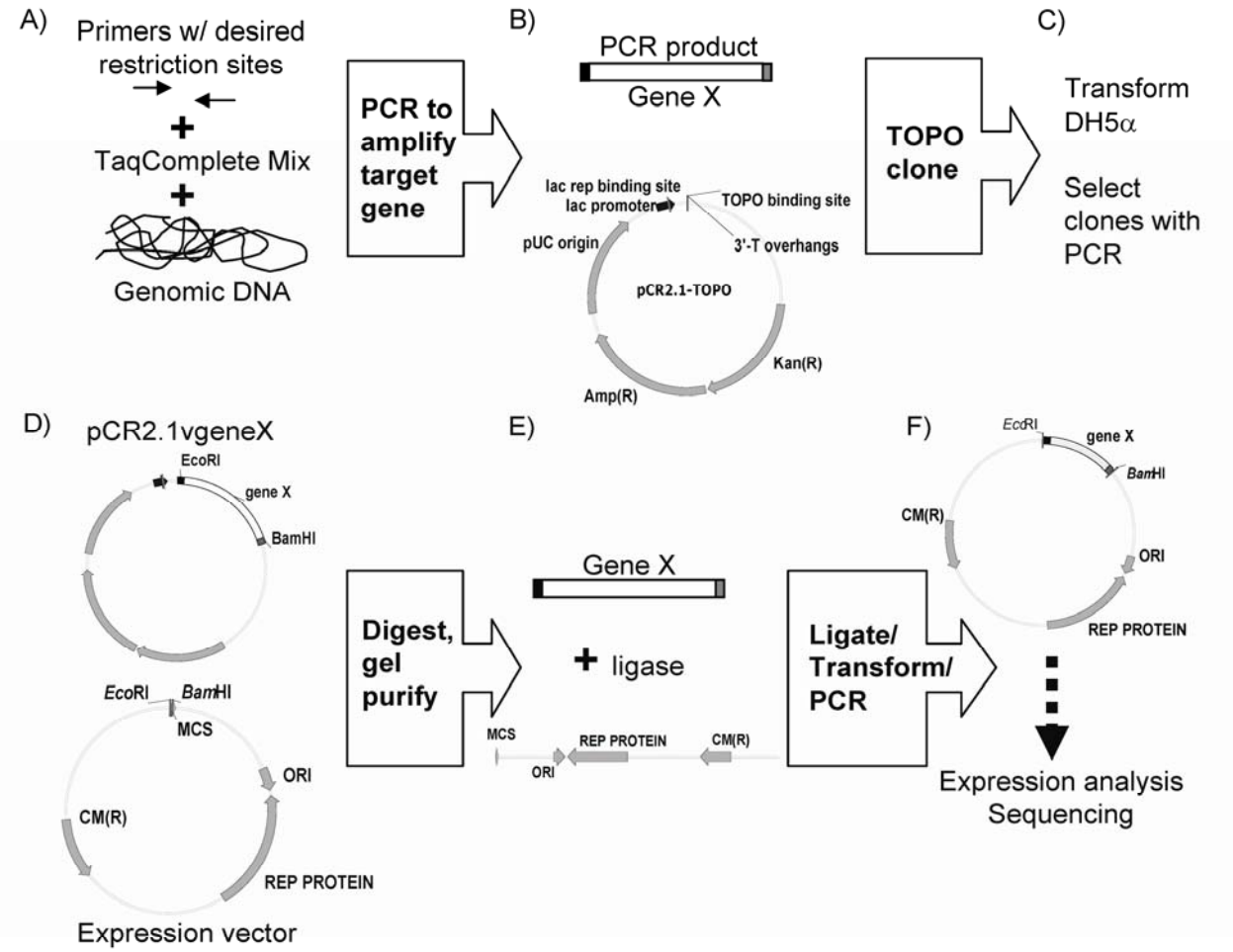

Figure 3.1 Diagram describing the general construction of recombinant vectors. Primers containing the desired restriction sites (to allow for in frame cloning into the final vector) are designed to amplify the gene of interest (gene X) (A). These products are then cloned into pCR2.1-TOPO (B) and transformants are selected using PCR (C). pCR2.1vgeneX and the appropriate expression vectors are then digested and the products are purified in agarose gels (D). Digested products are then ligated (E) and used to transform *E. coli.* Transformants are screened using PCR and positive clones are grown to purify DNA. Final vectors are tested for protein production and are sequenced to ensure that mutations have not been introduced during the cloning process (F).

Table 3.2 General information on vectors used in Chapter 4.0.

Vector	Description	Reference	Inducer	Resistance	DNA size (kbp)	rCPB2 size (kDa)
pJIR750	*C. perfringens* shuttle vector	Bannam and Rood, 1993		Cm^r	6600	
pJIR751	*C. perfringens* shuttle vector	Bannam and Rood, 1993.		Erm^r	6000	
pCR2.1 TOPO	*E. coli* cloning vector	Invitrogen		Amp^r, Kan^r	3900	
pCR2.1vcpb2h1	pCR2.1 TOPO with the *cbp2h1* gene (promoter and ORF)	Fisher *et al*., 2005		Amp^r, Kan^r	5100	
pCR2.1vcpb2h2	pCR2.1 TOPO with the *cbp2h2* gene (promoter and ORF)	Fisher *et al*., 2005		Amp^r, Kan^r	5200	
pB2h2v750	pJIR750 with the *cbp2h2* gene (promoter and ORF)	Fisher *et al*., 2005		Cm^r	7900	
pB2h1v751	pJIR750 with the *cbp2h1* gene (promoter and ORF)	Fisher *et al*., 2005		Erm^r	7200	
pCR2.1vcpb2S^{stop}	pCR2.1 TOPO with the *cpb2h1* ORF (with the secretion signal and stop signal)			Amp^r, Kan^r	4700	
pCR2.1vcpb2NS^{stop}	pCR2.1 TOPO with the *cpb2h1* ORF (no secretion signal, with stop signal)			Amp^r, Kan^r	4600	
pCR2.1vcpb2S	pCR2.1 TOPO with the *cpb2h1* ORF (with the secretion signal, no stop signal)			Amp^r, Kan^r	4700	
pCR2.1vcpb2NS	pCR2.1 TOPO with the *cpb2h1* ORF (no secretion signal, no stop signal)			Amp^r, Kan^r	4600	
pCR2.1vcpb2S^{gex}	pCR2.1 TOPO with the *cpb2h1* ORF (with the secretion signal and stop signal for pGex-2T)			Amp^r, Kan^r	4700	
pTrcHisB	*E. coli* expression vector with C-term 6xhis tag	Invitrogen	IPTG	Amp^r	4400	
pTrcHisvcpb2S	pTrcHisB with the *cpb2typ* gene (with secretion signal)	Jost *et al*., 2003	IPTG	Amp^r	5200	33.7
pMAL-c2	*E. coli* expression vector with C-term cytoplasmic targeted MBP	NEB	IPTG	Amp^r	6600	
pMAL-c2vcpb2S	pMAL-c2 with the *cpb2h1* ORF (with the secretion signal)		IPTG	Amp^r	7400	73.2
pMAL-c2vcpb2NS	pMAL-c2 with part of the *cpb2h1* ORF (no secretion signal)		IPTG	Amp^r	7300	70
pBAD	*E. coli* expression vector with N-term 6xhis tag	Invitrogen	L-arabinose	Amp^r	4100	
pBADvcpb2S	pBAD with the *cpb2h1* ORF (with the secretion signal, no stop signal)		L-arabinose	Amp^r	4900	33.7
pBADvcpb2NS	pBAD with the *cpb2h1* ORF (no secretion signal, no stop signal)		L-arabinose	Amp^r	4800	30.4
pBAD/Thio-TOPO	*E. coli* expression vector with C-term thioredoxin and N-term 6xhis tag	Invitrogen	L-arabinose	Amp^r	4500	
pBAD/Thiovcpb2S	pBAD/Thio TOPO with the *cpb2h1* ORF (with the secretion signal, no stop signal)		L-arabinose	Amp^r	5300	46.7
pBAD/ThiovcpbNS	pBAD/Thio TOPO with the *cpb2h1* ORF (no secretion signal, no stop signal)		L-arabinose	Amp^r	5300	27.4
pGEX-2T	*E. coli* expression vector with C-term GST tag	Amersham	IPTG	Amp^r	4900	
pGEXvcpb2S	pGEX-2T with the *cpb2h1* ORF (with the secretion signal)		IPTG	Amp^r	5700	59.7

Table 3.3 Primers used to amplify inserts for creating recombinant vectors (see Table 3.3).

	Primers (used to generate inserts for cloning)	
Vector	**Forward (5'-3')**	**Reverse (5'-3')**
pCR2.1vcpb2h1	ATCGAATTCGTAAAGGAATCCATAAAAATTTAGG	ATGGATCCGTTATAAATAAATATAATTCTCTAAAACC
pCR2.1vcpb2h2	ATTAGGATCCGATTTAATTTTCCATTATAGTGCTAGT	TATACTTAAGCTGGTAATTTTTTCTTTTATCGATG
pCR2.1vcpb2S^{stop}	ATGAAAAAATTAATAGTAAAAAGTACAATGATGCTTTTA	TTAATAACAATAACCCTCACCAAATACTT
pCR2.1vcpb2NS^{stop}	AATGAAGTGAATAAATACCAATCTGTAATGGTA	TTAATAACAATAACCCTCACCAAATACTT
pCR2.1vcpb2S	ATGAAAAAATTAATAGTAAAAAGTACAATGATGCTTTTA	ATAACAATAACCCTCACCAAATACTTTAATC
pCR2.1vcpb2NS	AATGAAGTGAATAAATACCAATCTGTAATGGTA	ATAACAATAACCCTCACCAAATACTTTAATC
pCR2.1vcpb2S^{gex}	TAATGAAAAAATTAATAGTAAAAAGTACAATGATGC	TTAATAACAATAACCCTCACCAAATACTT

3.2.2 DNA sequencing and bioinformatics

DNA sequencing was performed using either plasmids as DNA template (for example, to verify recombinant vectors) or PCR products (for example, sequencing of *cpb2* genes from different isolates). All sequencing, unless stated otherwise, was performed at the University of Pittsburgh

core sequencing facility (http://www.genetics.pitt.edu/services.html) using protocols recommended by the core facility. Sequencing primers (not found in the Appendix) and their targets are listed in Table 3.4 along with the primers used to amplify the *cpb2* gene from different *cpb2*-positive *C. perfringens* isolates. PCR products were prepared for sequencing using ExoSapIt (Amersham) to remove primers left over from the initial PCR reactions. Genes were sequenced with at least 2-fold coverage and DNA sequences were analyzed and edited using either BioEdit (57) or Vector NTI 10.1.1 (Invitrogen). Nucleotide and amino acid sequence alignments were performed using either BioEdit or ClustalW (ch.EMBnet.org). Phylogenetic analysis was performed using Phylip on the ClustalW server (http://www.ebi.ac.uk/clustalw/) and drawn with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Sequencing of the *cpe* and *cpe/cpb2* plasmids from F4969 and F5603, respectively, was performed at Wakayama Medical University by Dr. Kazuaki Miyamoto as described in (98) located in the Appendix.

Table 3.4 List of sequencing primers.

Primers		
Forward (5' - 3')	**Reverse (5' - 3')**	**Vector[a]/PCR product sequenced**
AGATTTTAAATATGATCCTACC	CAATACCCTTCACCAAATACTC	*cpb2* multiplex PCR fragment
GGTCGTCAGACTGTCGATGAAGCC	CGCCAGGGTTTTCCCAGTCACGAC	pMAL-c2vcpb2S
GGTCGTCAGACTGTCGATGAAGCC	CGCCAGGGTTTTCCCAGTCACGAC	pMAL-c2vcpb2NS
ATGCCATAGCATTTTTATCC	GATTTAATCTGTATCAGG	pBADvcpb2S
ATGCCATAGCATTTTTATCC	GATTTAATCTGTATCAGG	pBADvcpb2NS
TTCCTCGACGCTAACCTG	GATTTAATCTGTATCAGG	pBADThiovcpb2S
TTCCTCGACGCTAACCTG	GATTTAATCTGTATCAGG	pBADThiovcpb2NS
GGGCTGGCAAGCCACGTTTGGTG	CCGGGAGCTGCATGTGTCAGAGG	pGEX-2Tvcpb2S

[a]All vectors/inserts also were sequenced with the cloning primers (Table 3.4)

3.3 ANALYSIS AND DETECTION OF PURIFIED TOXINS AND TOXINS FROM CULTURE SUPERNATANTS

3.3.1 Detection of toxins from *C. perfringens* type A-D isolates

SDS-PAGE analysis followed by Western blotting was routinely used to detect toxins in culture supernatants. Table 3.5 lists the toxins detected, antibodies used, and the references where more specific details can be found including bacterial growth conditions, SDS-PAGE methods, and Western blotting methods (all references are included in the Appendix). The purification protocols for β toxin, ε toxin, and CPE (used to create standard curves for quantifying toxin levels in culture supernatants) are found in the papers included in the Appendix. A modified method for the purification of CPB2 is described in section 3.4.1.

3.3.2 Detection of bacterial surface-bound CPB2

To determine if CPB2 could be bound to the surface of *C. perfringens*, bacteria were grown to late-log phase in TGY media to maximize CPB2 production. Bacteria were then harvested by centrifuging 3 ml of culture at 13000 rpm in a microcentrifuge. Pellets were washed 3x with 1 ml of ice cold PBS and resuspended in 500 μl PBS for sonication. Bacteria were sonicated (Misonix Sonicator 3000) using 1 min pulses and disruption of bacteria was monitored using microscopy. After lysis, the solutions were centrifuged at 13000 rpm in a microcentrifuge to crudely fractionate bacterial membranes from soluble, cytosolic components.

Soluble material was mixed with 2x Laemmli buffer and membranes were resuspended in 1x Laemmli buffer. Solutions were boiled for 5 min and run on 12% SDS-PAGE. CPB2 Western blotting was then performed as described previously (49).

3.3.3 Detection of recombinant CPB2 from *E. coli*

3.3.3.1 pBAD-based vectors. Recombinant *E. coli* carrying pBAD based vectors were grown in 3 ml of LB($amp^{100\mu g/ml}$) broth overnight with shaking at 37°C. Following overnight growth, a 1/100 dilution was made in 10 ml of fresh LB($amp^{100\mu g/ml}$) and grown at 37°C with shaking until reaching an OD_{600nm} of 0.5. To induce expression of the fusion gene, filter sterilized L-arabinose (Sigma) dissolved in water was added to give a final concentration of 0.2%. Cultures were then grown with shaking at 37°C for another 4 hr. After induction, bacteria were pelleted by centrifugation and processed for Western blotting analyses by mixing with 1x Laemmli buffer and boiling for 5 min. Samples were run on 12% SDS-PAGE, transferred to nitrocellulose (BIO-RAD), and probed with anti-V5-HRP antibody (Invitrogen) as directed by the manufacturer.

3.3.3.2 pMAL/pGEX-based vectors. To detect production of recombinant *cpb2* from pGEX-2T and pMAL-2c based vectors, recombinant *E. coli* were prepared for induction as described in section 3.3.3.1. Expression was induced by the addition of either 0.3 mM IPTG (Fisher-Scientific) for pMAL-2c or 1 mM IPTG for pGEX-2T. Cultures were then grown for 2 hr at 37°C with shaking to allow for recombinant protein production. Bacteria were then harvested and prepared for SDS-PAGE by mixing with 1x Laemmli buffer and boiling for 5 min. Samples were run on 8% SDS-PAGE, which were then stained with Coomassie Brilliant blue (G-250, BIO-RAD) to detect recombinant protein or transferred to nitrocellulose for Western blotting with either anti-GST antibodies (Amersham) or anti-MBP antibodies (New England Biolabs).

Table 3.5 Summary of primary antibodies used in this dissertation.

Antibody Name	Type	Antigen	Properties	Dilution	Source	Reference
5B7	monoclonal	ε	Neutralizing(Western)	2mg/ml (1:1000)	Paul Hauer, Univ of Iowa	Sayeed *et al.* 2005
3C9	monoclonal	CPE	Neutralizing(Western)	2mg/ml (1:1000)	McClane Lab	Fisher *et al*. 2006
CPCN10A2	monoclonal	β	Neutralizing(Western)	2mg/ml (1:1000)	Paul Hauer, Univ of Iowa	Fisher *et al*. 2006
anti-α toxin	monoclonal	α	Neutralizing	2mg/ml	Paul Hauer, Univ of Iowa	Fisher *et al*. 2006
anti-CPB2(Popoff)	polyclonal (rabbit)	CPB2	Western	1:500	Michel Popoff, Pasteur Institute	Fisher *et al*. 2005
anti-CPB2(Fisher)	polyclonal (rabbit)	CPB2	Western	1:500	PRF&L (McClane lab)	Fisher *et al*. 2006
anti-CPE	polyclonal (rabbit)	CPE	Western	1:500	McClane Lab	Fisher *et al*. 2005
anti-V5-HRP	monoclonal	V5-epitope	Western	1:5000	Invitrogen	
anti-GST	polyclonal (goat)	GST	Western	1:500	Amersham	
anti-MBP	polyclonal (rabbit)	MBP	Western	1:10000	New England Biolabs	

3.4 PURIFICATION OF CPB2 AND BIOLOGICAL ACTIVITY ASSAYS

3.4.1 Purification of CPB2 from (pB2h1v751)ATCC3624 and (pB2h2v750)ATCC3624

Purification of CPB2h1 and CPB2h2 was performed as previously described with minor modifications (49). Starting from cooked meat stocks, 0.1 ml of (pB2h1v751)ATCC3624 or (pB2h2v750)ATCC3624 was inoculated into 10 ml of FTG (containing the appropriate antibiotic [Table 3.1]) and grown overnight at 37°C. One ml of the overnight culture was then used to inoculate 30 ml of TGY (with antibiotics), which was incubated at 37°C for 7 hrs. The 30 ml TGY culture was then used in its entirety to inoculate 3 L of TGY (with antibiotics) and incubated at 37°C for 9 hrs.

After 9 hrs, cultures were chilled to 4°C and the supernatant was harvested by centrifuging the cultures for 20 min at 10000g. Protein was then precipitated with ammonium sulfate (40%), which was added to the supernatant and allowed to mix slowly at 4°C for 1 hr. Supernatants were then centrifuged for 20 min at 10000g (at 4°C) and the precipitated protein was retained. Protein pellets were resuspended in 30 mM Tris-HCl (BIO-RAD), pH 7.5 and dialyzed overnight versus resuspension buffer (40 ml versus 8L total) at 4°C. Following dialysis, the protein solution was centrifuged for 20 min at 13000g (at 4°C) and then filter sterilized with a 0.45 μm PVDF (Millipore).

The filtered protein solution was loaded on a column packed with pre-equlibriated (30 mM Tris-HCl, pH 7.5) DEAE CL6B sepharose resin (25 ml column bed volume, [Sigma]). The loaded column was then washed with four bed volumes of dialysis buffer and protein was eluted with a gradient of 0.1 M NaCl (Fisher Scientific), 30 mM Tris-HCl, pH 7.5 using the AKTA Prime purification system (GE Healthcare). Fractions were assessed for the presence of CPB2 (using anti-CPB2 Western blotting) and biological activity simultaneously by treating 2 day old Caco-2 monolayers grown in a 96-well plate with 50 μl of each fraction (mixed with 50 μl of Caco-2 media). Fraction purity was measured using Coomassie Brilliant Blue staining. Fractions that contained biological activity (wells in which cells exhibited cytopathic effects), were positive for CPB2 by Western blotting, and contained a single band on Coomassie brilliant blue stained gels were pooled and concentrated using an Amicon Ultra-10000 (Millipore) centrifugal concentration device.

After concentration, the Lowry assay was used to determine the amount of protein in the final sample. A 10 μg sample of CPB2 was then run on 12% SDS-PAGE and stained with Coomassie brilliant blue to determine purity of the final product. CPB2 also was used for Western blotting to confirm its identity and applied to five day old Caco-2 monolayers seeded in six well cell culture dishes to ensure that the final preparation retained biological activity.

3.4.2 Cell culture assays performed with CPB2h1 and CPB2h2

Caco-2 cells, a human colon carcinoma cell line with both small intestine and colon-like properties were routinely used to assess the activity of CPB2 using different assays (described below). The conditions and media used to culture Caco-2 cells are described in (49). Caco-2 cell stocks were stored in Cell Freezing Medium-DMSO 1x (Sigma) in liquid nitrogen.

For simple cytotoxicity screens, Caco-2 cells were treated with either purified CPB2h1/ CPB2h2 or *C. perfringens* culture supernatants, as previously described (49). Cells were monitored for cytopathic effects using phase contrast microscopy (Zeiss Axiovert 25 microscope) and results were recorded using a Canon Powershot G5 digital camera. For cytotoxicity trials using recombinant CPB2 produced in *E. coli*, Caco-2 cells were grown in 6-well tissue culture dishes. Recombinant bacteria were grown and expression was induced as discussed in section 3.3.2. Following induction, bacteria were harvested from 10 ml cultures by centrifugation, resuspended in 1 ml of PBS, placed on ice, and lysed via sonication. Lysis was

monitored using light microscopy under oil immersion. After lysis, sonicates were clarified by centrifugation followed by filtering through a 0.45 μm PVDF filter. Clarified lysates were then applied to Caco-2 cells and cells were incubated at 37°C. Cells were observed up to 8 hrs for the development of cytopathic effects. Lysates prepared from *E. coli* carrying empty vectors were used as negative controls. All cell culture experiments were performed in at least triplicate.

3.4.3 The neutral red activity assay

3.4.3.1 Measuring cytotoxicity. The neutral red activity (NRA) assay is described in detail in (49). Briefly, the assay utilizes the ability of healthy cells to take up and retain the neutral red dye (Sigma) within lysosomes. In damaged cells (for example, due to toxin treatment), membrane integrity is lost and the neutral red is released into the media. Caco-2 cells grown until 60-70% confluent in 96-well plates are allowed to take up neutral red in the presence of HBSS at 37°C and are then treated with different amounts of toxin and allowed to incubate at 37°C for 2 hrs. Following treatment, the cells are washed to remove free dye and the remaining cells are solubilized to determine the amount of remaining dye (measured at 570nm) compared to control, non-toxin treated cells. A decrease in dye indicates a loss of viability. This assay was initially verified for CPB2 and Caco-2 cells in (49).

3.4.3.2 Heat lability of CPB2. The NRA assay also was used to determine if CPB2 was heat labile. For these studies, CPB2h1 was diluted in 100 μl of HBSS to give a final concentration of 400 ng/ml (the concentration required to reduce Caco-2 cell viability ~50% (49)). The amount of toxin required to reduce Caco-2 cell viability by 50% was used to allow for detection of increases (which cannot be observed at toxin concentrations reducing cell viability by 100%) and decreases in CPB2h1 activity due to heat treatment. The diluted toxin was incubated at either 22°C, 30°C, 37°C, 45°C, or 52°C for 0, 2, 5, 10, or 20 min and then added to Caco-2 cells containing neutral red. The percent of viable Caco-2 cells remaining after treatment for 2 hrs at 37°C was assessed as previously described (49).

3.4.3.3 Effect of reducing agents. Since some toxins are sensitive to oxidation and can be stabilized by the presence of reducing agents (14), the effects of reducing agents on CPB2 activity was explored. The remaining cytotoxic activity after treatment of CPB2 with various reducing agents was measured using the NRA assay. CPB2h1 diluted in 100 μl of HBSS to 400 ng/ml was incubated at 37°C for 0, 5, 10 or 30 min with or without 20 mM of BME (BIO-RAD), DTT (Sigma), or L-cysteine (Sigma). Reducing agents were dissolved or diluted in HBSS as necessary. Some samples were also incubated using the conditions stated above with shaking at 220 rpm on an orbital shaker. To determine if inactivated toxin could be "rescued" by the introduction of a reducing agent, select samples that were first shaken in the absence of reducing agents were then incubated with either 20 mM DTT, BME, or L-cysteine for 5 min at room temperature following shaking.

Following the stated incubation periods, CPB2h1 control (no incubation with shaking or reducing agents) and experimental samples were used to treat Caco-2 cells containing neutral red. Caco-2 cells were incubated at 37°C for 2 hrs and viability was determined as previously described (49).

3.4.4 Experiments performed with ^{125}I-CPB2h1

3.4.4.1 ^{125}I labeling of CBP2. As an alternative method for analyzing CPB2, CPB2h1 was labeled with ^{125}I using the IODO-GEN kit from (Pierce). The direct labeling method was used with minor modifications to label tyrosine residues with ^{125}I (ICN). CPB2h1 was chosen for ^{125}I labeling rather than CPB2h2 because CPB2h1 is more cytotoxic (using Caco-2 cells) and contains more Y residues than CPB2h2 (20 compared to 17). Prior to labeling with ^{125}I, the labeling procedure described below was performed with cold iodine to ensure that CPB2h1 would retain its cytotoxic activity following the mechanical manipulations and incubation time required for labeling. Cold labeling also provided an estimate of which fractions CPB2h1 would elute into following passage through the PD-10 desalting column (determined using CPB2 Western blotting).

Due to the lability of CPB2, a CPB2h1 toxin purification was scheduled to allow labeling to be performed immediately after concentration of purified toxin. This allowed CPB2h1 to be labeled without requiring thawing and subsequent re-freezing of the sample. Prior to labeling, a PD-10 desalting column (GE Healthcare) was pre-equilibriated with 2 column bed volumes of

PBS (~20 ml). A 500 μg amount of CPB2h1 was then brought up to 100 μl in PBS to give a final concentration of 5 μg/μl and stored on ice until labeling. This 100 μl aliquot of CPB2h1 was added to the IODO-GEN tube followed by the addition of 2.5 mCi of $Na^{125}I$ and incubated on ice for 15 min. The tube was gently shaken every 2 min to help facilitate the labeling reaction. After 15 min, the labeling solution was added to a 1.5 ml eppendorf tube containing 10 μl of 25 mM KI (Fisher Scientific) to quench the reaction. The quenched reaction mixture was then added to 2 ml of PBS and loaded on the PD-10 column. Fractions were eluted with PBS, collected (~six drops per glass tube), and roughly assessed for radioactivity with a Geiger counter. A radioactivity curve was drawn and the fractions within the labeling peak that coincided with the fractions in which CPB2h1 had eluted during the cold labeling trial were pooled and stored as 60 μl aliquots at -80°C.

To determine the amount of labeling per μg of protein, a Lowry was performed on the ^{125}I-CPB2h1 followed by trichloroacetic acid precipitation of an aliquot to separate labeled protein (which will precipitate) from free label (supernatant). The amount of ^{125}I in the sample was measured using a gamma counter (Cobra Quantum, Perkin Elmer) and the aliquots were determined to contain ~8000 CPM/μg ^{125}I-CPB2. The labeled toxin also was mixed with Laemmli buffer and boiled for 12% SDS-PAGE analysis. Gels were dried and exposed to autoradiography film (Fuji) at -80°C for up to 24 hrs to visually detect labeled toxin. Finally, the ^{125}I-CPB2h1 batch was tested on Caco-2 cells grown in 6-well plates to ensure that the samples were still cytotoxic.

3.4.4.2 Trypsin sensitivity of CPB2. The susceptibility of purified CPB2h1 to trypsin was assessed using ^{125}I-CPB2h1 and cold CPB2h1. To compare the trypsin sensitivity of CPB2h1 to CPE (which is trypsin resistant), 2 μg of ^{125}I-CPB2h1, 1 μg cold CPB2h1, or 1 μg ^{125}I-CPE (132) were treated with 4 ng of trypsin (Sigma) in a total volume of 100 μl of PBS at 25°C for 0, 5, or 15 min or 30 min at 37°C. To stop the reaction, 100 μl of 2x Laemmli buffer was added to the sample which was then boiled for 5 min. Samples were loaded on 12% SDS-PAGE. After electrophoresis, protein was detected using either autoradiography (^{125}I-labeled samples) or using CPB2 Western blotting (cold CPB2 samples).

3.4.5 ^{86}Rubidium release assays

The release of ^{86}Rb, a K^+ analog, into cell culture medium can be used as an indicator of membrane permeability alterations induced by the effects of pore-forming toxins on sensitive cell lines (45, 90). In this assay, confluent Caco-2 cells grown in 24-well tissue culture dishes are preloaded with 2 ml of prewarmed Caco-2 cell media containing 4 μCi of ^{86}Rb (ICN). Caco-2 cells are then incubated at 37°C for 3-5 hrs to allow for uptake of ^{86}Rb. The cells are then washed with warm HBSS to remove free ^{86}Rb and treated with 2 ml of HBSS containing CPB2h1. At the end of treatment (see below for different conditions), the HBSS is removed from the wells and the free ^{86}Rb is counted using a gamma counter. The amount of free ^{86}Rb due to toxin treatment is calculated as follows:

$$\text{Percent } ^{86}\text{Rb release} = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximal release - Spontaneous release}} \times 100$$

Spontaneous release is determined by counting the amount of ^{86}Rb released into the HBSS at the end of the experiment in untreated wells. The maximal release is calculated by counting the amount of ^{86}Rb released into media in control wells treated with 2 ml of cell lysis buffer (0.5% saponin [Sigma], 1 M citric acid [Sigma], 0.3 M sucrose [Fisher Scientific], 1 mM $MgCl_2$ [Sigma], 1 mM KH_2PO_4 [Fisher-Scientific], pH 6.8).

CPB2h1 was used to treat ^{86}Rb-loaded Caco-2 cells using a number of different experimental procedures. Initial experiments sought to determine dose response curves and time response curves by treating Caco-2 cells with HBSS containing 1, 5, or 10 μg of toxin. Cells were then incubated for 10, 20, or 30 minutes at which point the HBSS was removed and the amount of free ^{86}Rb was counted. All experiments were performed in a 37°C warm room with prewarmed solutions. Replicate dose response experiments also were performed using HBSS lacking either 1.7 mM Ca^{2+} ($CaCl_2$ [Fisher Scientific]), 1.8 mM Mg^{2+} ($MgCl_2$ [Sigma]), or both 1.7 mM Ca^{2+} and 1.8 mM Mg^{2+} to assess the effect of divalent cations on the biological activity of CPB2h1 (cells were treated for 10 min at 37°C).

3.4.6 Osmoprotection studies

Confluent Caco-2 monolayers grown in 6-well tissue culture dishes were treated with 2 ml of HBSS containing different concentrations of CPB2h1 or CPB2h2 (10 or 50 μg/ml) and either 0.3 M of PEG (~3000 Da, [Sigma]), 0.3 M sucrose (~342 Da, [Sigma]), or 0.3 M of dextran (~1500 Da, [Sigma]). Treated cells were then incubated at 37°C and monitored for the development of cytopathic effects using phase contrast microscopy. Control wells included cells treated with CPB2 without an osmoprotectant, cells treated with only an osmoprotectant, and cells with no treatment.

Since PEG caused large changes in cell morphology (cell swelling and heterokaryon formation), osmoprotectant studies were only continued with sucrose and dextran. Caco-2 cells grown in 6-well culture dishes were treated with CPB2h1 as described above, except after 1 hr of treatment (after the appearance of visible cytopathic effects in control CPB2-treated cells) the dextran was washed off of the CPB2h1/dextran treated cells and the control dextran treated cells and replaced with 2 ml warm HBSS. Cells were then returned to the 37°C incubator. Since sucrose did not provide protection (see Results), washing experiments were not performed with sucrose treated cells. As a positive control (sucrose should protect sensitive cells from CPE (90)), 10 μg of CPE was added to sensitive cells (Madine-Darby canine kidney cells) in the absence or presence of 0.3 M sucrose.

A second set of experiments with dextran involved pretreatment of Caco-2 cells with 1 ml of HBSS containing 20 μg/ml of CPB2h2 at 4°C for 30 min. Following this treatment, the cold HBSS was removed from all cells and replaced with either HBSS/0.3M dextran, HBSS, HBSS with 20 μg/ml CPB2h2, or HBSS with 20 μg/ml CPB2h2 and 0.3M dextran. The cells were then incubated at 37°C and monitored for cytopathic effect using phase contrast microscopy.

3.5 ANIMAL EXPERIMENTS

Animal experiments were not performed at the University of Pittsburgh. Polyclonal anti-CPB2 antibodies were developed by Pocono Rabbit Farms and Laboratories Incorporated as a

contract service. Rabbit skin test studies, intestinal loop studies, and mouse intravenous injection studies were performed by or under the supervision of Dr. Francisco Uzal (University of California, Davis) and his lab members. Dr. Uzal is a certified veterinary pathologist who has extensive experience studying both natural and experimental *C. perfringens* diseases of domestic animals. All animal studies were performed at AAALAC accredited facilities and studies performed at UC Davis had approval from the IACUC of the University of California, Davis (Permit #04-11593) and the California Animal Health and Food Safety Laboratory, University of California, Davis (Permit #34).

3.5.1 Development of rabbit polyclonal anti-CPB2 antibodies

Anti-CPB2 serum was required for use in CPB2 Western blotting procedures. To facilitate this work, rabbit polyclonal antibodies were produced by Pocono Rabbit Farms and Laboratories Incorporated (PRF&L). Prior to the start of this contract work, active CPB2h1 purified from (pB2h1v751)ATCC3624 as described in section 3.4.1 was assessed in a rabbit intradermal injection model to ensure that the amount of active toxin to be used for the immunization procedures would not be lethal for the rabbit. The use of active toxin rather than a CPB2h1 toxoid was preferred since 1) inactivated toxin may not be as immunogenic as active toxin, and 2) toxoids may present different epitopes than native toxins.

Intradermal challenge trials were performed by injecting 1, 10, or 100 μg of CPB2h1 diluted in 30 μl PBS (without an adjuvant) or saline (negative control) at multiple sites in two different New Zealand White rabbits (Charles Rivers). Injections were performed on day 1, day 7, and day 14. Treated rabbits were observed after injections for development of signs of disease or lesions, and biopsy samples were taken 1 day after each injection for histology. To determine if the inoculated rabbits developed an immune response towards CPB2, preimmune serum samples (taken at time zero) and postimmune serum samples (taken on day 15) were used as primary antiserum in a CPB2 Western blotting assay. For Western blotting, 200 ng of purified CPB2h1 was mixed with Laemmli buffer and boiled for 5 min and then run on 12% SDS-PAGE, transferred to nitrocellulose, and probed as described in (49).

Since the intradermal injection of 100 μg of CPB2h1 did not induce major pathology in the rabbits, the PRF&L standard rabbit immunization procedure was used to produce anti-CPB2h1 polyclonal antibodies (Table 3.6). Prior to immunization, preimmune serum from 4

rabbits was screened for reactivity against CPB2 or other *C. perfringens* proteins using a Western blot approach. Nitrocellulose containing supernatant proteins from (pCPB2h1v751)ATCC3624 (grown under conditions to maximize CPB2 production) separated on 12% SDS-PAGE was probed with the preimmune serum. No background immunoreactivity was observed and two rabbits were chosen at random for antibody production.

Table 3.6 Protocol for producing rabbit polyclonal antibodies against CPB2h1.

Day	Procedure	Antigen (CPB2h1)	Injections	Adjuvant
-7	Pre-bleed			
0	Intradermal injection (ID)	100 μg	10-20 sites	Complete Freund's Adjuvant
14	ID	100 μg	10-20 sites	Incomplete Freund's Adjuvant (IFA)
28	Subcutaneous injection	100 μg	1 site	IFA
42	Test bleed			
56	Subcutaneous injection	50 μg	1 site	IFA
70	Exsanguination			

Following exsanguination, terminal bleeds were placed into small aliquots and stored at -80°C. The final bleeds were then assessed for immunoreactivity against both CPB2 variants (h1 and h2) via Western blot using supernatants from ATCC3624, (pB2h1v751)ATCC3624, and (pB2h2v750)ATCC3624 grown in TGY and run on 12% SDS-PAGE. The new antibodies were used at a final dilution of 1:500.

3.5.2 Rabbit intestinal loop studies with purified CPB2h1 and *cpb2*-positive and *cpb2*-negative *C. perfringens* isolates

For all intestinal loop experiments, rabbits were first tranquilized using intramuscular injections of xylazine and acepromazine followed by buprenorphine for analgesia. Anesthesia was then induced using ketamine hydrochloride (i.v.) and maintained with isofluorhane (inhalation). The abdomens were opened to expose the small and large intestines and loops averaging 20 mm in

length were created. Care was taken to not restrict blood flow to the intestinal segments and empty loops were used between consecutive experimental and control loops. Loops were then injected with a 1 ml volume of different treatment solutions (with soybean trypsin inhibitor) and the intestines were placed back inside the abdominal cavity, which was then loosely sutured. Rabbits remained anesthetized during the entire procedure and were monitored for breathing and heart rate. After a defined period of time, the rabbits were euthanized and the loops were removed from the abdominal cavity. Loops were scored for fluid accumulation (length to volume ratio), gross pathology and histopathology compared to control loops containing sterile Ringer's solution (for purified toxin trials) or sterile TGY media (for bacterial culture trials). For histopathology, tissue samples were immersed in 10% buffered formalin (pH 7.2) for 24 hrs and then embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin and analyzed under light microscopy.

To study the effects of CPB2h1 *in vivo*, intestinal loops (made in the small intestine and colon) were injected with 300 μg of CPB2h1 (with soybean trypsin inhibitor) and then incubated for 6 hrs. Experiments were typically performed using at least two loops per rabbit and two rabbits per experiment.

Rabbit intestinal loops also were challenged with filter sterilized (using 0.45 um PVDF filters) culture supernatants, whole bacterial cultures (bacteria and supernatants), or washed bacterial cultures (resuspended in 1 ml of Ringer's solution) from F5603, (pCPB2h1v751) ATCC3624, (pCPB2h2v750)ATCC3624, or (pJIR750)ATCC3624. For these experiments, bacteria were grown in TGY until reaching late-log phase. Intestinal loops were injected with 1 ml samples and incubated for 6 hrs. Following incubation, loops were process as described above for toxin-treated loops.

3.5.3 Mouse intravenous lethality experiments

To dissect the roles of individual toxins produced by type B-D isolates in lethality, a mouse intravenous injection model was used to determine the LD_{50}/ml of culture supernatants from a large number of isolates (22, 147). The LD_{50}/ml for each isolate was then correlated with toxin concentrations to assess the importance of each toxin in lethality. Toxins deemed important by this correlation analysis were then neutralized and the remaining lethality of select supernatants was assessed using the mouse i.v. lethality model. The detailed methods for these experiments

can be found in the papers included in the Appendix (48, 121). Mice also were injected i.v. (via the tail vein) with purified CPB2h1 to determine a rough LD_{50} (22).

4.0 RESULTS

4.1 ESTABLISHING AN ASSOCATION BETWEEN THE *Clostridium perfringens* BETA2 TOXIN AND GASTROINTESTINAL DISEASE IN HUMANS AND DOMESTIC ANIMALS

The results of experiments designed to determine the initial validity of my hypothesis, that AAD and SD isolates produce an accessory toxin that is not produced by food-poisoning isolates, are described in detail in ((49, 98) [Appendix]). A summary of those results will be discussed below in section 4.1.1 and 4.1.2. Studies performed to address the mechanism of action of CPB2 will be reviewed in Section 4.2. Finally, the results from experiments designed to address the potential role of CPB2 in veterinary gastrointestinal diseases will be discussed in section 4.3. Detailed results for section 4.3 involving type C and type D isolates can be found in the manuscripts located in the Appendix (48, 121).

4.1.1 The production of *Clostridium perfringens* beta2 toxin by human gastrointestinal disease isolates

The initial goal of this dissertation was to address the main thrust of my hypothesis, that the production of an accessory toxin (namely, CPB2) by CPE-positive nonfoodborne human GI disease isolates (causing AAD and SD) carrying a plasmid *cpe* gene, could explain the more severe symptoms of patients suffering from AAD/SD compared to the symptoms of patients with CPE-associated food poisoning. For this hypothesis to be valid, it was necessary to initially ask whether AAD and SD isolates did indeed carry an accessory toxin gene (49). PCR-

based assays determined that ~80% of AAD and SD isolates carried the *cpb2* gene in opposition to only 11% of food-poisoning isolates being positive for *cpb2*. Interestingly, within *cpb2*-positive AAD/SD isolates, all *cpe*/IS*1151* plasmid isolates were positive for *cpb2* whereas only ~70% of AAD/SD isolates carrying a *cpe*/IS*1470*-like plasmid were positive for the *cpb2* gene by PCR.

Results from experiments that used pulsed field gel electrophoresis (PFGE) followed by Southern blotting (SB) to assess the localization of the *cpb2* gene in AAD/SD isolates (with either DIG-labeled *cpe* or *cpb2* probes) indicated that the *cpb2* and *cpe* toxin genes co-localize to an ~75 kb plasmid in *cpe*/IS*1151* plasmid isolates. However, in isolates carrying a *cpe*/IS*1470*-like plasmid, the *cpe* and *cpb2* genes are on two different-sized plasmids of ~75 kb and ~50 kb, respectively. Partial sequencing of the *cpe*/IS*1151* plasmid from F5603 determined that the *cpe* and *cpb2* toxin genes are on the same plasmid (~19 kb apart). An overlapping PCR assay that directly connects these two toxin genes was then developed and used to assess the relationship between *cpe* and *cpb2* in other isolates. Results from this PCR survey, in agreement with PFGE/SB results, indicated that the *cpb2* gene is on the *cpe*/IS*1151* plasmid in most isolates, supplying a partial explanation as to why *cpb2* is strongly associated with AAD/SD isolates.

Sequencing of the *cpb2* gene from AAD/SD isolates and the two *cpb2*-positive FP isolates identified two human *cpb2* variants, named *cpb2h1* and *cpb2h2* (where h stands for human). The *cpb2h1* variant shares ~97% identity and 96% similarity with the amino acid and nucleotide sequences of the “atypical” *cpb2* gene identified in veterinary disease isolates (73) (reported while (49) was in press). The *cpb2h2* variant was more similar to the “typical” *cpb2* sequence originally reported by Gibert *et al.* (52) and shares 92% identity and 90% similarity at the level of amino acid and nucleotide sequences, respectively.

A second obvious requirement for CPB2 to be involved in human GI disease is the production of CPB2 by *cpb2*-positive isolates. While *in vitro* production of CPB2 does not necessarily indicate that CPB2 is produced *in vivo*, it at least indicates that a functional gene is present in *cpb2*-positive isolates. Western blotting experiments performed with vegetative culture supernatants demonstrated that most (95%) *cpb2*-positive isolates produced detectable levels of CPB2 *in vitro*. Both CPB2h1 and CPB2h2 variants were detected via Western blotting using rabbit polyclonal antibodies developed against the typical CPB2 (CPB2h2-like) variant.

Since the production of CPE and CPB2 together in the lumen of the intestine would be predicted to cause increased pathology (and thus more severe symptoms) compared to CPE alone, the presence of CPB2 in sporulating culture supernatants also was measured using

Western blotting. These experiments demonstrated that both CPE (produced only during sporulation (131)) and CPB2 could be present in the supernatants of sporulating cultures. Sporulation of cultures was assessed using microscopy and CPE was detected using a Western blot approach.

Collectively, these results support the hypothesis that, in opposition to FP isolates, the majority of AAD/SD isolates carry and produce an accessory toxin (CPB2). CPB2 was found to exist as two different amino acid sequence variants, CPB2h1 and CPB2h2, and the presence of *cpb2* in plasmid *cpe* isolates was explained, in part, by its presence on the *cpe*/IS*1151* plasmid. Further work was then performed to characterize the *cpe*/*cpb2* plasmids in AAD/SD isolates to determine if other plasmid-encoded virulence factors were present in these isolates.

4.1.2 Sequencing and analysis of *Clostridium perfringens cpe* and *cpe/cpb2* plasmids

Prior to this dissertation, sequence information for the *cpe* plasmids (both IS*1151* and IS*1470*-like) was only available for an 8 kb region immediately surrounding the *cpe* gene (26, 97). PFGE/SB experiments had determined that the *cpe* plasmids were ~100 kb in size, suggesting that the sequence of large regions of these plasmids remained unknown (49). Furthermore, sequencing of the 19 kb *cpb2*-*cpe* region from the *cpe/cpb2/*IS*1151* plasmid of isolate F5603 (pCPF5603), followed by subsequent PCR analysis, had indicated that this region was absent from *cpe*/IS*1470*-like plasmids (Appendix) (49). To determine if differences between the IS*1470*-like and IS*11151* plasmids extended beyond this 19 kb region and to identify other potential virulence factors, two *cpe/cpb2/*IS*1151* plasmids (one completely [pCPF5603] and one partially [pCPF4013]) and one *cpe*/IS*1470*-like plasmid (pCPF4969) were sequenced. The plasmid sequences were then used to design primers for use in PCR reactions to assess the conservation of genes and gene order in different *C. perfringens* virulence plasmids using long-range PCR assays (products were >2 kb in length), short-range PCR assays (which amplified regions inside ORFs), and overlapping PCR assays. The detailed results for these experiments can be found in (98) (Appendix).

Briefly, sequencing of the pCPF5603 and pCPF4969 plasmids and subsequent PCR assays revealed that the *cpe* plasmids are composed of a ~30 kb conserved region that encodes two putative DNA methylases (a DNA cytosine methylase [*dcm*] and a DNA adenosine methylase [*dam*]), a cluster of ~10 genes that are weakly similar (10-20% at the amino acid

level) with ORFs found in the conjugative transposon Tn*916* (widely dispersed across Gram-positive species (37)), and a cluster of ~6 genes with strong similarity (>80%) to genes encoded on the *C. perfringens* plasmid pCP13 (part of the sequenced genome of strain 13 (125)). The Tn*916*-like gene cluster contains ORFs that are known to be required for the conjugative transfer of Tn*916*. Recently, this gene cluster was identified in the *C. perfringens* conjugative plasmid pCW3 (encoding tetracycline resistance) and gene knockout construction has demonstrated that genes within the Tn*916*-like gene cluster are necessary for conjugative plasmid transfer (11). This region and the genes within this region have therefore been renamed as transfer clostridial plasmid genes (or *tcp*). The pCP13-like gene cluster contains a gene encoding a putative collagen adhesion, *cna*.

The non-conserved region of pCPF5603 contains a large cluster of metabolic genes with similarity to genes encoded on the strain 13 chromosome, a cluster of 4 genes (including *cpb2h1*) with similarity to genes found on pCP13, the *cpb2* gene, and two putative mobile DNA elements (IS*1151* and a putative transposase) that flank the non-conserved region of DNA. The non-conserved region of pCPF4969 contains two putative bacteriocins, a VirR/VirS homolog, and putative mobile elements that also flank the non-conserved DNA region.

Sequencing of the *cpb*-encoding virulence plasmid from a type C isolate and partial sequencing of the *etx*-encoding virulence plasmid from a type D isolate by the Institute for Genome Research (Ian Paulsen and Gary Myers, unpublished [TIGR DARPA/NIAID 1U01AI499 21-01]) and long-range PCR (98) designed using the *cpe* plasmid sequences indicated that most *C. perfringens* virulence plasmids carry the large conserved region including the *tcp* region. Independent annotation of the type C and D plasmids determined that, like pCPF5603 and pCPF4969, the non-conserved DNA regions are also flanked by putative mobile elements (Figure 4.1). Furthermore, it is interesting that all three toxin genes *cpe, etx,* and *cpb* are present adjacent to the *dcm* gene and that the *tcp* region is always present between the *dcm* and *dam* genes, indicating that these locations may be a hot spot for insertions. Similar to the *cpe* gene in pCPF5603, the *etx* and *cpb* genes are also located adjacent to an IS*1151* element.

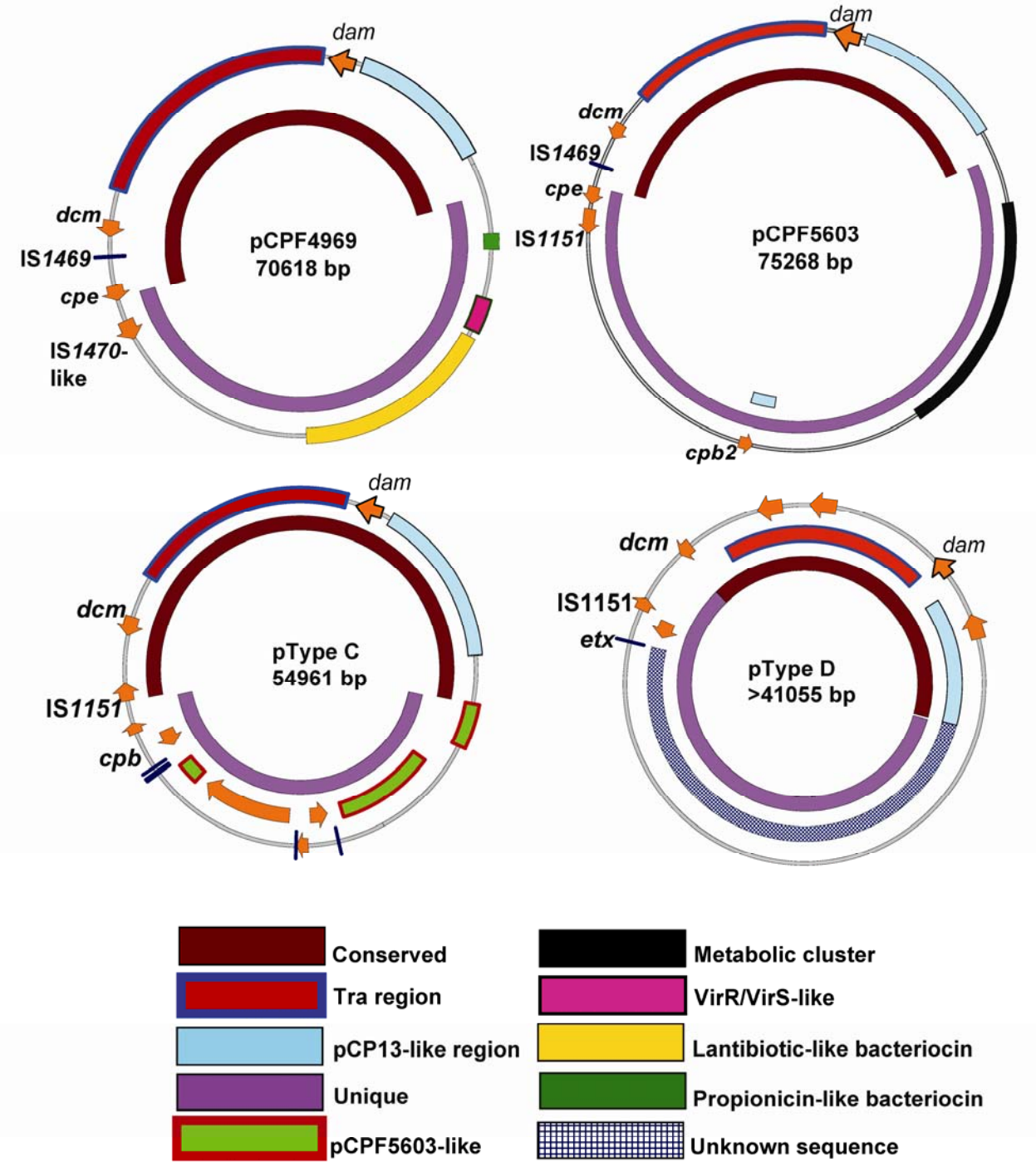

Figure 4.1 A graphic comparison of four sequenced *C. perfringens* virulence plasmids. A key to the different plasmid regions is shown at the bottom of the figure.

4.1.3 Production of CPB2 by non-type A gastrointestinal disease isolates from domestic animals

The *cpb2* gene is the most widely dispersed non-chromosomal toxin gene in *C. perfringens* and can be found in all types A-E (27). Most evidence supporting a role for CPB2 in disease comes from epidemiological studies with the strongest link between CPB2 and GI disease in domestic animals being for porcine and equine species (Table 1.3). However, CPB2 can be found commonly in some healthy animal species (such as poultry) (27). One possible explanation for the lack of correlation between the presence of the *cpb2* gene and disease in some animals may be the lack of CPB2 production by those isolates. Consistent with this hypothesis, it also has been reported that non-porcine veterinary disease strains carrying an atypical *cpb2* gene do not produce detectable levels of CPB2 (27, 73). However, a number of findings indicate that veterinary disease isolates carrying an atypical *cpb2* gene should be able to produce CPB2. These findings include: 1) human disease isolates naturally carrying a *cpb2h1* gene (which is similar to the atypical *cpb2* gene, Table 4.1 and Figure 4.2) produce detectable levels of CPB2h1 (49), 2) a naturally *cpb2*-negative type A isolate transformed with a vector carrying a 1.2 kb DNA insert carrying the *cpb2h1* gene can produce CPB2h1 (49), and 3) the putative promoter region (based on the known promoter region of the typical *cpb2* gene) cloned in front of a promoterless gene can drive expression of the foreign gene and allow production of the foreign protein (34).

Table 4.1 Amino acid (A) and nucleotide sequence (B) similarity charts comparing typical and atypical CPB2 sequences with *cpb2h1* and *cpb2h2* sequences.

A)

CPB2 "type"	typical		CPB2h2	CPB2h1	atypical
	CWC245	Strain 13	F4859	F5603	JGS1902
CWC245	100	92	92	63	62
Strain 13		100	97	63	63
F4859			100	64	63
F5603				100	96
JGS1902					100

B)

cpb2 "type"	typical		*cpb2h2*	*cpb2h1*	atypical
	CWC245	Strain 13	F4859	F5603	JGS1902
CWC245	100	95	90	70	71
Strain 13		100	93	70	71
F4859			100	68	68
F5603				100	97
JGS1902					100

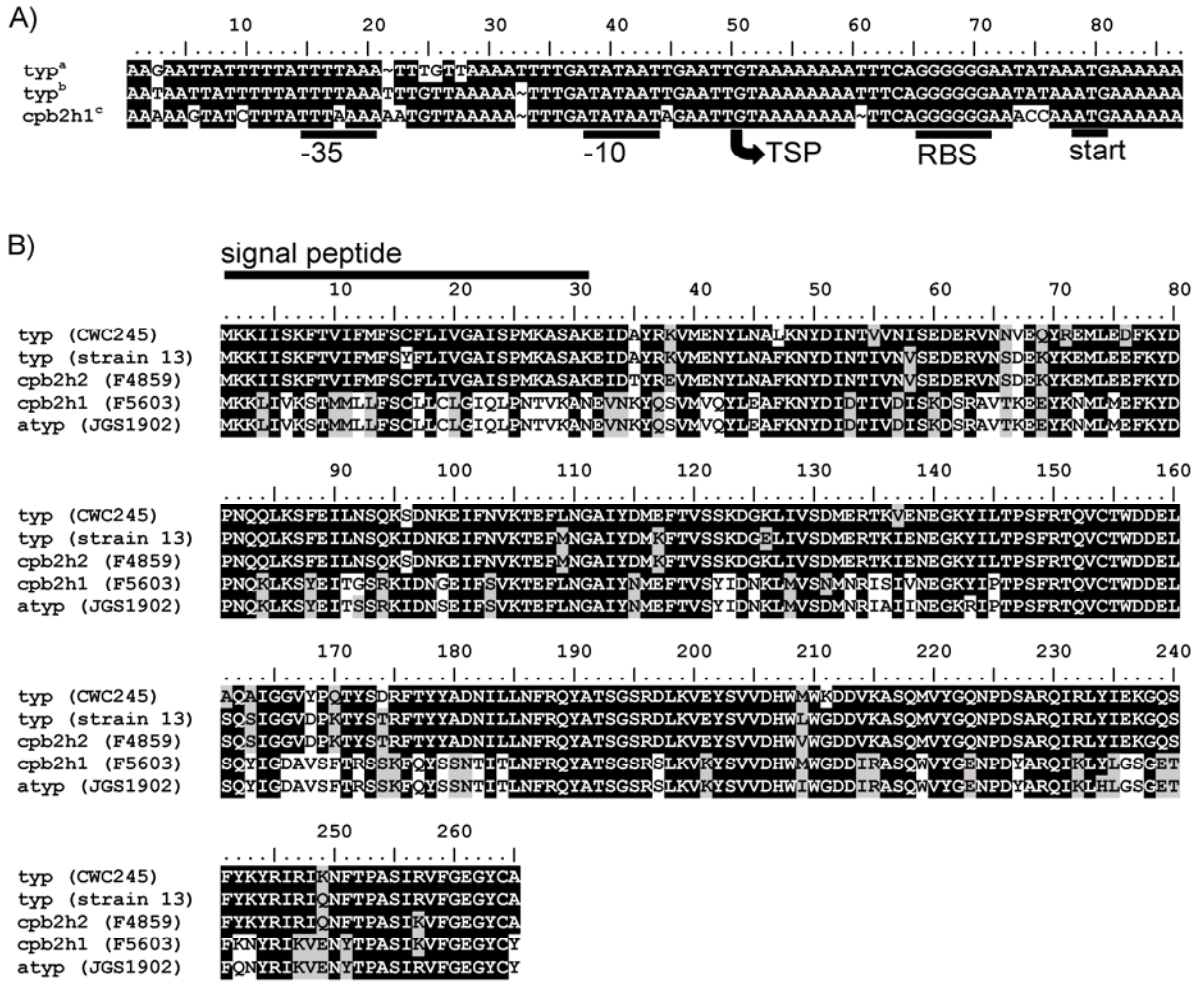

Figure 4.2 Nucleotide and amino acid sequence alignments comparing variant *cpb2* genes. In (A), the promoter region from strain 13([a]) which has been mapped by promoter extension PCR (104), is compared to the promoter from a second typical *cpb2* gene (CWC245[[b]]) and the *cpb2h1* gene from F5603([c]). The -35, -10, transcriptional start site (TSP), putative ribosomal binding site (RBS), and start codon are indicated. The *cpb2* ORFs from atypical and typical strains were then translated and compared in B. The signal peptide is indicated by the line at the top of the amino acid sequence alignment. Shading was performed in panels A and B using a 50% identical threshold.

Furthermore, in the reports in which the atypical CPB2 toxin was not detected in culture supernatants, isolates were grown for either 24-48 hrs (27) or 48 hrs (73). While the authors were able to detect production of typical CPB2 using these conditions, pilot experiments detecting CPB2h1 and CPB2h2 in the supernatants of (pB2h1v751)ATCC3624 and (pB2h2v750)ATCC3624 indicated that CPB2 production peaks during late-log phase and then decreases during stationary phase (Figure 4.3). It should be noted that our laboratory uses different anti-CPB2 antibodies (two independent rabbit polyclonal antibodies versus a goat polyclonal or a mouse monoclonal), different media for growth (TGY versus BHI), and different methods of concentration (centrifugal filters versus chloroform/methanol), and a different growth stage (late log phase versus late stationary phase) to detect CPB2 in culture supernatants.

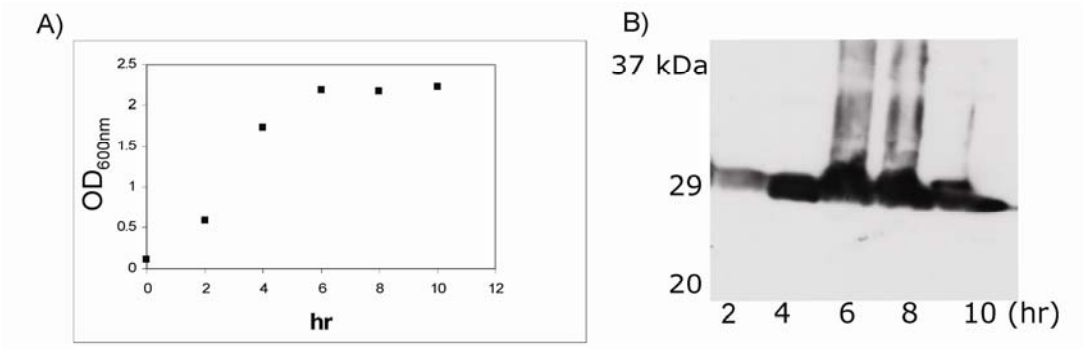

Figure 4.3 CPB2 production versus growth. A growth curve (A) and CPB2 Western blot (B) were performed to determine when CPB2 production is maximized during bacterial growth. Samples were removed every two hours and assessed for turbidity (OD_{600nm}) and levels of CPB2.

To determine if our collection of non-type A isolates contained atypical *cpb2* gene sequences, a portion of the *cpb2* gene ORF was sequenced from a number of type B-D isolates (at least 4 of each type) and aligned with typical and atypical *cpb2* genes (Figure 4.5). These sequencing results agreed with previous findings that most (or all) non-porcine disease isolates carry an atypical *cpb2* gene. Interestingly, two type C isolates (see CN5388, Figure 4.4) carrying an atypical *cpb2* gene had deletions within the *cpb2* ORF that would result in a truncated CPB2.

CPB2 Western blot analysis results from type B-D isolates carrying typical or atypical *cpb2* sequences demonstrated that most isolates could produce detectable levels of CPB2 ((48, 121) Appendix and Section 4.3.2.3 for type B isolates). The two type C isolates with deletions present in their *cpb2* genes failed to produce detectable levels of CPB2 (Table 4.2).

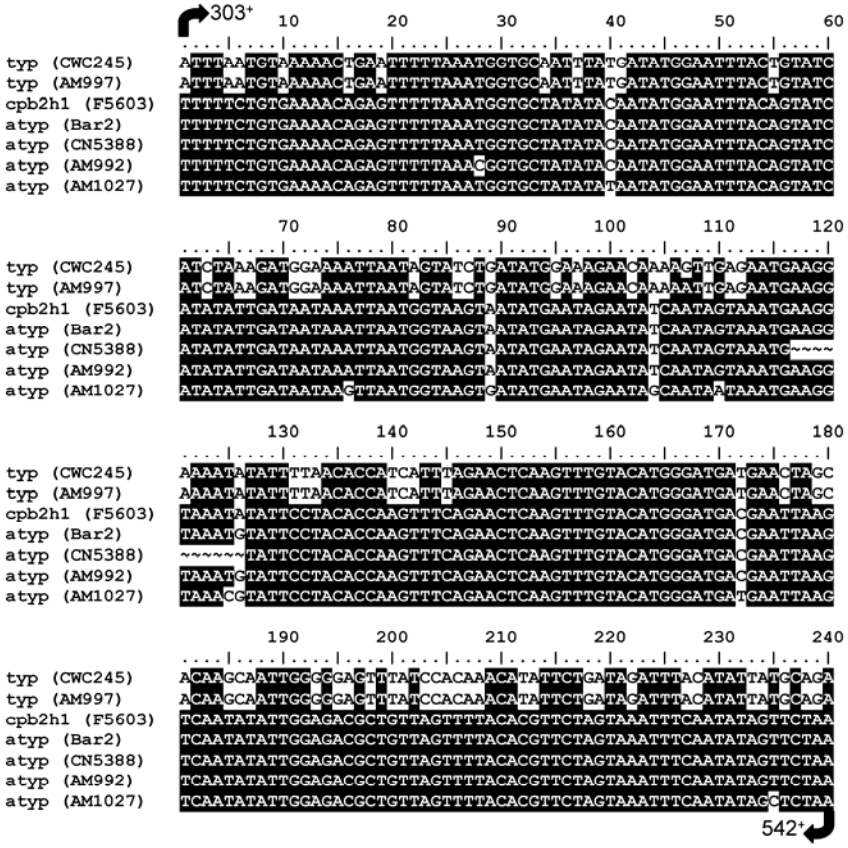

Figure 4.4 Nucleotide sequence alignment of *cpb2* genes from non-type A isolates. A portion of the *cpb2* ORF was amplified and sequenced to determine the *cpb2* type. The sequenced region includes nucleotides 303 to 542 within the *cpb2* ORF (indicated by the 5' and 3' arrows) and the *cpb2* type is indicated prior to the isolate name. CWC245 (type C, typical porcine disease isolate) is not listed in Table 3.1 and was sequenced by Gibert *et al*. (52).

4.1.4 Summary of section 4.1 findings

Results from section 4.1 have demonstrated that most human AAD and SD isolates produce CPB2, in contrast to most FP isolates. These studies also showed that the association between *cpb2* and AAD/SD isolates is due, in part, to the presence of *cpb2* on the *cpe* plasmid in *cpe*/IS*1151* positive isolates. Similar to *cpe*-negative/*cpb2*-positive isolates, the *cpb2* gene was only localized to plasmids in AAD and SD isolates and the type of *cpb2* gene present in AAD/SD isolates correlates with its presence on an ~50 kb plasmid (the *cpb2h2* gene found in *cpe*/IS*1470*-like isolates) or an ~75 kb plasmid (the *cpb2h1* gene found in *cpe*/IS*1151* isolates). Lastly, the *cpe*/IS*1151*/*cpb2* and *cpe*/IS*1470*-like plasmids appear to be capable of conjugative transfer based on the presence of the *tcp* gene cluster.

With regard to veterinary disease isolates, typical and atypical *cpb2* variants were identified and these variants are similar to the human *cpb2h2* or *cpb2h1* genes, respectively. CPB2 Western blotting was able to detect both typical and atypical CPB2 in culture supernatants [(48, 121) and section 4.3.2], conflicting with previous findings from another group. It remains possible that the different culturing conditions and methods used for detection of CPB2 explain these contrasting results.

4.2 UNRAVELING THE MECHANISM OF ACTION OF THE *Clostridium perfringens* BETA2 TOXIN (*IN VITRO* STUDIES)

Since the experimental results in section 4.1 were consistent with CPB2 playing a role in human gastrointestinal disease (and animal gastrointestinal disease) efforts were made to understand how CPB2 could be acting in pathogenesis. Initial studies focused on identifying and characterizing a cell culture model in which the mechanism of action of CPB2 (CPB2h2 and CPB2h1) could then be explored.

Prior to this dissertation it was known that CPB2 was cytotoxic for I407 and CHO cells and that treated cells developed phase-brightened margins and showed signs of cell rounding (52). Enzyme activity studies found that CPB2 does not possess UDP-glucosylation or ADP-

ribosylation activity and FITC-phalloidin staining of treated cells did not show striking disorganization of actin filaments as seen with clostridial toxins that inhibit actin polymerization (52). However, little follow up work has been reported on the mechanism of action of CPB2 and the previous studies were performed with typical CPB2.

4.2.1 Identifying a cell culture model for studying CPB2 *in vitro*

Preliminary experiments concentrated on identifying a cell culture model for studying the mechanism of action of CPB2 (previous studies were performed with CHO and I407 cells (52, 71)). Since I407 cell-lines have been reported to be derived from normal embryonic intestinal tissue that was contaminated with HeLa cells (1), we sought to assess the activity of CPB2 on a more established intestinal cell culture model. Caco-2 cells, which are routinely used as a model of the intestinal epithelium and to study the mechanism of action of intestinally-active toxins (including CPE (128)), were tested for their sensitivity to CPB2. These early studies were performed with naturally *cpb2*-positive AAD/SD isolates, CPB2 producing *C. perfringens* transformants ([pB2h1v751]ATCC3624 and [pB2h2v750]ATCC3624), and purified CPB2h1 and CPB2h2. Results from these findings are reported in detail in (49) (Appendix).

Briefly, Caco-2 cells were sensitive to CPB2h1 and CPB2h2, with cell death occurring rapidly (within 1 hour with a 10 μg/ml dose) (49). Previous studies with I407 cells used 20 μg/ml of typical CPB2. The cytopathic effects included condensation of nuclei, cell swelling, membrane bleb formation, and detachment of cells from the cell culture dish. Cells treated with CPB2h1 or CPB2h2 did not exhibit different morphology upon toxin treatment. Neutral red viability assays were then performed to determine if the amino acid sequence differences between CPB2h1 and CPB2h2 affected the biological activity of these toxins towards Caco-2 cells. This assay found that the TCD_{50} (toxin dose required to kill 50% of the monolayer) for CPB2h1 was lower than CPB2h2 with a TCD_{50} of 400 ng/ml for CPB2h1 compared to 6 μg/ml for CPB2h2, respectively.

Since CPB2h1 was more cytotoxic than CPB2h2 for Caco-2 cells, most of the remaining studies performed in the following subsections were performed with CPB2h1 because 1) it would be easier to detect small differences in activity (for example in heat lability studies and divalent cation studies) and 2) the more active CPB2h1 toxin would allow experiments to be performed with less toxin. Since only small amounts of CPB2h1 and CPB2h2 (~300 μg/L) are

obtained during purification from the *C. perfringens cpb2* transformants, the second point was an important consideration.

4.2.2 Use of the neutral red activity assay to study CPB2h1 heat lability and sensitivity to reducing agents

4.2.2.1 Heat lability of CPB2.

Using the neutral red cell viability assay to measure CPB2h1 activity, experiments were performed to determine whether CPB2 was heat labile (toxins are commonly classified as being heat-labile versus heat-stabile [for example, the ETEC heat-stable and heat-labile toxins]). Loss of cytotoxicity after heat treatment also is consistent with activity resulting from the action of a proteinaceous substance rather than the action of a non-protein contaminant (such as LPS in Gram-negative bacteria).

For heat lability experiments, CPB2h1 was diluted to 400 ng/ml in HBSS and incubated at different temperatures ranging from 22°C to 52°C for 20 min. Typically, heat stability is judged by the ability of a toxin to survive heating at 56°C for 20 min, which are the conditions traditionally used to inactivate serum complement proteins. After incubating at different temperatures and times, the toxin samples were applied to Caco-2 cells grown in 96-well plates that had been preloaded with neutral red. The Caco-2 cells were then incubated for 2 hr and viability of the treated cells was compared to untreated control cells. CPB2h1 was used at a concentration of 400 ng/ml since this amount of toxin would be predicted to kill ~50% of the monolayer after a 2 hr incubation at 37°C. This amount of cell killing would allow decreases and increases in CPB2 activity to be detected.

Results for these heating experiments are shown in Figure 4.5. CPB2h1 lost all activity after being incubated for 20 min at 52°C (Figure 4.5 A). This result is consistent with CPB2h1 being a heat labile toxin and is similar to other *C. perfringens* toxins which also are heat labile (54, 117, 133). Surprisingly, CPB2h1 also showed a loss of activity after 20 min of treatment at 37°C, but was stable at 22°C and 30°C for at least 20 min.

Stability at 37°C also was explored under conditions in which toxin samples were incubated either with or without shaking (Figure 4.5 B). Shaking at 240 rpm rapidly inactivated CPB2h1 with all activity lost within 5 min of treatment. This result indicates that CPB2h1 may also be sensitive to oxygen (introduced by aeration during shaking).

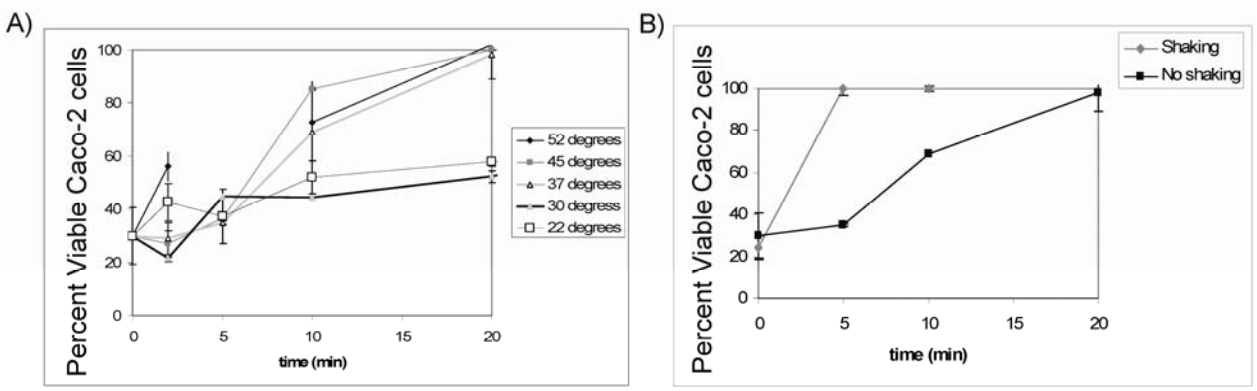

Figure 4.5 The effects of heat and shaking on CPB2h1 activity. 400 ng/ml of CPB2h1 diluted in HBSS was incubated at different temperatures for different time periods and the remaining cytotoxicity after treatment was measured using the neutral red viability assay with Caco-2 cells (A). The effect of shaking on CPB2h1 incubated at 37°C also was assessed using the neutral red viability assay (B). Activity is reported as the percent of viable cells.

4.2.2.2 Effect of reducing agents. The loss of CPBh1 cytotoxicity after shaking was not surprising in the context of previous observations that samples lost activity after rigorous vortexing or with repeated inversion of sample tubes. These results are consistent with CPB2 being sensitive to oxidizing conditions. Some pore-forming toxins, including PFO and listeriolysin O (LLO) produced by *Listeria monocytogenes*, lose activity or are inactive in the presence of oxygen (14). These toxins are members of a larger class of toxins known as the thiol-activated toxins (also refered to as the cholesterol-dependent cytolysins) that are sensitive to oxidizing conditions and can be inactivated by excessive agitation in an O_2-rich environment. This oxygen sensitivity is thought to result from the change in the oxidation state of a single cysteine residue in these toxins. Treatment of inactivated toxins with a reducing agent such as DTT will reactivate the toxin and removal of the cysteine residue using site-directed mutagenesis results in a toxin that is no longer sensitive to oxidizing conditions (123).

Unlike the thiol-activated toxins, all CPB2 variants possess two conserved cysteine residues at positions 154 and 264 and either zero, one, or two cysteines within the signal peptide (Figure 4.2). The presence of two conserved cysteine residues would suggest that the tertiary structure of CPB2 contains a single disulfide bridge. This configuration would not result in a free thiol group that would be sensitive to oxidizing conditions. However, since structural analysis of CPB2 has not been performed it is possible that the cysteine residues may be free and exposed to the solvent. Therefore, the ability of reducing agents to restore or protect CPB2h1 activity from oxidation due to shaking was explored.

CPB2h1 diluted to 400 ng/ml in HBSS was shaken at 37°C with or without 20 mM of the reducing agents DTT, BME, or L-cysteine. These samples were then tested for activity using the neutral red activity assay (Figure 4.6 A). All samples showed loss of activity approaching 100% after 5 minutes of treatment and the starting (time 0) activity of the CPB2h1 preparations varied from the control sample (no reducing agent) with the possible exception of samples treated with DTT, which had a starting activity similar to the control sample. The DTT-treated samples also were the only samples to retain some cytotoxic activity throughout the 30 minutes of treatment (samples retained ~20% more activity than the control sample or the BME and L-cysteine treated samples).

In separate experiments performed to test whether the addition of reducing agents after inactivation of CPB2h1 (by shaking) could rescue toxin activity, samples were shaken as before at 37°C except after shaking 20 mM of DTT, BME, or L-cysteine was added to each sample. Samples were incubated for 5 min at room temperature and were then tested for any remaining cytotoxicity using the neutral red assay. Results for each reducing agent are shown in Figure 4.6 (B-D). With the exception of DTT, which was able to rescue some activity at the 5 min time point compared to the control sample, none of the other reducing agents were able to restore activity. Collectively, these experiments demonstrate that CPB2h1 activity does not seem to be preserved in the presence of different reducing agents and that CPB2h1 activity also can not be rescued by reducing agents.

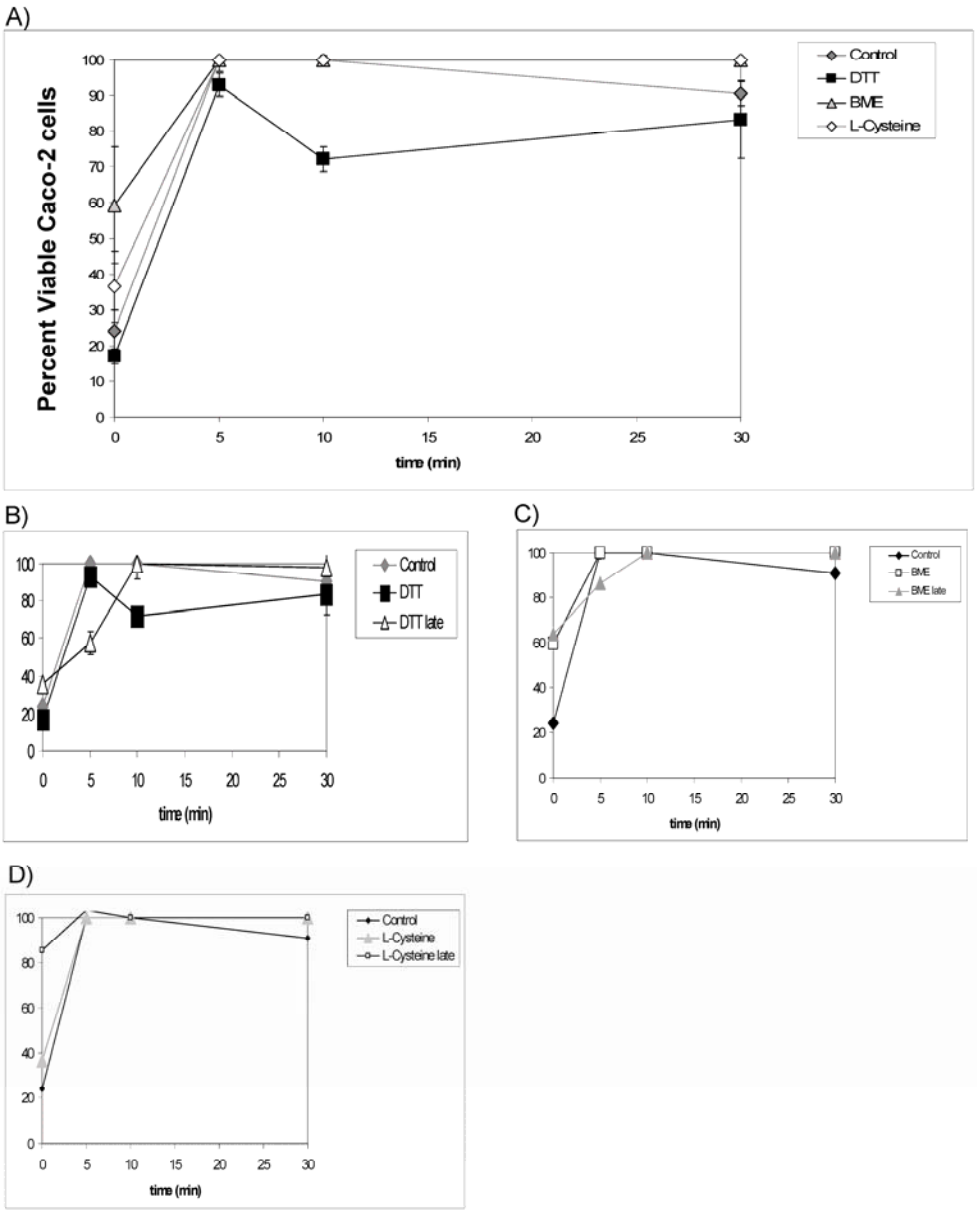

Figure 4.6 The effects of different reducing agents on the activity of CPB2h1. 400 ng/ml of CPB2h1 was shaken at 37°C for different time points with or without 20 mM reducing agents (A). Samples were then assessed for activity on Caco-2 cells using the neutral red viability assay. Samples shaken without reducing agents also were incubated with different reducing agents for 5 min at room temperature after shaking (B-D) and the activity of each sample was measured using the neutral red viability assay. Activity is reported as the percent of viable Caco-2 cells after 2 hrs of treatment.

4.2.3 Sensitivity of CPB2h1 to trypsin

Toxins acting within the mammalian intestine are subject to proteolysis due to the presence of enzymes including trypsin and chymotrypsin. Some toxins have evolved mechanisms to act in the presence of trypsin, by 1) requiring trypsin-digestion for activation (ε toxin) (96), 2) showing increased activity after minimal proteolysis (CPE) (55, 78), and/or 3) having a relatively trypsin-resistant tertiary and quaternary structure (CPE and ε toxin are highly trypsin resistant after an initial proteolysis event) (131). However, some toxins (such as β toxin) that are initially secreted into the intestinal lumen remain sensitive to deactivation due to proteolysis, yet are still associated with disease (possibly under ideal conditions, such as low trypsin levels).

Initial work by Gibert *et al.* (52) determined that typical CPB2 is sensitive to trypsin. Treatment with small amounts of trypsin resulted in initial degradation of CPB2 into an ~24 kDa fragment (with 16 ng/ml) and then two fragments of ~15 kDa and ~13 kDa. Treatment with doses higher than 16 ng/ml resulted in two fragments of ~15 kDa and ~13 kDa without the ~24 kDa intermediate. Cytotoxicity studies with trypsin-treated CPB2 found that no activity remained after proteolysis.

Since degradation fragments may not be recognized by antibodies (normally used to detect CPB2h1 via Western blot), CPB2h1 was labeled with ^{125}I to allow for detection of toxin fragments using autoradiography. The iodination labeling method (^{125}I labels tyrosine residues) was chosen because our lab has successfully used this method for labeling CPE (79). There are ample tyrosine residues within the secreted portion of CPB2h1 (Figure 4.2) and the tyrosine residues are equally dispersed at roughly 10 amino acid increments. This equal dispersal of labeled residues should allow for detection of proteolytic products regardless of the cleavage location. This broad labeling is also an advantage over fluorescent probe labels such as NBD, which typically label a single residue and would not give information about all fragment sizes.

To ensure that the labeling process (which requires gentle mixing of the labeling solution) would not affect CPB2h1 activity, the iodination procedure was performed with cold iodine (see methods). Toxin undergoing a cold labeling treatment was still active (based on its ability to damage Caco-2 monolayers) after being collected from the desalting column. CPB2h1 was then purified and immediately labeled after purification to avoid undergoing an extra freeze thaw step. To ensure that the ^{125}I CPB2h1 was labeled, hot toxin (and ^{125}I CPE) was run on 10% SDS-PAGE which was then dried and exposed to film to detect labeled toxin (Figure 4.7 A). In

contrast to ^{125}I CPE, a large portion of the labeled CPB2h1 was present at the dye front, suggesting that the iodination process may have damaged CPB2h1. Similar to CPE which is known to aggregate at high concentrations, labeled CPB2h1 species also were present at the top of the gel (at the 5 μg dose). Labeled toxin still retained activity after iodination based on the ability of the ^{125}I CPB2h1 preparation to damage a Caco-2 monolayer (Figure 4.7 B-E).

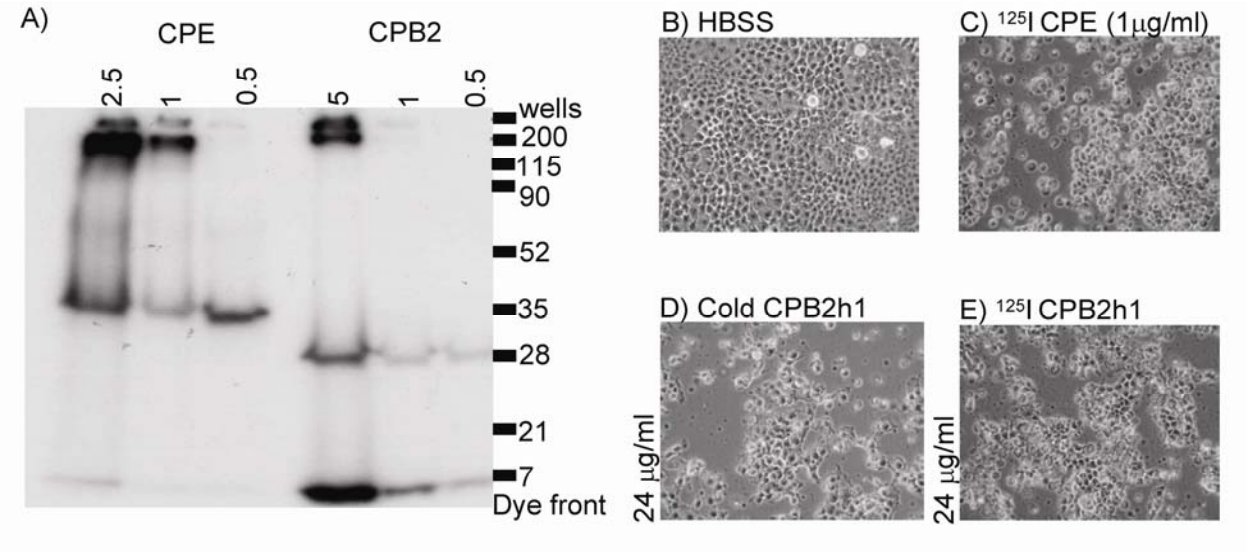

Figure 4.7 Characterization of ^{125}I CPB2h1. ^{125}I labeled CPE (lanes 1-3) and CPB2 (lanes 4-6) were run on 10% SDS-PAGE to observe labeled toxin (A). Toxin was detected using autoradiography. The amount of toxin loaded is indicated at the top of the gel and the location of molecular weight markers (in kDa) are shown to the right of the gel. Labeled toxin also was tested for cytotoxicity on Caco-2 monolayers (B-E). Toxin was diluted in HBSS ([toxin] is listed with each micrograph) and used to treat Caco-2 monolayers at 37°C for 1 hr. Pictures were taken at 200x and the toxin used for treatment is listed at the top of each micrograph.

^{125}I CPB2h1 and cold CPB2h1 were then subjected to treatment with trypsin. Initially, either 2 μg of hot toxin or 1 μg of cold toxin were treated with 4 ng of trypsin at room temperature for 0, 5, or 15 min (and 37° for 30 min for hot toxin, only). Samples were then

boiled and run on 12% SDS-PAGE to separate degradation products. Hot toxin was detected using autoradiography and cold toxin was detected using CPB2 Western blot (Figure 4.8 A and B). As a positive control for trypsin treatment, hot CPE also was digested with trypsin. Consistent with previous reports, CPE was initially cleaved to a slightly smaller fragment (possible due to removal of the first 30 N-terminal amino acids) and then remained relatively trypsin resistant (left 4 lanes in A) (55, 112).

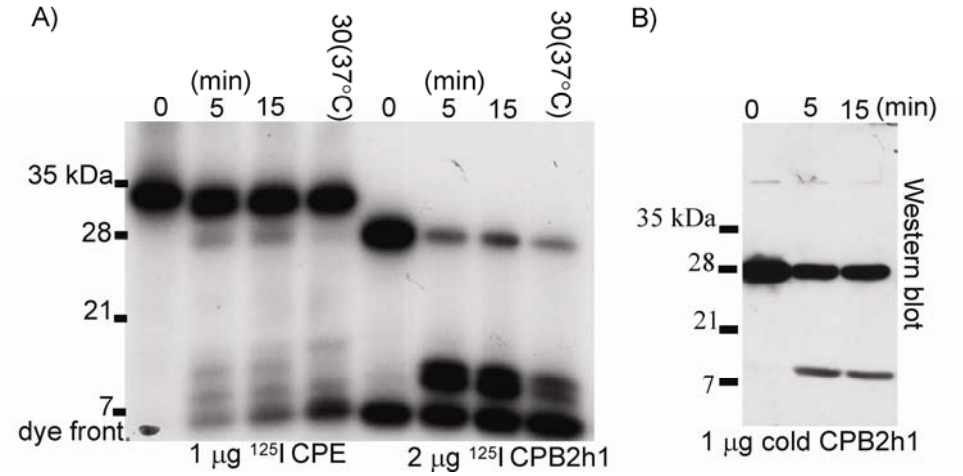

Figure 4.8 Sensitivity of CPB2h1 to trypsin. Purified ^{125}I CPB2h1 (A) and purified cold CPB2h1 (B) were treated with 4 ng of trypsin at room temperature (A and B) or at 37°C for 30 min (A). Samples were then boiled and run on 12% SDS-PAGE gels. Hot toxin was detected using autoradiography and cold toxin was detected via CPB2 Western blotting. Toxin samples are listed at the bottom of gels and treatment time is listed at the top of gels in A and B. ^{125}I CPE was included as a positive control. The location of molecular weight standards are shown to the left of each gel (parts A-B).

In contrast to ^{125}I CPE, ^{125}I CPB2h1 was very sensitive to proteolysis by trypsin. The majority of CPB2h1 was cleaved into two smaller fragments similar to what was observed for treatment of typical CPB2 by Gibert *et al*. (52) after only 5 min incubation with trypsin at room

temperature (Figure 4.8 A). These fragments were still present after 15 min of treatment. Trypsinization of CPB2h1 for 30 min at 37°C resulted in a reduction in the amount of full length CPB2h1 and in the amount of the two cleavage products with a subsequent increase in the radioactivity present at the dye front. Treatment of cold CPB2h1 with trypsin also resulted in the production of detectable cleavage products after only 5 min. Unlike hot CPB2h1, cold CPB2h1 appears to be slightly more resistant to trypsin (based on the increased amount of full length toxin after the 5 min and 15 min incubation periods). This may be due to slight structural alterations that may have occurred to CPB2h1 during iodination. Those structural alterations could have made potential trypsin cleavage sights more accessible. Furthermore, only one cleavage product is apparent in the Western blot of trypsin treated cold toxin. This may be due to 1) the failure of CPB2 antibodies to detect the other trypsin-fragment due to the absence of recognizable epitopes, 2) the creation of different cold CPB2h1 fragments that are too close in size to be separated under these SDS-PAGE conditions, or 3) the production of only one large fragment upon trypisn treatment (with the other half of CPB2h1 being degraded into fragments that migrate with the dye front). Interestingly, CPB2 Western blotting could not detect any CPB2h1 fragments at the dye front.

Comparing primary sequences of CPB2 versus other *C. perfringens* toxins using the EXPASY peptide cutter (http://ca.expasy.org/tools/peptidecutter/) to predict trypsin cleavage sites (using the 100% prediction requirement thresh hold) found that CPB2 variants contain on average one predicted trypsin site every 11 amino acids. CPE and ε toxin, which are both relatively trypsin resistant, had predicted trypsin cleavage sites every 16 or 19 amino acids, respectively. Similar to CPB2, β toxin (which also is trypsin sensitive) contained a predicted trypsin cleavage site every 13 amino acids. While sites predicted to be present in proteins based on primary sequence may be hidden from trypsin when a protein is folded into its tertiary structure, a trend between the experimental trypsin sensitivity of these mature toxins and the number of predicted trypsin sites within their primary sequences does exist. Trypsin sites located within the secretion signals (not present in the mature toxin) were ignored for this analysis.

4.2.4 Measuring the effects of CPB2 treatment on cell membrane permeability (^{86}Rubidium and osmoprotection assays)

4.2.4.1 ^{86}Rubidium assay. The cytopathic effects seen after CPB2 treatment of Caco-2 cells, including rapid cell death and membrane bleb formation suggest that CPB2 is a membrane active toxin. Given the propensity of *C. perfringens* to produce membrane active toxins (including PFO, CPE, α, ε, and β toxins), a similar mechanism of action for CPB2 would not be surprising. Since membrane active toxins typically cause alterations in membrane permeability, the affects of CPB2h1 on Caco-2 cells preloaded with ^{86}Rb was tested. ^{86}Rb is a K^+ analog that can be loaded into the cytoplasm of healthy cells by incubating cells with media containing ^{86}Rb. Cells are then treated with toxin and the release of ^{86}Rb above background levels (due to spontaneous release of ^{86}Rb) into the media indicates that membrane permeability alterations have occurred. Results are reported as % ^{86}Rb release compared to control cells (which are used to determine maximal and spontaneous ^{86}Rb release).

Initial pilot experiments were performed with CPE to demonstrate that the assay was functional. These experiments agreed with previous reports demonstrating that CPE causes ^{86}Rb release from Caco-2 cells in a dose and time dependent manner (data not shown) (90). Before time and reagent intensive ^{86}Rb experiments were carried out with CPB2h1, basic studies were then performed using a fixed dose (1 μg/ml) and a fixed time point (15 min) to determine if toxin treatment resulted in release of ^{86}Rb from Caco-2 cells. The results from this study demonstrated that ^{86}Rb is released above background (spontaneous) levels from CPB2h1 treated Caco-2 cells presumably through alterations in membrane permeability (data not shown).

Experiments were then executed to more carefully study the kinetics of ^{86}Rb release in CPB2h1-treated Caco-2 cells and to determine the effects of different doses of CPB2h1 on ^{86}Rb release. Results from these kinetic and dose response studies are shown in Figure 4.9 (A and B). Similar to previous studies reporting the affects of CPE on ^{86}Rb release from CPE-sensitive cell lines, ^{86}Rb release from Caco-2 cells treated with CPB2h1 increased with longer time periods of treatment (A) and with increasing doses of toxin (B). Typically for this assay, the amount of toxin required to induce the release of 50% ^{86}Rb from sensitive cells is used to gauge the activity of a toxin. For CPE, a dose of 600ng/ml to1 μg/ml can be expected to cause release of 50% ^{86}Rb after 10 min of treatment (90, 132). For CPB2h1, a 1 μg/ml dose required ~18 min

to cause the release of 50% ^{86}Rb (Figure 4.9 A). After 30 min of treatment all doses (500 ng to 10 μg/ml) resulted in release of ≥ 50% ^{86}Rb.

Utilizing the ^{86}Rb release assay, the effect of divalent cations on CPB2h1 activity was explored. Caco-2 cells were treated with either 0.5, 1, or 10 μg/ml of CPB2h1 for 10 min in HBSS supplemented with physiological levels of Ca^{2+} alone, Mg^{2+} alone, or Ca^{2+} and Mg^{2+}. Compared to control cells (treated with buffer containing Ca^{2+} and Mg^{2+}) which required ~3 μg/ml of CPB2h1 to cause release of 50% ^{86}Rb, a 0.75 μg/ml or 2 μg/ml dose of CPB2h1 was required to cause release of 50% ^{86}Rb in the absence of Ca^{2+}, or in the absence of Mg^{2+}, respectively (Figure 4.9 C). These 4-fold or 1.5-fold differences were in contrast to CPE whose activity (based on binding studies and ^{86}Rb release studies) is not affected by the presence or absence of divalent cations. The only *C. perfringens* toxin reported to be sensitive to divalent cations is α toxin, which requires Ca^{2+} for binding to the membrane of sensitive cells (103). At a high toxin dose (10 μg/ml) the divalent cation content of treatment buffers did not seem to affect the amount of ^{86}Rb released from the Caco-2 cells.

The 4-fold decrease in toxin required to reach a 50% ^{86}Rb release for cells treated with CPB2h1 in the absence of Ca^{2+} was further investigated using Caco-2 monolayers to see if morphological differences could be seen in cells treated with CPB2h1 in the presence or absence of Ca^{2+}. Caco-2 cells were grown until confluent in 6-well cell culture dishes, loaded with ^{86}Rb, and then treated with 10 μg/ml of CPB2h1 at 37°C. Cells were monitored for morphological changes under phase contrast microscopy and photographed after 30 and 60 min (Figure 4.10 B-G). Consistent with Figure 4.9 C results, a 10 μg/ml dose of CPB2h1 caused release of ~100% ^{86}Rb after 30 min of treatment regardless of the presence or absence of Ca^{2+} (Figure 4.10 A). However, the morphology of cells treated with CPB2h1 in the absence of Ca^{2+} showed greater morphological damage after 30 min (more cells with condensed/refractive nuclei and membrane bleb formation) and 60 min (disruption of the Caco-2 monolayer) of treatment compared to cells treated with buffer containing Ca^{2+}. These differences paralleled the observed differences between ^{86}Rb release with Ca^{2+} buffer and Ca^{2+} free buffer measured after 10 min using low doses of toxin (0.5 and 1 μg/ml). Caco-2 cells incubated with Ca^{2+} free buffer for 30 and 60 min (Figure 4.10 D and G) were morphological the same as Caco-2 cells incubated with buffer containing Ca^{2+} (Figure 4.10 H).

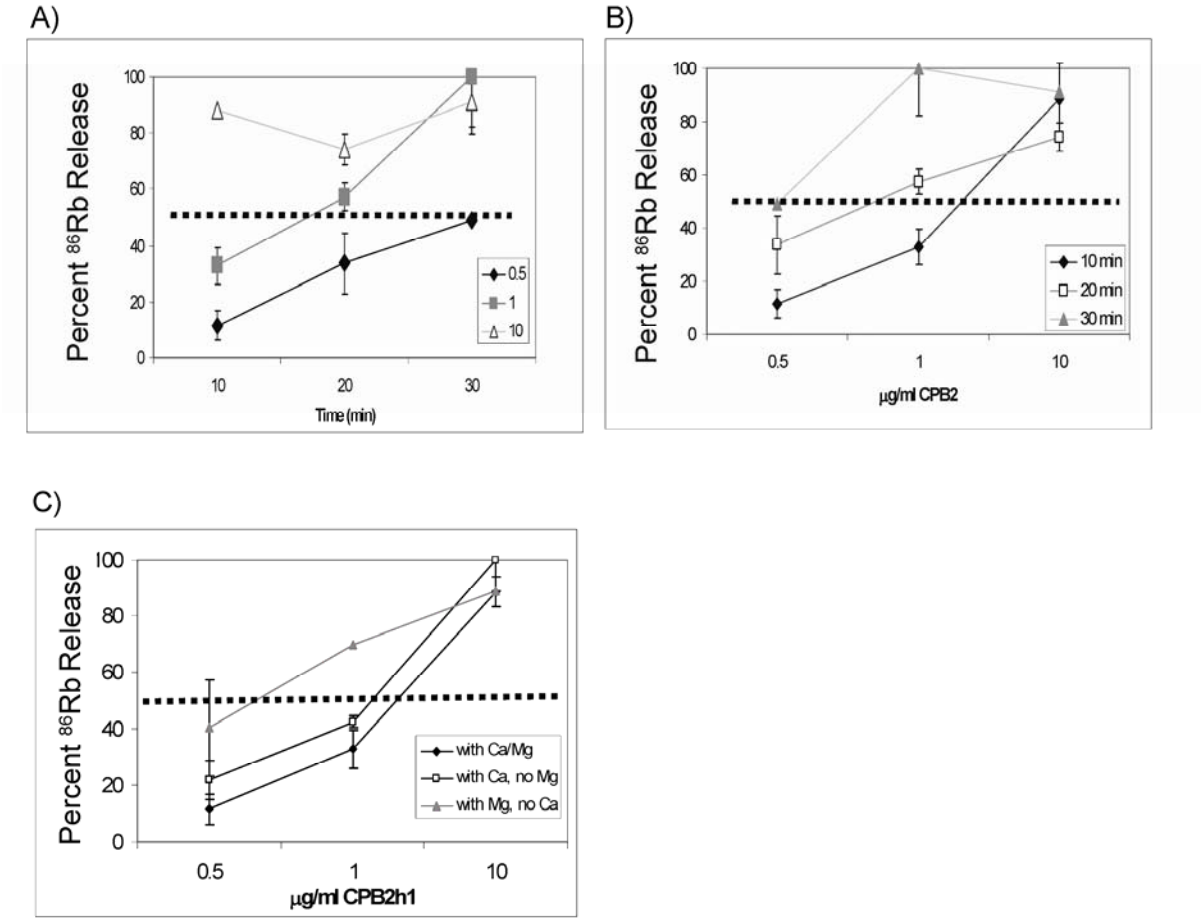

Figure 4.9 CPB2h1-induced membrane permeability alterations in Caco-2 cells measured by ^{86}Rb release. Caco-2 monolayers preloaded with ^{86}Rb were treated with 0.5, 1, or 10 μg/ml of CPB2h1 for 10, 20, or 30 min at 37°C to generate time (A) and dose (B) response curves. The amount of ^{86}Rb released into the supernatants was measured using a gamma counter and reported as the percent ^{86}Rb release. In C, the effect of extracellular ions on CPB2h1 activity was measured. Either 0.5, 1, or 10 μg/ml of CPB2h1 was prepared in HBSS media that contained 1.7 mM Ca^{2+} and 1.8 mM Mg^{2+}, 1.7 mM Ca^{2+} with no Mg^{2+}, or 1.8mM Mg^{2+} with no Ca^{2+} and used to treat Caco-2 monolayers as in part A for 10 min. The dotted line in A and B indicates the 50% Rb^{86} release point.

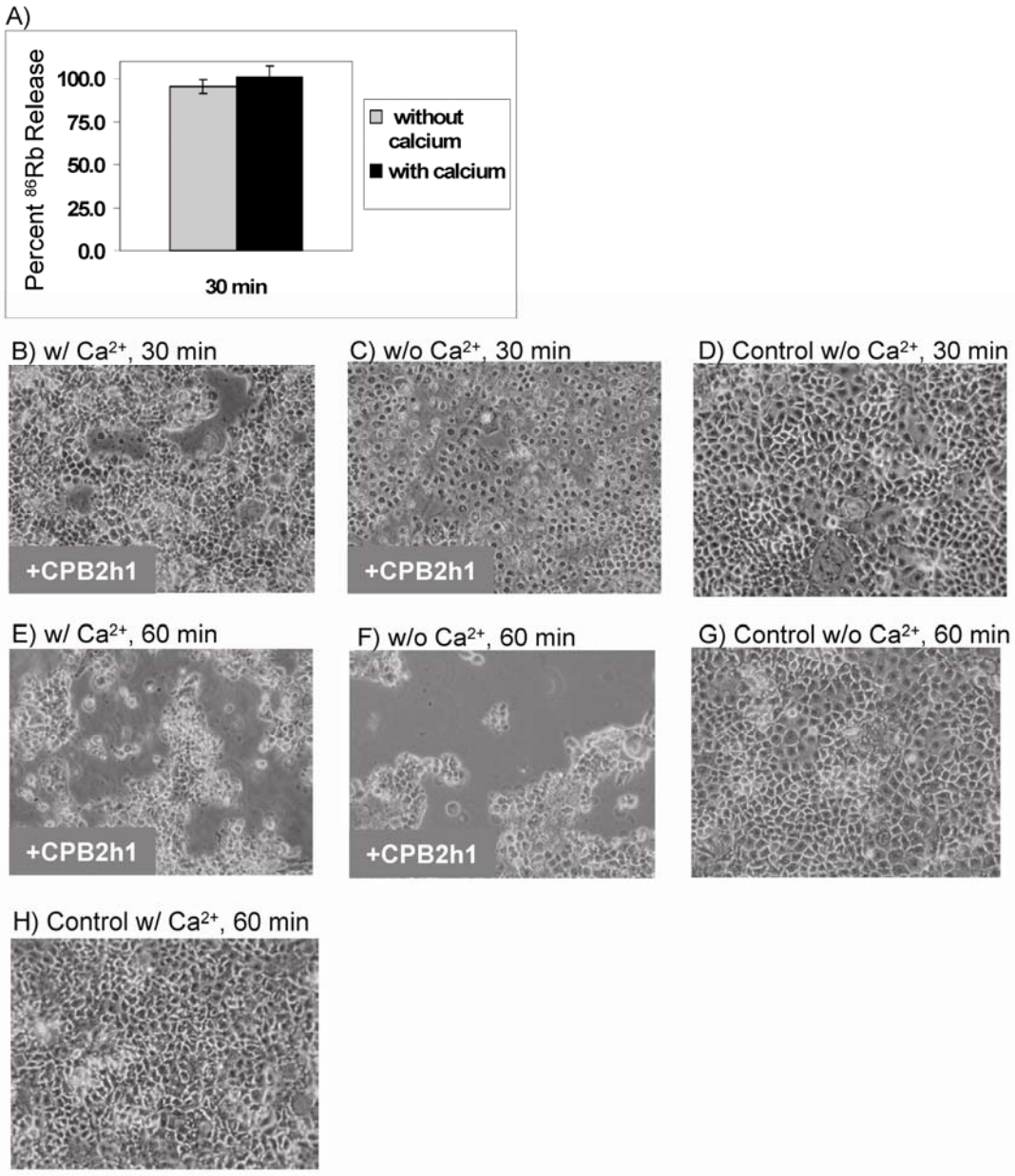

Figure 4.10 The effect of Ca^{2+} on CPB2h1 activity. Caco-2 cell monolayers loaded with ^{86}Rb were treated with 10 μg/ml of CPB2h1 in HBSS with or without 1.7 mM Ca^{2+}. In A, the percent ^{86}Rb release from cells treated for 30 min with CPB2h1 in the presence or absence of Ca^{2+} is shown. The morphology of cells treated with CPB2h1 in the presence (B and E) or the absence (C and F) of calcium are shown after 30 or 60 min. Control cells treated with HBSS without Ca^{2+} are shown in D and G after 30 or 60 min and with Ca^{2+} after 60 min in H. Micrographs are shown at 200x.

4.2.4.2 Osmoprotection assay. Since the membrane permeability of Caco-2 cells is affected by CPB2h1 treatment, a second set of experiments was performed to further tease apart its mechanism of action. One mechanism by which a toxin can disrupt membrane permeability is through pore-formation. Consistent with observed cytopathic effects, pore-formation would result in rapid cell death (compared to toxins that act intracellularly) as water moves into cells (following the osmotic potential), resulting in swelling and bursting of the cell membrane (visualized as swollen cells and the formation of membrane blebs). Since pore-formation primarily disrupts osmotic equilibrium, the presence of osmotic stabilizers in cell culture media (to maintain an osmotic potential of ~0, i.e. no net movement of water) can transiently protect cells from death by preventing the influx of water. However, osmotic stabilizers will only be effective if they are larger than the pore, which prevents the stabilizer from entering the cell due to passive diffusion down the concentration gradient, so the osmotic equilibrium remains close to 0. If the use of an osmotic stabilizer protects cells from challenge with toxin, the size of osmotic stabilizers can be varied to approximately characterize the “size” of the pore.

Initial osmoprotection experiments were performed with sucrose (~342 Da), which can act as an osmotic stabilizer for cells treated with CPE (90, 92). As a positive control, MDCK cells (which are sensitive to CPE (136)) were treated with 10 μg/ml of CPE in the presence or absence of 0.3M sucrose (Figure 4.11 A-C). Cells treated with CPE in the presence of 0.3M sucrose appeared morphologically similar to untreated MDCK cells whereas cells treated with 10 μg/ml CPE without sucrose showed extensive cytopathic effects after 30 min of treatment. These results are consistent with previous findings and indicate that the sucrose protection assay was functioning properly. Cytotoxicity was measured visually using phase contrast microscopy.

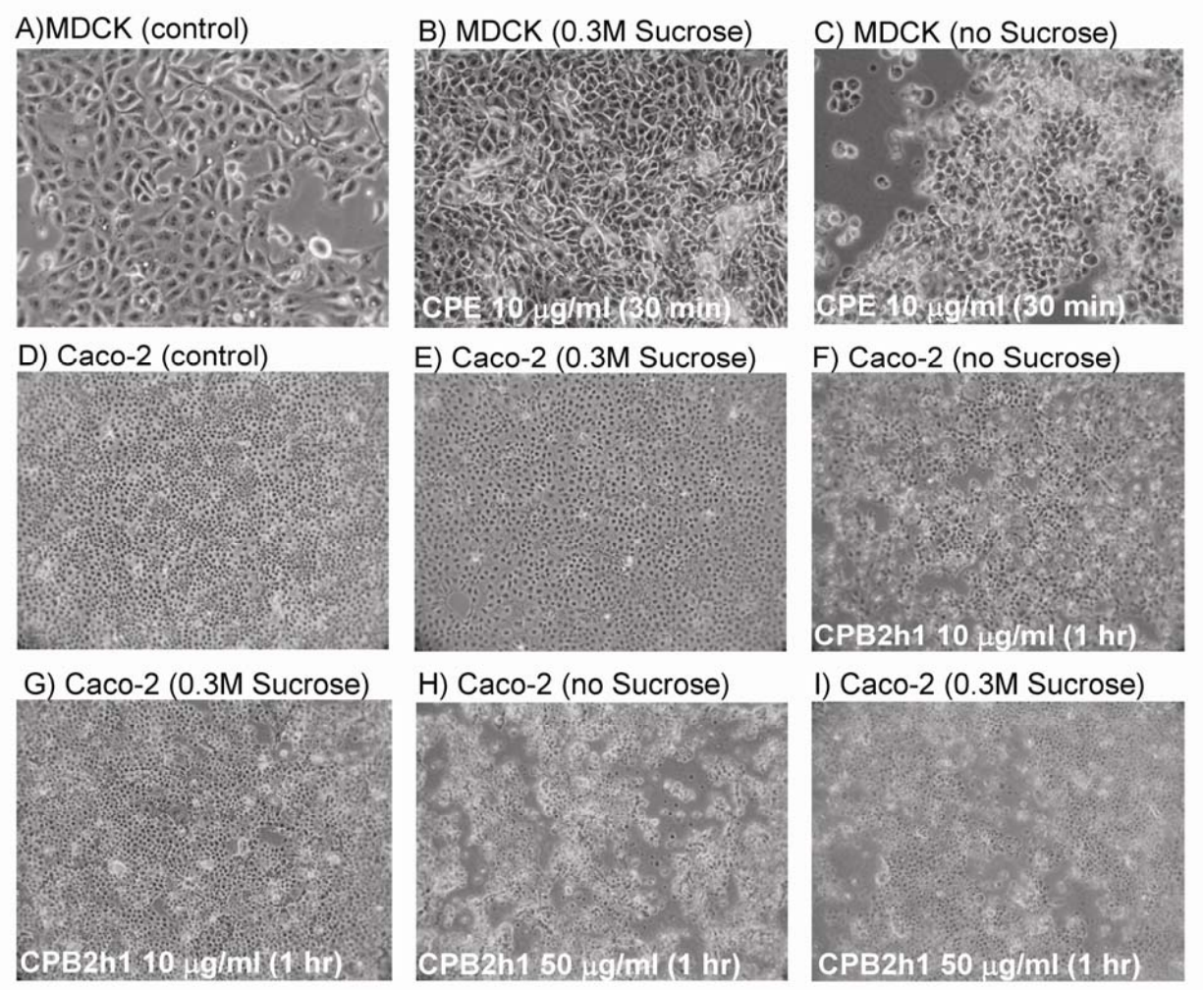

Figure 4.11 The osmotic stabilizer sucrose does not protect Caco-2 cells from CPB2h1 cytotoxicity. As a positive control, MDCK cells were challenged with 10 μg CPE in the presence or absence of 0.3M sucrose at 37°C for 30 min (A-C). Cell types and culture conditions are listed at the top of each micrograph and [toxin] are listed at the bottom of each micrograph. Caco-2 cells were challenged with either 10 μg/ml of CPB2h1 (F and G) or 50 μg/ml of CPB2h1 (H-I) and incubated at 37°C for 1 hr with or without 0.3M sucrose. Cell damage was assessed using phase contrast microscopy and pictures were taken at 200x (except for A – 400x).

Treatment of Caco-2 cells with a low dose of CPB2h1 (10 μg/ml) in the presence of 0.3M sucrose only offered minor protection for challenged cells compared to cells treated in the absence of sucrose (4.12 F and H). Cells treated with CPB2 in the presence of sucrose still showed signs of swelling and membrane bleb formation and would detach from the monolayer. Caco-2 cells treated with a high dose of CPB2h1 (50 μg/ml) with sucrose were not protected from CPB2h1 cytotoxicity and showed extensive damage that was similar to non-sucrose toxin treated cells. Cells were observed at 30 min and 1 hr (micrographs were taken at 1hr) and all toxin-treated wells looked similar regardless of the presence or absence of sucrose at both time points.

These results indicated that sucrose could not osmotically protect cells from CPB2h1 treatment suggesting either that CPB2h1 is not a pore-forming toxin or that sucrose is too small to offer protection, i.e. sucrose may be small enough to diffuse through a CPB2h1 pore-complex. To determine whether a larger molecule could protect cells, similar experiments were performed with different-sized polyethylene glycol (PEG) molecules. Not surprisingly, owing to the use of PEG as an agent to induce cell fusion, PEG greatly affected the morphology of cells and prohibited easy discrimination of healthy and toxin-affected cells showing cytopathic effects. Furthermore, ^{86}Rb release experiments performed with HBSS media containing PEG indicated that PEG was negatively affecting CPB2h1 activity. ^{86}Rb release was reduced 10-fold in the presence of PEG (data not shown) despite the fact that PEG should not affect the diffusion of small molecules from the cytoplasm to the media (90).

Dextran was then explored as an osmotic stabilizer due to its inert nature and the commercial availability of different sized dextran polymers. Experiments were first performed with the smallest dextran available, D1500 (average size ~1500 Da). Caco-2 cells were treated with 20 μg/ml of CPB2h1 dissolved in HBSS with or without 0.3M dextran and incubated at 37°C (Figure 4.12). Cells were observed for damage at 30 min intervals. After 1 hr, cells treated with CPB2h1 without dextran showed substantial damage including detachment from the monolayer and membrane bleb formation. In contrast, cells treated with CPB2h1 and dextran did not show signs of damage. Monolayers were mostly intact (open areas in the micrograph were non-confluent prior to treatment) and membrane blebs were not present along open areas in the monolayer. Dextran treatment of cells did cause minor morphological alterations (compare panels A and B). However, removal of HBBS/dextran from cells and replacement with HBSS resulted in morphologically normal cells after 30 min (E). To ensure that dextran had not interfered with CPB2h1 binding, cells treated with toxin plus dextran were washed and HBSS was added to wells. Cells were then incubated at 37°C and monitored for damage (F and I). Cell

damage in these washed wells was apparent 30 min after washing away the dextran and was very obvious after an overnight incubation. Cells treated with HBSS or HBSS/dextran and incubated overnight did not show signs of damage (Figure 4.12 G and H).

Follow up experiments were performed to determine if dextran could protect cells containing toxin bound to their membranes. For these studies, CPB2h2 was substituted for CPB2h1. Caco-2 cells were treated with either HBSS or 20 μg/ml CPB2h2 and allowed to incubate at 4°C for 30 min to allow toxin to bind. No morphological alterations were observed after this cold treatment (Figure 4.13 A and B). After 30 min, the media was removed and cells were washed with HBSS to remove unbound toxin. HBSS alone (C), HBSS supplemented with 0.3M dextran (D), HBSS containing 20 μg/ml of CPB2h2 (added to cells pretreated with HBSS alone at 4°C [E]), or HBSS containing 20 μg/ml of CPB2h2 and 0.3M dextran (added to cells pretreated with HBSS alone at 4°C [F]) was then added to cells and incubated at 37°C. Cells were observed after 1 hr of incubation at 37°C for morphological damage. Cells pretreated with toxin (at 4°C) followed by dextran and cells treated simultaneously with dextran and toxin appeared healthy after the 1 hr incubation. However, cells pretreated with toxin (at 4°C) and then incubated at 37°C and cells treated with toxin immediately before the 37°C incubation exhibited typical signs of CPB2 intoxication. Cells pretreated with toxin at 4°C showed slightly less damage than cells treated with toxin and incubated at 37°C without washing. This difference could be due to reduced toxin binding at 4°C. Since cells did not show damage after 30 min of toxin treatment at 4°C, experiments were performed to determine if an overnight treatment resulted in damage to cells. Caco-2 cells were incubated with either Caco-2 media or Caco-2 media containing 20 μg/ml CPB2h2 overnight at 4°C. Morphological changes consistent with CPB2 activity were observed in cells treated with CPB2h2 overnight at 4°C, but not in cells treated with media alone (Figure 4.13 G and H).

These results suggest that molecules of ~1500 Da can protect CPB2h1 or CPB2h2 treated cells from death by stabilizing osmotic equilibrium, i.e. cells treated with toxin in the presence of dextran or that had dextran applied after toxin was allowed to bind did not exhibit signs of cell swelling or membrane bleb formation. Removal of dextran from toxin-treated cells, followed by adding HBSS back to those cells, resulted in morphological changes consistent with CPB2-induced cell death. This suggests that dextran was not interfering directly with the action of CPB2. The ability of dextran to protect cells that had pre-bound toxin (in the absence of dextran, Figure 4.13) further supports the claim that dextran was not interfering with CPB2 binding and activity.

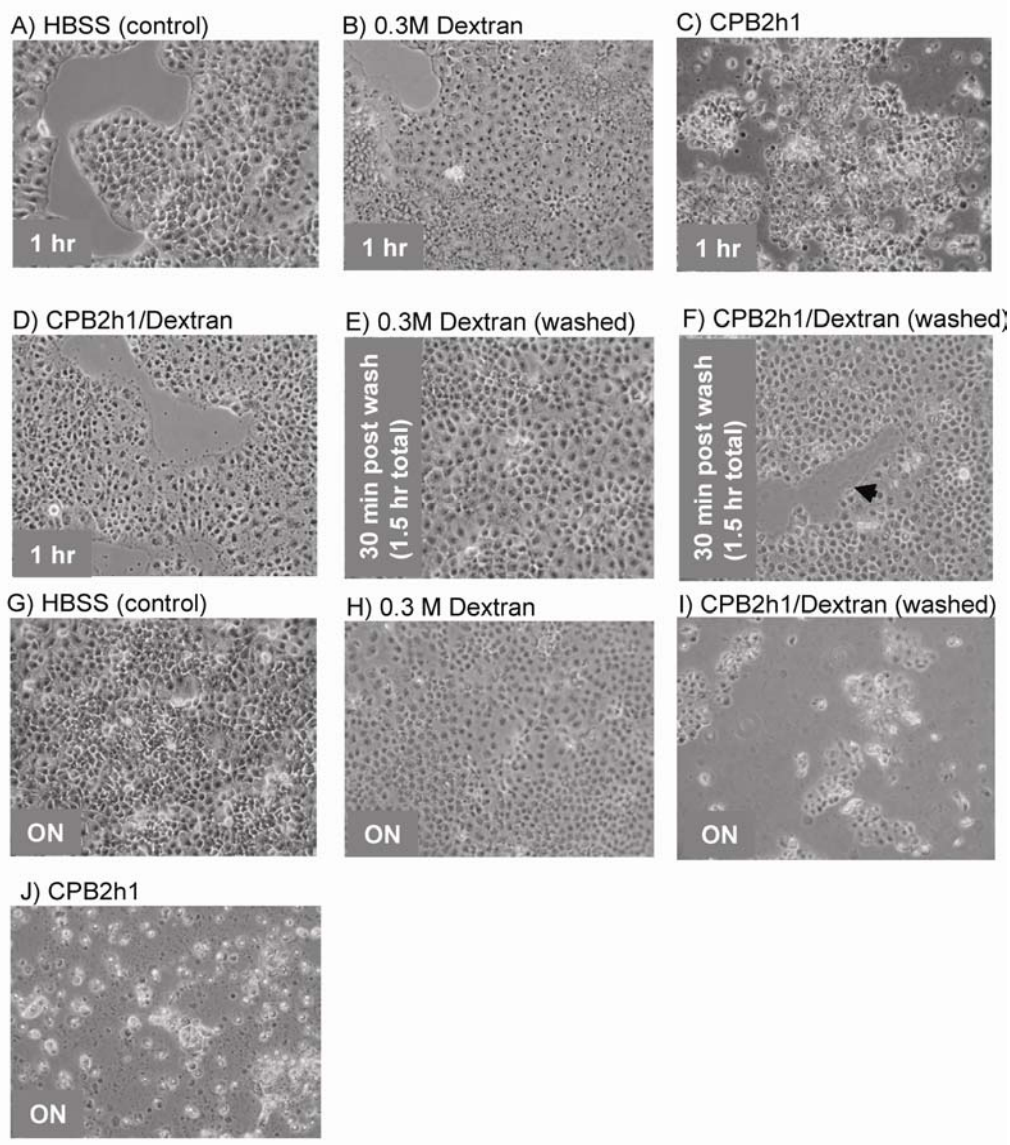

Figure 4.12 Protection of Caco-2 cells from CPB2h1 using dextran. Caco-2 cells were treated with 20 μg/ml of CPB2h1 in the presence (D) or absence of 0.3M dextran (C) and incubated at 37°C. After 1 hr of incubation, cells were viewed under phase contrast microscopy for damage. The dextran was then removed and replaced with HBSS (F) and incubated at 37°C and damage was monitored after an additional 30 min (F) or overnight (ON, I). Control cells were treated with HBSS (A) or 0.3M dextran (B). Dextran-treated cells were washed and HBSS was added back to cells in (E) as a control for (F). Micrographs G-J depict cells treated overnight at 37°C. Treatment buffers are listed at the top of each picture and incubation times are listed at the bottom, left corners. Micrographs are shown at 200x.

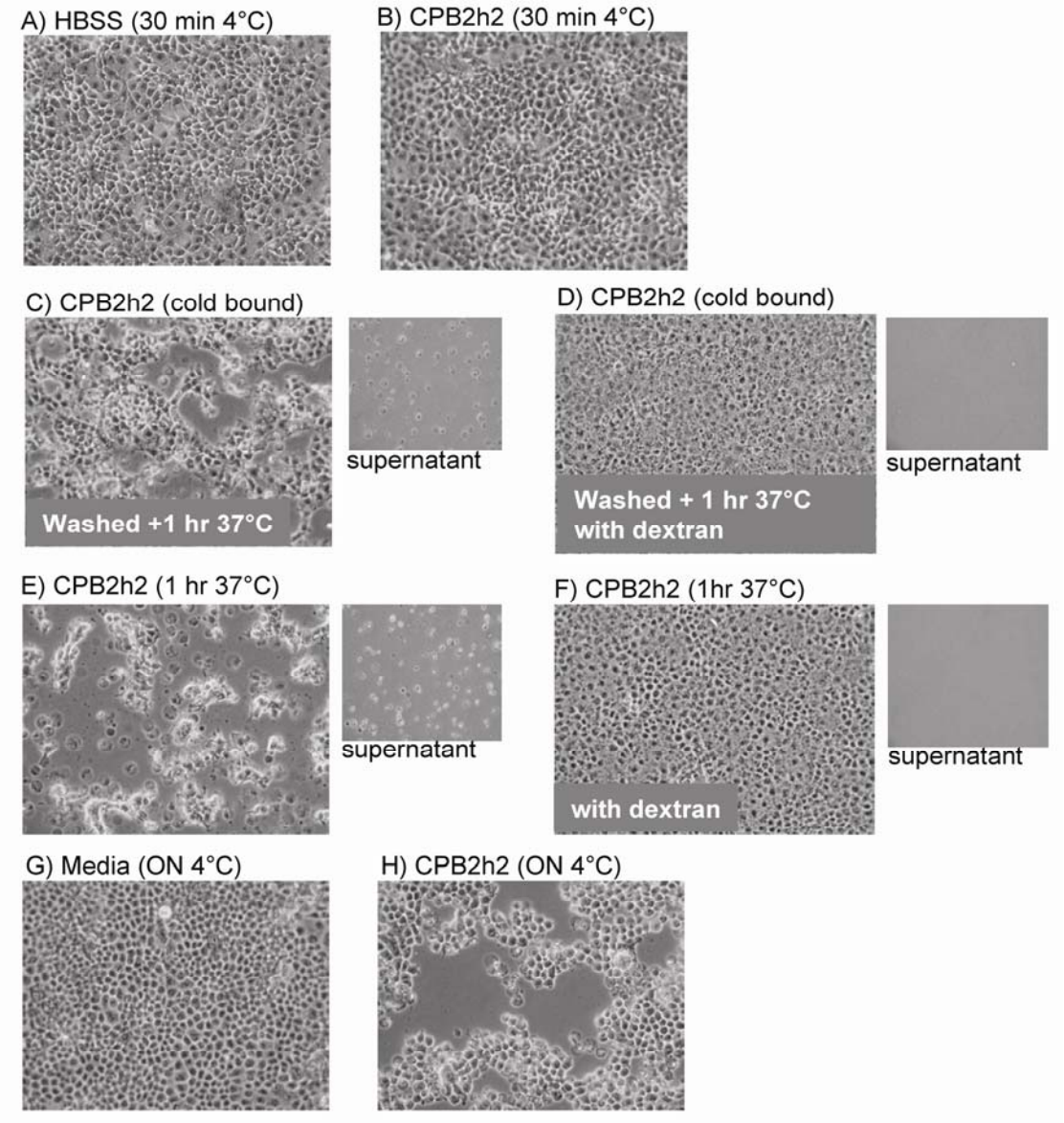

Figure 4.13 Dextran protection of Caco-2 cells treated with CPB2h2 at 4°C. Caco-2 cells were treated with or without 20 μg/ml CPB2h2 and incubated at 4°C for 30 min (A and B, pictures taken after 30 min). Media was then removed and replaced with either HBSS or HBSS with 0.3M dextran and cells were incubated at 37°C for 1 hr (C and D). Control cells were treated with CPBh2 with or without 0.3M dextran for 1 hr at 37°C without the 4°C binding step (E and F). After 1 hr, supernatants were removed and photographed along with remaining monolayers (C-E). Caco-2 cells were also incubated overnight (ON) at 4°C with or without toxin. All pictures were taken at 200x. Treatment conditions are listed with each panel.

4.2.5 Attempts to develop a recombinant *E. coli* system to produce CPB2h1

To facilitate future work with CPB2, studies were performed to identify an *E. coli*-based recombinant expression system for CPB2. *E. coli* is widely used to produce foreign proteins of interest and numerous recombination systems have been developed for use in *E. coli* that allow proteins to be over-produced compared to wild type protein levels. Those systems also have been designed to allow the addition of protein tags to aid in purification and detection of recombinant proteins. Furthermore, the development of a *cpb2*-expressing system in *E. coli* would facilitate the use of molecular techniques, such as site-directed mutagenesis, that are useful for performing structure-function studies. While possible to perform structure-function studies using *C. perfringens*, *E. coli* is much easier to work with and cloning strains are available that contain no background cytotoxicity (unlike *C. perfringens* which produces multiple toxins).

The basic approach undertaken to develop a recombinant *cpb2* expression system was to clone the *cpb2* gene with or without its secretion signal behind an *E. coli* vector-encoded promoter. This promoter was then used to drive production of CPB2 with various protein tags located at the N- or C-terminus of CPB2. The production of the recombinant CPB2 was verified using Coomassie Brilliant blue staining or immunological detection methods and recombinant strains were screened for cytotoxicity on Caco-2 cells (similar to studies using CPB2 from *C. perfringens*).

The *cpb2* gene with or without its signal peptide was successfully cloned in pBAD, pBAD/THIO, and pMAL-2c vectors. A recombinant vector carrying the full *cpb2* ORF also was constructed using pGEX-2T. The basic properties of these vectors and the resulting fusion product can be found in Table 3.2. Unfortunately, the limited trials performed with *E. coli* lysates containing recombinant CPB2h1 were unable to detect cytotoxic activity on Caco-2 cell monolayers. These results may indicate that the recombinant CPB2 1) was not active due to the presence of its signal peptide, 2) was not active due to the presence of the vector-encoded tag, 3) was not folded properly in *E. coli*, and hence not in an active conformation, 4) was not present at a high enough concentration to kill cells, and/or 5) requires *C. perfringens* cofactors to be active.

4.2.6 Summary of section 4.2 findings

Results indicating that CPB2h1 and CPB2h2 are cytotoxic for Caco-2 cells suggest that CPB2 has the potential to damage the human intestinal epithelium. The rapid death of Caco-2 cells after CPB2 treatment and the formation of membrane blebs along the surface of effected cells suggest that CPB2 is a membrane-active toxin. Experiments demonstrating a CPB2h1 dose and time-dependent release of ^{86}Rb from toxin-treated Caco-2 cells also is consistent with CPB2 functioning as a membrane active toxin that disrupts membrane permeability. This mechanism of action was further supported by osmoprotection experiments, which demonstrated that toxin-treated cells could be protected from cell death by adding an osmostabilizer (dextran ~1500 Da) to treatment media. While these experiments do not prove that CPB2 acts as a pore-forming toxin, they are consistent with the results expected for cells treated with a pore-forming toxin.

Collectively, previous literature and the results reported in sections 4.2 indicate that CPB2 is a rather labile toxin. CPB2 is heat labile, sensitive to oxidation and trypsin, and has been reported by at least two groups to lose activity during the purification process due to unknown reasons (52, 148). These findings indicate that CPB2 may require ideal conditions (for instance low trypsin levels) to cause disease. It also is possible that *cpb2*-positive isolates adhere to the epithelium of intestines and locally secrete CPB2 which could overcome the lability issues of CPB2 by allowing it to interact immediately with target cells before it is destroyed by the harsh conditions present within the lumen of the intestine.

4.3 STUDYING THE EFFECTS OF THE *Clostridium perfringens* BETA2 TOXIN *IN VIVO*

This section will discuss the results of *in vivo* experiments performed with the aim of better understanding the role that CPB2 plays during pathogenesis. Previous work with CPB2 (mistakenly believed to be CPB (71)) reported that purified (typical) CPB2 caused fluid accumulation at low doses and necrosis without fluid accumulation at high doses in a guinea pig ileal loop model. More recent work has demonstrated that typical CPB2 is lethal in a mouse i.v. injection model (LD_{50} 3 μg/ml) (52) and that typical and atypical CPB2 variants are lethal in a

mouse intraperitoneal injection model (using ~200 μg of either typical or atypical CPB2 (148)). This section will report findings pertaining to attempts to study purified CPB2h1 in a rabbit disease model and also will summarize results of mouse i.v. lethality experiments performed with non-type A culture supernatants. The driving force behind this aim lays in 1) the debate in the *C. perfringens* field on the actual role and importance of CPB2 in the gastrointestinal diseases of domestic animals and 2) our hypothesis that CPB2 plays a role in human GI disease.

4.3.1 Studies of the effects of CPB2h1 in rabbit intradermal and intestinal loop models

Rabbits were chosen for preliminary studies with CPB2 because 1) the effect of CPE in rabbit ileal loops is well understood (ideal for studying CPB2-producing AAD/SD isolates) and 2) the rabbits are relatively easy to purchase, house, and use in surgeries.

4.3.1.1 Intradermal. Early CPB2 Western blot procedures were performed with antibodies from a source in France (Michel Popoff, Pasteur Institute). Due to the limitations of this source, a goal of this study was to generate our own source of CPB2 antibodies. CPB2h1 purified from the *C. perfringens* transformant (pDFB2h1v751)ATCC3624 was used to immunize rabbits for the production of rabbit polyclonal CPB2 (immunization performed by Pocono Rabbit Farms & Laboratories Inc). However, since it was not known if the dose of CPB2h1 to be used for inoculations would harm the rabbits (i.e. we did not know if the dose would be lethal), rabbits were initially challenged with different doses of CBP2h1 injected via an intradermal route. Prior to the intradermal skin challenge, the activity of the CPB2h1 preparation was verified via cell culture (always performed before animal trials) and mouse i.v. lethality (LD_{50} of 30 μg).

Rabbits were injected with 1, 10, or 100 μg of CPB2h1 at multiple sites at 1, 7, and 14 days. The rabbits also were injected at separate sites with saline as a negative control. One day after each injection (days 2, 8, and 15) biopsies were performed at injection sites and tissues were processed for histochemistry. Hemotoxylin and eosin-stained biopsies for rabbit control sections (panels A and D, saline injected) and experimental sections (panels B, C, and E, 10 μg CPB2h1) harvested after 1 day are shown in Figure 4.14. Compared to the control section in (A), the CPB2h1-treated sections in B and C have immune cell infiltrates and show signs of

edema. Tissue surrounding the vasculature of treated animals also shows signs of edema and infiltration of leukocytes (E) compared to a control section (D). Serum samples also were taken from each rabbit before the initial injections (day 0) and after the final injection (day 15) to determine if an immune response was mounted against CPB2h1. These serum samples were used in place of standard anti-CPB2 antibodies (from Michel Popoff) in the CPB2 Western blot. Only serum samples taken after challenge with CPB2h1 were able to detect purified CPB2h1 run on 12%SDS-PAGE (Figure 4.14 F). However, blots developed using our new serum required a much longer exposure time (30 min) than our original serum (10 sec) to detect CPB2 by chemiluminescence. Possible reasons for this difference in sensitivity include 1) the rabbits treated with CPB2h1 in our study were not co-injected with Freund's complete/incomplete adjuvant and 2) normal immunization procedures allow at least 1 month for rabbit antibody production to increase titres.

Sections taken at all time points from control and experimental injection sites also were scored for a number of properties including necrosis and edema. Individual results are reported in Figure 4.15 A-G and the total score for each category is shown in panel H. Only rabbit biopsies from regions treated with CPB2h1 exhibited signs of damage and the development of a localized immune response, i.e. saline-treated regions were always unresponsive. Furthermore, scores were higher in regions injected with higher doses of CPB2h1 indicating that a positive dose response occurred.

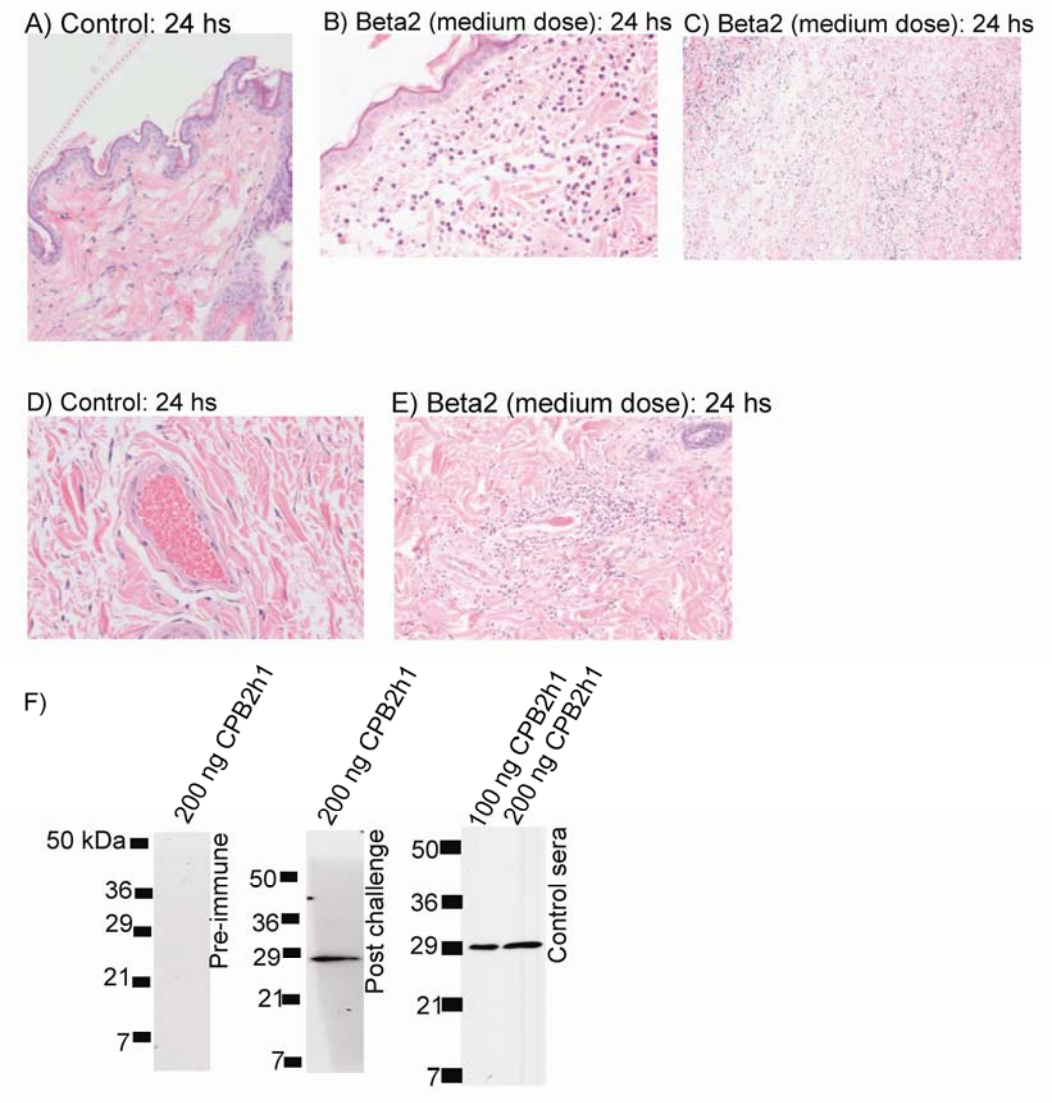

Figure 4.14 The effects of intradermal (i.d.) injection of CPB2h1 into rabbits. Rabbits were injected i.d. with either 100, 10 or 1 μg doses of purified CPB2h1 at 1, 7, and 14 days to determine the effects of CPB2h1 activity within the rabbit dermal tissue. Tissue biopses were taken from areas injected with CPB2h1 or saline. Control (A and D) and experimental (B, C, and E) sections were then processed for H and E staining. Rabbit serum samples were also tested for anti-CPB2 antibodies prior to challenge and after the 14 day challenge (serum was collected at day 15). The serum was used to detect purified CPB2h1 run on 12% SDS-PAGE using the standard CPB2 Western blot procedure. Results are shown in panel F for pre-immune sera, post-challenge sera, and for control sera (antibodies previously developed to detect CPB2). The migration of molecular weight markers (kDa) is shown to the left of each blot.

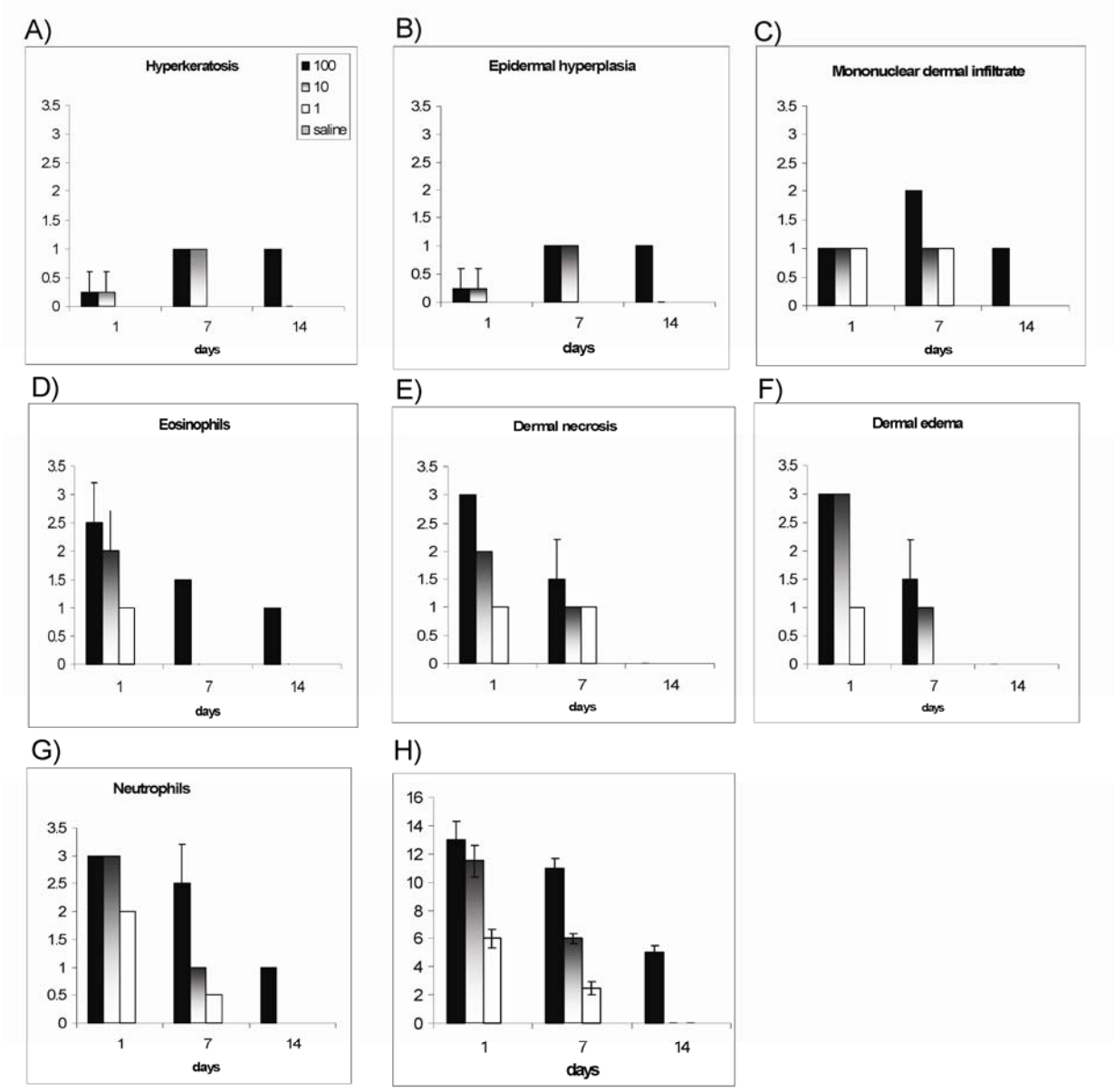

Figure 4.15 Histopathology scores of tissue sections from rabbits treated with intradermal inoculations of CPB2h1. Tissue sections from rabbits inoculated intradermally with 100, 10, or 1 μg of CPB2h1 or saline (negative control) at 1, 7 and 14 days were scored for visual pathology (such as necrosis) and infiltration of various immune cells (such as neutrophils). The characteristic measured is indicated at the top of A. Panel H represents the sum of all the categories (A-G) for the different challenge doses and time periods.

The results from the intradermal challenge studies indicated that we could follow the vaccination schedule recommended by Pocono Rabbit Farms & Laboratories Inc and that we could use active CPB2h1 toxin. This was important since toxin inactivation procedures such as formaldehyde treatment can destroy epitopes. Immunization of rabbits with purified CPB2h1 resulted in polyclonal antibodies that could detect CPB2(h1 and h2) from *C. perfringens* transformants and wild type *cpb2*-positive *C. perfringens* (Figure 4.16). These antibodies also were specific for CPB2 as they failed to react with proteins from the *cpb2*-negative *C. perfringens* transformant carrying an empty vector.

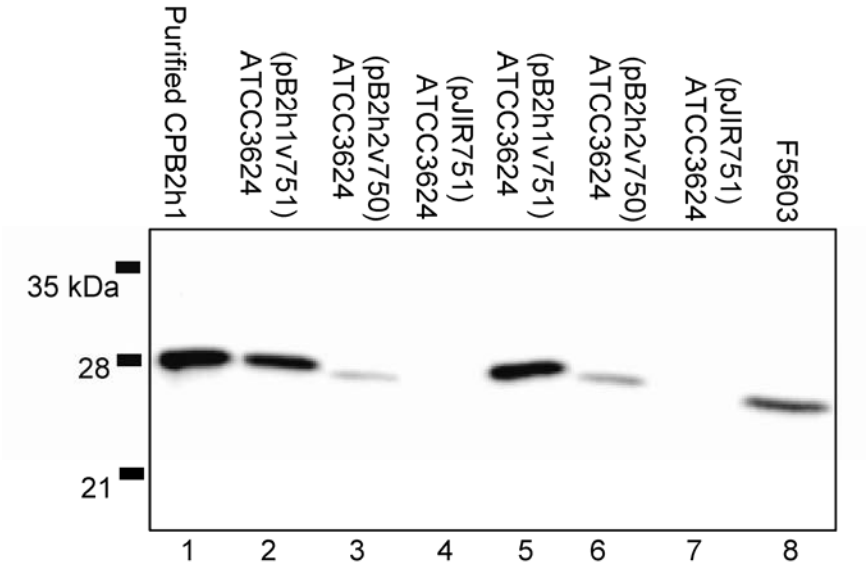

Figure 4.16 Detection of CPB2h1 and CPB2h2 via Western blot with new CPB2 antisera. Purified CPB2h1 or supernatants from *cpb2*-positive (lanes 1-3, 5-6, and 8) or *cpb2*-negative isolates (lanes 4 and 7) were run on 12% SDS-PAGE and protein was detected using the new rabbit polyclonal anti-CPB2 antibody. The migration position of molecular weight markers is shown to the left of the blot. Supernatants shown in lanes 2-4 and 5-7 were obtained from independently grown cultures.

4.3.1.2 Intestinal loops. To enable the future study of isogenic *cpb2* toxin knockout mutants, purified CPB2h1 or variations of *cpb2*-positive *C. perfringens* isolates (including supernatants, washed bacteria, and whole cultures [bacteria plus supernatants]) were tested in a rabbit ileal and colonic loop model. Initial experiments focused on studying purified CPB2h1. Loops were injected with 300 μg/ml in the presence of soybean trypsin inhibitor and loops were incubated for 6 hrs. After this time period, the rabbits were anesthetized and the loops were scored for fluid accumulation and damage (gross pathology and histopathology). While damage was apparent in some rabbits (Figure 4.17), these differences were inconsistent (performed on 3 separate occasions with two rabbits and multiple loops each time). When present, damage in ileal loops consisted of desquamation of the intestinal villi, most prominently occurring at the tips of the villi. In the colon, excess mucous was detected in the lumen along with villus blunting and desquamation. Negative control loops did not show any damage. Similar to previous reports for guinea pig ileal loops treated with high doses of typical CPB2 (which showed necrosis without fluid accumulation (52)), fluid accumulation was not apparent in CPB2h1 treated loops.

Due to the instability of CPB2h1 (which could account for the inconsistent rabbit pathology results) and more recent literature results indicating that CPB2 may be localized around *C. perfringens* at lesion sites, further experiments in rabbit ileal loops were performed with bacteria and supernatants. Immunohistochemistry of lesion sections from horses diagnosed with CPB2-associated gastrointestinal disease have shown that CPB2-specific staining typically localizes to rod shaped bacilli ((9, 148) and Figure 4.18 A). These staining results suggest that CPB2 may be localized to the bacterial surface, which could indicate that bacteria are required to model a wildtype infection. Notably, transfer of toxins from bacteria to target cells has been demonstrated for VacA from *Helicobacter pylori* (67).

Therefore, prior to further *in vivo* experiments, work was done to explore whether CPB2 is attached to the surface of *C. perfringens.* To test this hypothesis, the *C. perfringens* transformants (pDFB2h1v751)ATCC3624 and (pJIR751)3624 were grown in TGY until late log-phase. Bacteria were washed 3x with HBSS to remove free protein and then lysed via sonication. Bacterial lysates were then separated into lysate and pellet fractions were run on SDS-PAGE. CPB2 Western blot analysis of these fractions demonstrated that bacteria-associated CPB2h1 could survive repeated washings indicating that CPB2h1 may be firmly attached to the bacterial surface (Figure 4.18 B). Interestingly, immunoreactive bands consistent with full length and processed CPB2h1 were identified.

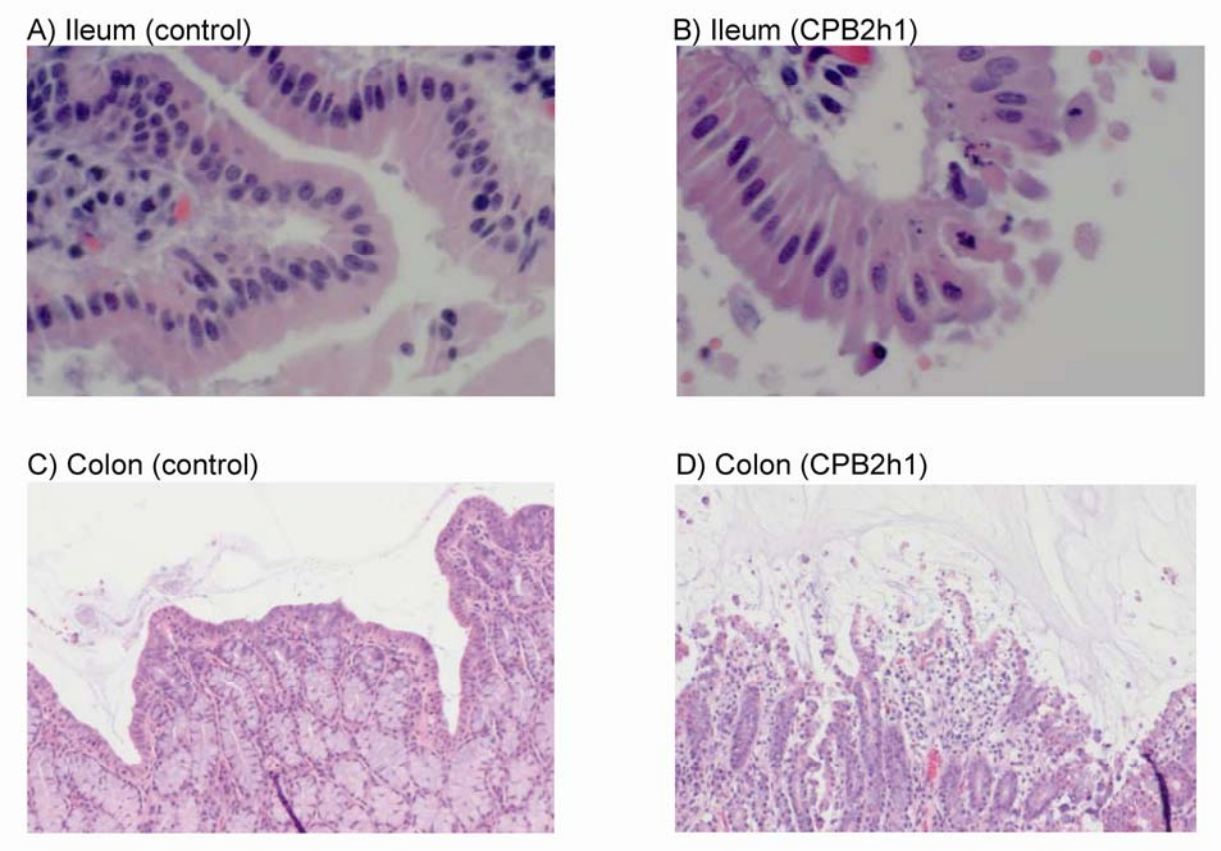

Figure 4.17 Hemotoxylin and eosin stained sections from rabbit intestinal loops treated with CPB2h1. Rabbit ileal and colonic loops were treated with Ringer's solution (A and C) or 300 μg/ml of CPB2h1 (B and D) and incubated for 6 hr. Rabbits were then euthanized and tissue was examined for gross pathology and embedded in paraffin and sectioned for H and E staining.

To assess the effects of CPB2-producing live bacteria on the rabbit intestine, *C. perfringens* bacteria and supernatants were injected into rabbit ileal and colonic loops. Preliminary results from these experiments have not identified damage or fluid accumulation. However, these early experiments have only been carried out with two rabbits (for short periods, < 6hr) and the intestinal loops were not run in duplicate. Experiments using more subjects/loops and various time periods are currently being pursued.

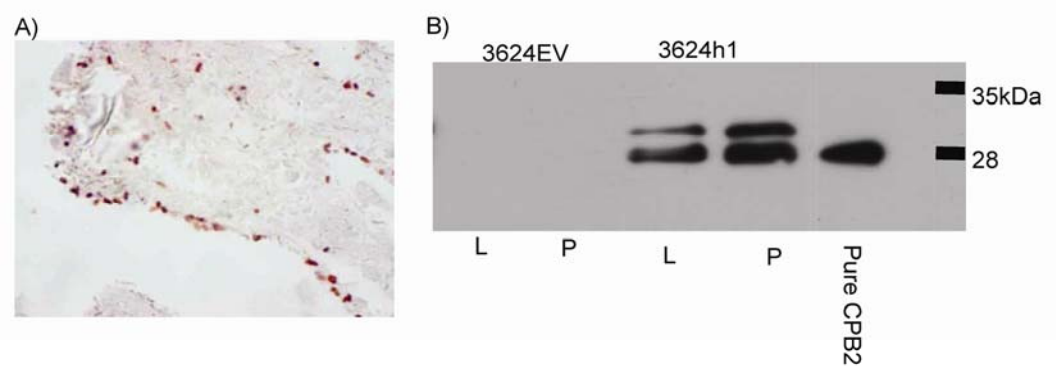

Figure 4.18 Surface localization of CPB2. Immunohistochemical detection of CPB2 in intestinal sections obtained from a goat suffering from CPB2-associated GI disease (A). CPB2 was detected in paraffin sections using an immunoperoxidase method with rabbit polyclonal anti-CPB2 antibodies. In B, localization of CPB2 to bacteria was further explored using *C. perfringens* transformants (pDFB2h1v751)ATCC3624 (CPB2-positive) and (pJIR751)3624 (negative control). Bacteria were grown to late log-phase and then bacteria were harvested and washed 3x with HBSS, lysed using sonication, and then separated into crude bacterial lysate (L) and pellet (P) fractions using centrifugation. Protein was run on 12% SDS-PAGE and detected using CPB2 Western blot. The names of isolates and fraction run in each lane are listed at the top or bottom of the blot, respectively. Purified CPB2 was run as a positive control. The location of molecular weight markers are shown to the right of the blot.

4.3.2 Determining the primary lethal components of non-type A vegetative culture supernatants using a mouse intravenous injection model

As mentioned throughout the introduction, one of the primary questions in the field of *C. perfringens* pathogenesis is which toxin(s) is important in gastrointestinal diseases caused by non-type A isolates. Most studies exploring the role of toxins produced by non-type A isolates have focused on studying the activity of individual, purified toxins using *in vitro* cell culture systems. While informative, studies with purified toxins in cell culture do not fully address the role of toxins during pathogenesis (also a valid criticism of the CPB2 research reported in section 4.2). For example, while purified β toxin is potent when administered i.v., studies have

shown it is not sufficient to mimic a natural type C infection in intestinal loop models (which requires the use of culture supernatants with or without bacteria) (134). Furthermore, most researchers believe that animals that die due to type B-D infections succumb to the lethal effects of ε and/or β toxins circulating systemically (134). However, it is not known how these toxins cross the intestinal barrier and enter into the circulation. These issues raise the question as to whether accessory toxins, such as CPB2 or α toxin, may play a role in the pathogenesis of non-type A infections by helping to initiate damage within the intestinal tract, facilitating the movement of ε and/or β toxins across the mucosa. While CPB2 is clearly not required for non-type A disease (owing to its absence from some non-type A disease isolates), the ability of CPB2 to damage intestinal-like cells *in vitro* and the association of *cpb2*-positive type A isolates with enteritis in some species (mainly porcine) suggests that when present, CPB2 could exacerbate non-type A infections.

While the ideal method for studying the role of toxins *in vivo* involves the creation of isogenic knockout mutants, the creation of isogenic knockouts in *C. perfringens* (until more recently (34)) has been extremely difficult (often requiring 1-2 years to construct a knockout). Currently, there have been only 2 *in vivo* pathogenesis studies utilizing isogenic toxin knockout mutants (created using allelic exchange) in the *C. perfringens* literature (7, 119). Furthermore, the basic work required to select candidates for knockout construction including the screening of a panel of isolates for toxin genes, toxin production levels, and lethality (and especially for *C. perfringens,* transformability) has not been performed with non-type A isolates, which as a group are rather poorly characterized. Hence, the studies that will be described in the following subsections were designed to address these basic questions. Isolates were screened for their toxin genotype and phenotype (which is not assessed by the current multiplex PCR typing scheme) and their lethal properties were assessed using a mouse i.v. challenge model.

While useful in identifying knockout candidates, these studies were not simply used to characterize isolates, but by correlating lethality with toxin production levels and through antibody neutralization studies we were able to determine which toxins are primarily important in i.v. lethality. The results from these studies have already impacted vaccines targeted for domestic animals and have served as a springboard for our future pathogenesis studies. Published results from type D and type C studies will be briefly discussed in subsections 4.3.2.1 and 4.3.2.2, respectively. More detailed discussion of results for type D and C studies can be found within the papers included in the Appendix (48, 121). Detailed results for experiments involving type B isolates will be reported in subsection 4.3.2.3 and a summary of the toxin phenotyping results for all non-type A studies are located in Table 4.2.

Table 4.2 Toxigenic properties of non-type A *C. perfringens* isolates

Isolate name	Type (Toxin Genotype)	ε[a] µg/ml	stdev	β[b] µg/ml	stdev	CPB2[c] µg/ml	stdev	CPE[d] µg/ml	stdev	α[e] U/mg protein x 10^{-3}	stdev	PFO[f] log2	stdev	*pfoA* PCR[g] +/-
1[h]	B (*cpb2atyp*)	18.8	2.8	7.0	2.2	13.5	0.2			2.5	0.9	1.5	0.1	
2	B (*cpb2atyp*)	4.4	1.3	8.6	1.6	9.0	2.8			2.3	0.2	3.6	0.4	+
3	B (*cpb2atyp*)	<1		17.7	6.2	<1				Nd[i]		3.5	0.4	
4	B (*cpb2atyp*)	10.3	3.4	<1		6.0	2.1			0.9	0.2	ND		+
5	B (*cpb2*)	6.5	2.7	8.8	3.4	5.5	1.0			1.3	0.1	ND		+
6	B (*cpb2*)	8.1	0.0	12.2	3.1	5.7	1.8			1.1	0.2	1.6	0.4	
7	B (*cpb2*)	1.2	0.0	32.4	8.9	7.1	3.5			0.9	0.1	3.6	0.0	
8	B (*cpb2*)	1.3	0.1	13.5	7.3	9.3	0.5			0.5	0.0	2.5	0.0	
9	B (*cpb2*)	1.2	0.0	8.6	5.7	5.4	2.5			1.0	0.1	4.4	0.5	
10	B (*cpb2*)	13.7	5.2	15.8	6.2	7.4	0.4			1.3	0.1	2.5	0.2	
11	B (*cpb2*)	15.3	4.1	18.3	3.9	10.8	2.1			0.7	0.1	3.6	0.4	
12	B (*cpb2*)	7.4	0.0	23.3	9.4	9.3	0.5			1.2	0.1	ND		+
13	B (*cpb2*)	8.0	0.4	36.6	8.1	14.0	4.0			1.8	0.2	3.8	0.1	
14	B (*cpb2*)	4.7	1.1	34.1	11.7	7.7	0.8			0.8	0.1	ND		+
15	B (*cpb2*)	2.7	0.6	5.3	0.6	9.0	0.3			0.9	0.0	1.6	0.0	
16	B (*cpb2*)	4.7	1.3	19.5	6.8	8.5	1.5			1.2	0.2	2.1	0.4	
17	B (*cpb2*)	4.3	0.9	11.0	3.0	4.6	1.6			0.8	0.0	ND		+
18	B (*cpb2*)	4.0	0.1	23.1	3.9	3.5	0.9			1.7	0.3	2.3	0.4	
19	B (*cpb2*)	<1		<1		<1				0.9	0.2	ND		+
AM930	C			11.7	2.0					1.0	0.1	1.8	0.1	+
AM931	C			8.9	3.0					1.1	0.1	2.2	0.3	
AM933	C			9.0	2.4					1.2	0.2	1.8	0.0	
CN5383	C			23.5	5.3					3.6	0.5	1.6	0.1	
AM950	C			45.9	8.1					5.6	0.9	4.8	0.1	
AM951	C			4.2	2.9					2.2	0.9	2.8	0.0	
AM952	C			23.6	7.1					6.3	0.7	2.6	0.1	
AM953	C			24.1	4.8					5.1	1.2	4.2	0.3	
AM954	C			15.3	4.3					3.6	0.2	3.4	0.2	
AM955	C			18.9	5.8					5.5	1.0	3.9	0.8	
AM956	C			6.1	0.8					4.1	1.4	2.3	0.4	
AM958	C			12.8	5.5					9.2	2.4	3.1	0.4	
AM961	C			13.0	6.0					6.0	0.3	2.9	0.6	
AM965	C			4.6	0.1					ND		1.9	0.4	
AM966	C			13.6	0.4	ND				0.7		1.9	0.3	
AM969	C			<1						ND		1.5	0.1	
AM989	C			<1		ND				ND	0.1	2.0	0.6	
AM990	C			<1						ND		1.7	0.2	
AM991	C			<1		ND				0.9	0.1	1.6	0.1	
AM999	C			30.9	19.3	ND				9.3	2.1	3.6	0.0	
Bar3	C (*cpb2atyp*)			16.2	7.1					2.3	0.3	ND		+
NCTC10719	C (*cpb2*)			2.7	0.6	0.9	0.1			0.7		ND		+
JGS1495	C (*cpb2typ*)			3.5	2.7	5.6	0.5			0.8	0.0	2.7	0.2	
JGS1504	C (*cpb2*)			4.1	0.5	5.7	5.7			0.7		3.7	0.2	
JGS1070	C (*cpb2*)			2.1	0.4	0.1	0.0			1.3	0.5	3.6	0.1	
JGS1071	C (*cpb2typ*)			2.5	0.3	7.8	3.6			0.9	0.4	3.1	0.7	
JGS1075	C (*cpb2*)			7.4	6.6	2.6	2.4			0.8		2.7	0.1	
JGS1076	C (*cpb2*)			4.0	2.6	13.8	3.8			1.1		2.9	0.5	
JGS1659	C (*cpb2*)			<1		0.4	0.3			ND		4.3	0.6	
AM962	C (*cpb2typ*)			6.0	2.2	1.9	1.0			1.2	0.2	1.9	0.4	
AM963	C (*cpb2*)			7.4	4.8	0.4	0.1			0.7		3.8	0.1	
AM964	C (*cpb2*)			2.3	0.6	0.6	0.1			0.7	0.2	2.4	0.0	
AM968	C (*cpb2atyp*)			3.0	0.2	2.8	0.4			0.0		1.7	0.1	
AM988	C (*cpb2atyp*)			<1		<0.1				0.8	0.2	1.9	0.5	
AM992	C (*cpb2atyp*)			6.6	0.0	1.0	0.1			0.7		2.4	0.3	
AM993	C (*cpb2atyp*)			<1		0.9	0.1			0.7		2.5	0.1	
AM994	C (*cpb2atyp*)			<1		7.0	0.7			0.0		2.7	0.1	
AM995	C (*cpb2*)			5.6	0.2	<0.1				0.9	0.2	2.7	0.7	
AM997	C (*cpb2typ*)			<1		0.5	0.1			0.8	0.1	ND		+
CN5388	C (*cpe, cpb2atyp*)			3.0	1.4	ND		<0.05		0.9		1.8	0.2	+
AM1035	C (*cpe*)			22.3	5.4			10.5	8.4	0.7	0.1	ND		-
AM1039	C (*cpe*)			8.3	3.3			6.7	3.0	1.4	0.5	ND		-
AM1043	C (*cpe*)			5.0	1.0			10.4	3.2	0.8	0.3	ND		-
AM1046	C (*cpe*)			14.8	4.4			11.2	3.5	5.2	0.8	ND		-
AM1048	C (*cpe*)			40.4	4.4			11.2	3.5	2.7	0.3	ND		-

Table 4.2 (continued)

Isolate name	Type (Toxin Genotype)	ε[a] µg/ml	stdev	β[b] µg/ml	stdev	CPB2[c] µg/ml	stdev	CPE[d] µg/ml	stdev	α[e] U/mg protein x 10^3	stdev	PFO[f] log2	stdev	*pfoA* PCR[g] +/-
1	D	25.0	5.5							1.9	1.0	ND		
2	D	2.0	0.1							0.8	0.2	2.7	0.2	
3	D	13.0	0.3							2.1	0.3	ND		
4	D	<1.7								1.4	0.9	ND		
5	D	<1.7								1.9	0.3	ND		
6	D	<1.7								1.2	0.3	2.8	0.1	
7	D (*cpb2atyp*)	<1.7				ND				0.7	0.1	ND		
8	D (*cpb2atyp*)	5.0	0.1			0.3	0.1			0.0		1.6	0.2	
9	D	<1.7								1.0	0.6	3.8	0.1	
10	D	4.0	0.1							1.1	0.3	ND		
11	D	<1.7								3.3	1.2	3.2	0.5	
12	D	<1.7								3.3	1.6	3.5	0.4	
13	D	<1.7								1.4	0.3	ND		
14	D (*cpe*)	<1.7								0.9	0.1	2.6	0.1	
15	D (*cpe*, *cpb2atyp*)	<1.7				0.2	0.1			4.9	2.5	2.6	1.0	
16	D (*cpe*, *cpb2*)	<1.7				ND				1.0	0.3	3.5	0.2	
17	D	<1.7								0.4	0.1	3.5	0.5	
18	D (*cpe*)	4.0	0.6							2.0	0.2	ND		
19	D	5.0	1.7							0.7	0.1	ND		
20	D (*cpe*, *cpb2atyp*)	3.0	0.1			1.1	1.3			1.8	0.1	1.6	0.1	
21	D	8.0	0.9							1.5	0.4	1.9	0.0	
22	D	53.0	8.3							4.2	0.9	5.5	0.1	
23	D	17.0	3.3							1.6	0.5	0.0		
24	D	2.0	0.1							0.8	0.1	3.3	0.4	
25	D	2.0	0.3							1.7	0.3	0.0		
26	D	6.0	3.1							2.5	0.6	2.7	0.1	
27	D	8.0	0.3							1.3	0.0	ND		
28	D	10.0	0.6							1.5	0.4	ND		
29	D	6.0	3.9							0.6	0.1	1.8	0.0	
30	D (*cpe*)	52.0	8.1							2.0	0.3	3.7	0.0	
31	D	5.0	1.0							1.2	0.1	1.5	0.2	
32	D	3.0	0.6							0.9	0.1	2.7	0.2	
33	D (*cpe*)	5.0	1.3							1.1	0.1	ND		
34	D (*cpe*, *cpb2atyp*)	<1.7				3.7	0.4			0.9	0.3	3.6	0.1	
35	D	4.0	0.1							3.5	1.6	2.9	0.3	
36	D (*cpe*)	2.0	1.6							4.6	0.8	3.5	0.1	
37	D	3.0	0.1							4.4	0.9	4.6	0.1	
38	D	2.0	0.9							5.1	0.6	2.9	0.1	
39	D	<1.7								2.5	0.6	3.9	0.1	

[a]ε toxin values for type B isolates were determined in this study. ε toxin values for type D isolates were determined by Sameera Sayeed (121).

[b]β toxin values for type B isolates were determined in this study. β toxin values for type C isolates are from (48).

[c]CPB2 values for type B isolates were determined in this study. CPB2 values for type C and D isolates are from (48, 121) .

[d]CPE values from type C isolates are from (48), while CPE values for type D isolates are from (121) and were determined by Sameera Sayeed.

[e,f]α toxin and PFO values were determined by Rachael Poon and Victoria Adams and are reported in this study (type B), type C (48), and type D (121).

[g]PCR results are listed for an assay designed to detect the presence of a portion of the *pfoA* ORF ((48, 121) and this study).

[h]Isolate names for type B and D isolates, which produce ε toxin, are not listed due to the inclusion of ε toxin on the USDA/CDC overlap select agent list.

[i]ND stands for not detected.

4.3.2.1 Type D. Death resulting from type D diseases is typically attributed to ε toxemia. However, as mentioned, it is not clear how ε toxin crosses the intestinal barrier. To explore the role of toxins in type D disease and to help identify suitable candidates for knockout construction, the toxin genotype and phenotype of a large collection of type D isolates was determined. Vegetative supernatants from these isolates were then assessed for their lethality using a mouse i.v. injection model. The detailed results from this study are described in (121) included in the Appendix.

Briefly, the toxin genotypes and phenotypes for type D isolates were generally the same, and approximately 20% of the type D isolates were found to encode and produce CPB2. By correlating the lethality with toxin concentrations, ε toxin was deemed the most important toxin in the mouse i.v. injection model. Further analysis of type D supernatants, including trypsin-treatment (to activate ε toxin) and antibody neutralization studies further supported the primacy of ε toxin activity in mouse lethality. No correlation between CPB2, PFO, or α toxin levels and lethality were found. However, the i.v. injection model does not completely mimic natural disease, which initiates in the intestines leaving the possibility that other toxins (such as CPB2) may contribute to pathogenesis in disease initiated in the gut.

4.3.2.2 Type C. Studies similar to those discussed for type D isolates also were performed for type C isolates. Detailed results for these experiments are reported in (48) located in the Appendix. Briefly, most type C isolates were able to produce their entire arsenal of toxins and a large number (~40%) of Type C isolates were able to produce CPB2. β toxin was determined to be the main contributor to lethality based on correlation analysis and toxin neutralization studies. Similar to type D studies, CPB2, PFO, and α toxin levels did not correlate with lethality.

However, since models mimicking type C disease in the intestines require more than just purified β toxin, a secondary factor must still be required to initiate disease in a normal host.

4.3.2.3 Type B. Type B isolates, which produce the two most potent *C. perfringens* toxins, i.e. ε toxin and β toxin, should potentially be the most lethal *C. perfringens* type. The production of these lethal toxins (along with α toxin, PFO, and potentially CPB2) also makes the pathogenesis of type B infections complicated to understand. To select candidates for the construction of isogenic toxin knockout mutants and to try and tease apart some of the basics of type B pathogenesis, studies were performed with a similar scope as the type D and C manuscripts. This work has been submitted to Infection and Immunity for review.

Multiplex PCR analysis of our collection of type B isolates determined that all of our type B isolates carry the *cpb2* gene in addition to their typing toxin genes (*plc, etx,* and *cpb*). Importantly, it also was shown that the *cpb2* multiplex PCR assay works better for detecting atypical *cpb2* genes when used by itself rather than in the multiplex PCR assay. Subsequent phenotypic analysis of those type B isolates using Western blot (to detect β toxin, ε toxin, and CBP2) and activity assays (to detect PFO and α toxin) to quantify toxin in late-log phase vegetative culture supernatants demonstrated that most isolates produced their full toxin repertoire. The few exceptions to this rule failed to produce detectable levels of PFO or PLC. Those isolates failing to produce PFO or α toxin do appear to carry at least a portion of the toxin gene ORF based on either multiplex PCR results (*plc*) or separate PCR analysis (*pfoA*). Toxin production in general was found to vary substantially between isolates (results are shown in Figure 4.19).

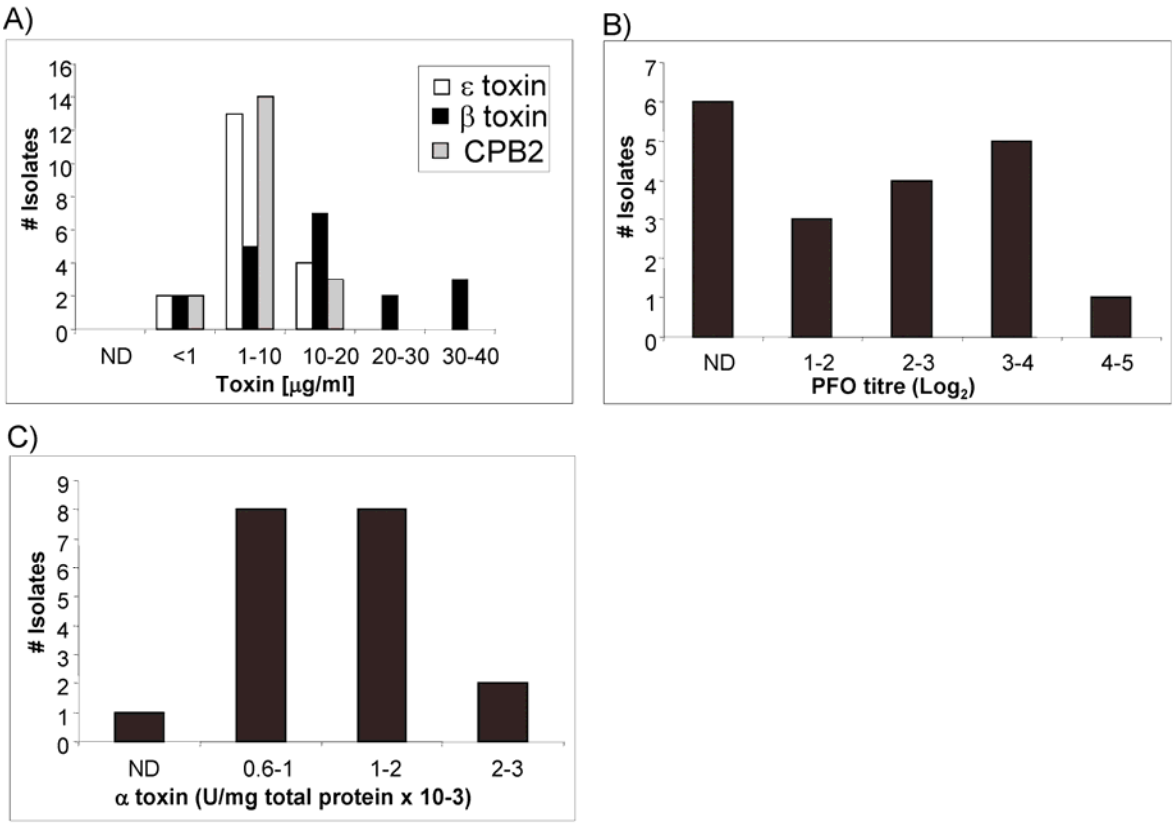

Figure 4.19 Toxin levels in late-log phase vegetative type B supernatants. β toxin, ε toxin, and CPB2 were quantified with Western blot using purified toxin to create standard curves. Toxin levels are reported as μg/ml toxin (A). PFO was quantified using a doubling dilution assay with horse red blood cells to measure PFO-specific hemolysis. PFO values are reported as the Log_2 titre (B). Lastly, α toxin levels were measured using an egg-yolk hydrolysis assay and amounts of α toxin are reported as U/mg total protein x 10^{-3} (C).

After the toxin phenotype of isolates had been determined, the lethality of their vegetative supernatants was assessed using a mouse i.v. injection model to determine the relative LD_{50}/ml of each isolate. Cultures were grown using the same conditions for quantifying toxin levels and were then filter-sterilized. Since ε toxin requires treatment with trypsin to be activated, the lethality of supernatants with and without trypsin pretreatment was determined.

The LD_{50}/ml results for trypsin and non-trypsin (Figure 4.20) treated supernatants were then correlated with their toxin levels to assess the contribution of individual toxins to lethality. Under trypsin conditions, ε toxin levels correlated best with LD_{50}/ml with an R^2 of 0.46 while β toxin levels correlated best with LD_{50}/ml values for non-trypsin treated supernatants with an R^2 of 0.62. The R^2 values for CPB2, PFO, and α toxin did not correlate with lethality.

Purified, activated ε toxin is approximately ~3 fold more lethal than purified β in a mouse i.v. lethality model (Table 1.2). Given this difference in activity, it was surprising that supernatants were less lethal after trypsin treatment (Figure 4.21). This is in contrast to type D supernatants where the LD_{50}/ml of most isolates greatly increased after trypsin treatment (121). These results may indicate that the lethal activity caused by β toxin (and other toxins) in non-trypsin treated samples is more lethal than the ε toxin activity plus any residual activity (from other toxins) left over after trypsin treatment.

To further analyze the role of type B toxins in our mouse i.v. challenge model, neutralization studies were performed with neutralizing MAb against ε and β toxins. Under non-trypsin conditions only supernatants pre-treated with β toxin MAb were neutralized. However, after trypsin treatment, both β toxin MAb and ε toxin MAb were required to neutralize the lethal properties of cultures supernatants. The neutralizing properties and specificity of the MAb used in this study were verified in experiments using semipurified β toxin (from a type C isolate) or purified ε toxin (from a type D isolate). The need to neutralize β toxin in trypsin treated supernatants was surprising because similar trypsin treatment conditions abolished the β toxin-specific lethal activity in type C supernatants, i.e. trypsin-treated type C supernatants no longer required β toxin MAb to neutralize the remaining lethality (48). Neutralization results are summarized in Table 4.3.

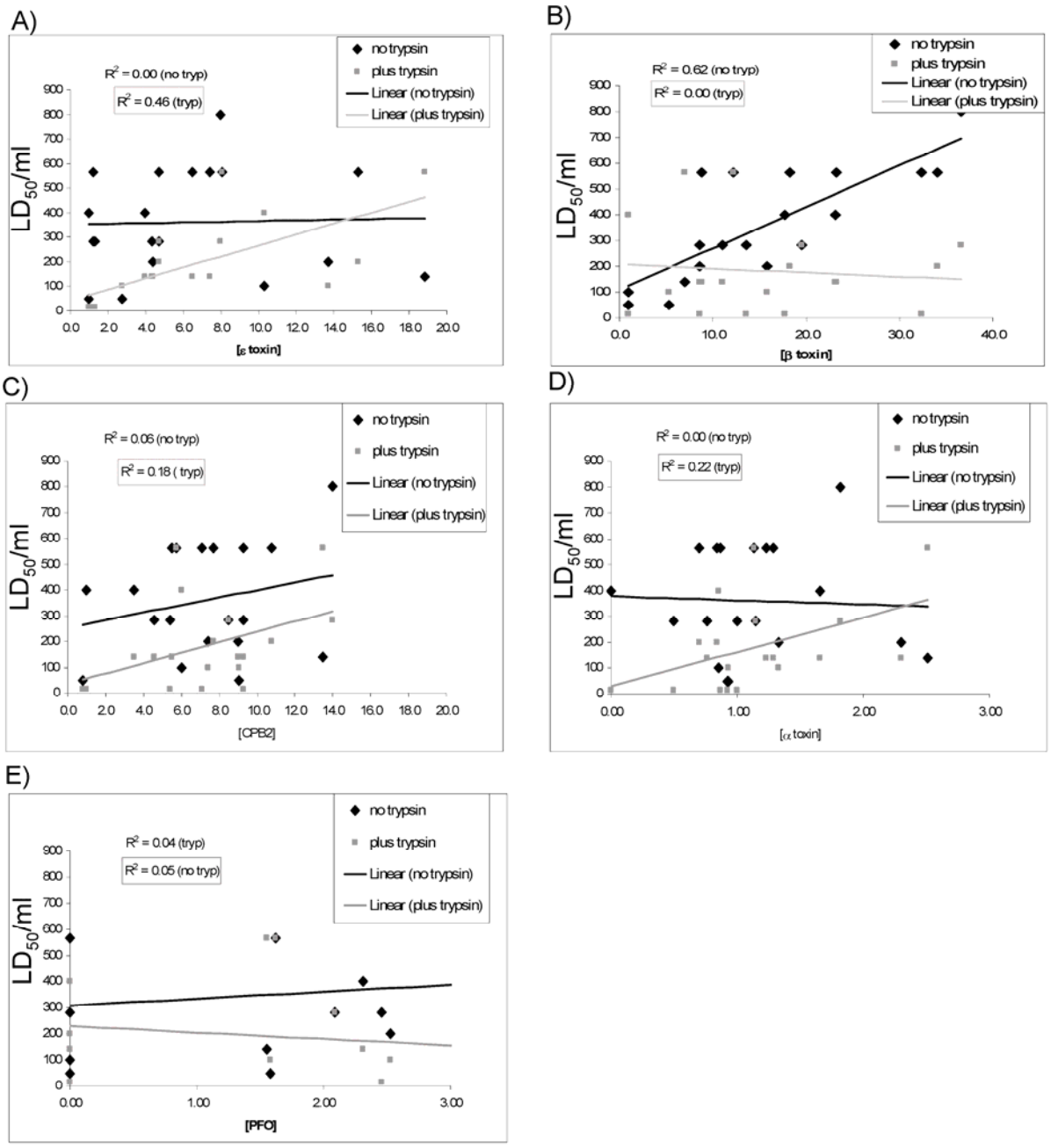

Figure 4.20 Correlation of toxin production versus LD_{50}/ml of trypsin and non-trypsin treated type B vegetative supernatants. The LD_{50}/ml of type B supernatants was determined using a mouse i.v. injection model. These values were compared to levels of ε toxin (A), β toxin (B), α toxin (C), PFO (D), and CPB2 (E) and a best fit line based on a linear equation was determined. R^2 values are reported for each graph (trypsin R^2 in gray boxes).

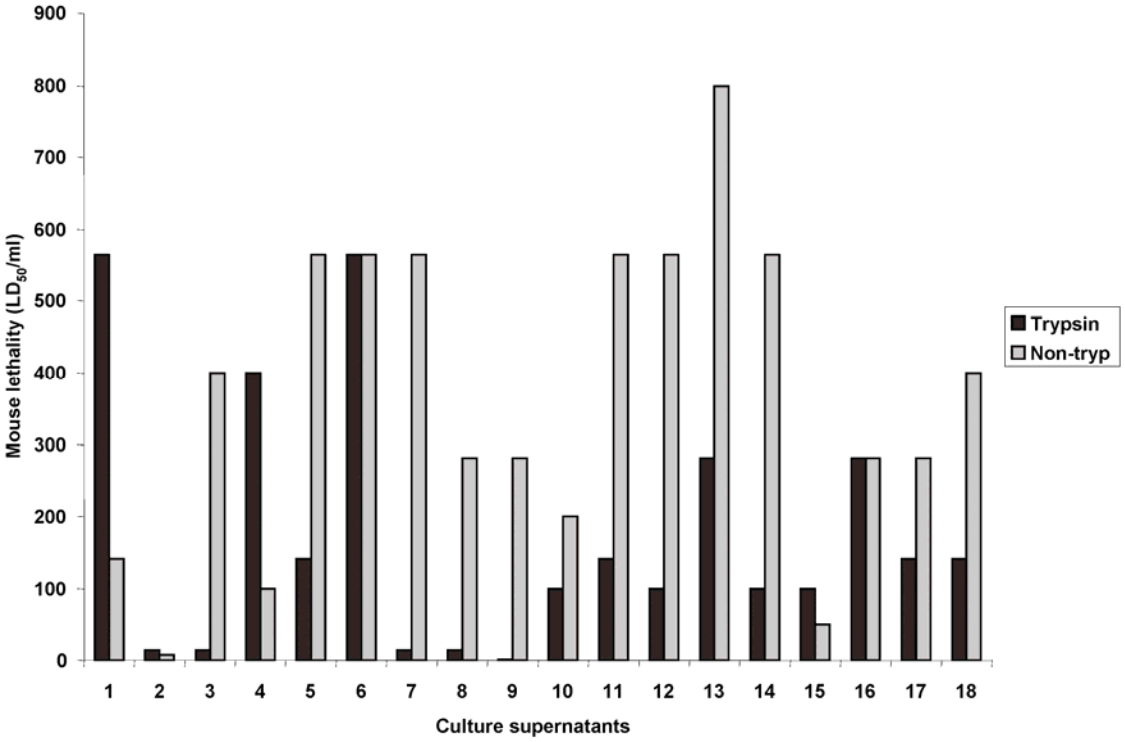

Figure 4.21 LD_{50}/ml values for trypsin treated versus non-trypsin treated type B supernatants. LD_{50}/ml values were determined using a mouse i.v. injection model.

Table 4.3 Neutralization of type B supernatant lethality using MAb.

		Supernatant lethality neutralized by MAb:							
		No Trypsin			Trypsin				
Isolate	Phenotype	Anti-β toxin	Anti-ε toxin	Anti-α toxin	Anti-β toxin	Anti-ε toxin	Anti-β+ Anti-ε toxin	Anti-α toxin	Anti-α+ Anti-ε toxin
3	$ETX_{<1.0}$,$CPB_{17.7}$,$CPB2_{<1.0}$,PLC_{ND},$PFO_{3.5}$	Y	N		N	N	Y		
6	$ETX_{8.1}$,$CPB_{12.2}$,$CPB2_{5.7}$,$PLC_{1.13}$,$PFO_{1.6}$	Y	N	N	N	N	Y	N	N
7	$ETX_{1.2}$,$CPB_{32.4}$,$CPB_{27.1}$,$PLC_{0.87}$,$PFO_{3.6}$	Y	N	N	N	N	Y	N	N
8	$ETX_{1.3}$,$CPB_{13.5}$,$CPB_{29.3}$,$PLC_{0.5}$,$PFO_{2.5}$	Y	N		N	N	Y		
9	$ETX_{1.2}$,$CPB_{8.6}$,$CPB_{25.4}$,$PLC_{1.0}$,$PFO_{4.4}$	Y	N	N	N	N	Y	N	N
10	$ETX_{13.7}$,$CPB_{15.8}$,$CPB_{27.4}$,$PLC_{1.3}$,$PFO_{2.5}$	Y	N		N	N	no data		
12	$ETX_{7.4}$,$CPB_{23.3}$,$CPB_{29.3}$,$PLC_{1.2}$,PFO_{ND}	Y	N		N	N	no data		
13	$ETX_{8.0}$,$CPB_{36.6}$,$CPB2_{14.0}$,$PLC_{1.8}$,$PFO_{3.8}$	Y	N	N	N	N	Y	N	N
14	$ETX_{4.7}$,$CPB_{34.1}$,$CPB_{27.7}$,$PLC_{0.84}$,PFO_{ND}	Y	N		N	N	Y		
15	$ETX_{2.7}$,$CPB_{5.3}$,$CPB2_{9.0}$,$PLC_{0.9}$,$PFO_{1.6}$	Y	N		N	N	Y		
16	$ETX_{4.7}$,$CPB_{19.5}$,$CPB_{28.5}$,$PLC_{1.15}$,$PFO_{2.1}$	Y	N	N	N	N	Y	N	N

4.3.3 Summary of section 4.3 results

The rabbit ileal loop model is a well-established model for studying the *in vivo* action of CPE. Since our initial goal was to study the role of CPB2 during *in vivo* pathogenesis using single and double *cpe* and *cbp2* isogenic toxin knockout mutants, rabbits were chosen as the initial animal model to study the effects of CPB2. Early studies performed with the overall goal of determining safe conditions for producing antibodies demonstrated that purified CPB2h1 caused minimal damage when injected intradermally. Changes included necrosis, edema, and leukocyte infiltration. This same CPB2h1 toxin prep also caused damage to the intestinal epithelium when injected into ileal and colonic intestinal loops. However, the results with purified CPB2h1 were inconsistent, possibly due to the lability of this toxin. Further *in vivo* efforts are currently being directed towards studying the effects of whole cultures in intestinal loops.

The role of CPB2 in non-type A disease was explored using a mouse i.v. lethal injection model. This model determined that the typing toxins from type B-D isolates are primarily responsible for lethality when supernatants are injected i.v. However, since these diseases normally initiate in the intestines, further work in more natural models of animal disease will be required to fully access the role of CPB2 in non-type A disease.

5.0 DISCUSSION

This chapter will add perspective and relevance to the discoveries reported in the previous sections. It should be noted that sections 5.1 and 5.3 will focus on tying together data that has mostly been reported in the attached manuscripts (located in the Appendix) (48, 49, 98, 121) and is intended to give a global outlook (see manuscripts for more focused discussions).

5.1 THE EVOLUTION OF *CPB2*: WHY THE DIVERGENCE?

The multitoxigenic pathogen *Clostridium perfringens* causes both histotoxic infections and enterotoxaemias in humans and domestic animals (113, 134). With the exception of gas gangrene (7), the interplay of different toxins in *C. perfringens* pathogenesis is poorly understood and has hindered development of more effective vaccines (particularly for domestic animals). Current vaccines are based on supernatant toxoids, with or without killed bacteria, and require repeated administration due to waning immunity (134, 138, 145, 146, 150). Ideally, vaccines should only contain those factors required to elicit a protective immune response since the presence of more antigenic molecules could take precedence over more critical epitopes that are required for developing a protective immune response. However, the production of multiple toxins by most *C. perfringens* isolates, as documented in my studies, makes it especially difficult to discern which components need to be neutralized by the immune system to prevent disease.

More recently, a new *C. perfringens* toxin, the beta2 toxin, has added to this complexity and currently there is much debate in the field as to whether CPB2 should be included in vaccines prepared for use with domestic animals. Research linking CPB2 to disease has been primarily epidemiological in nature, though limited *in vitro* and *in vivo* experiments have demonstrated that CPB2 is cytotoxic for certain cell lines (49, 52) and lethal when administered either i.v. (this study and (52)) or i.p. (148) in a mouse disease model. However, the presence of *cpb2*-positive isolates in healthy animals (particularly in poultry (129)) suggest that CPB2 may only cause disease in some species and/or may require specific conditions to cause disease (such as disrupted intestinal flora, preexisting damage to the GI tract, and/or low levels of proteolytic enzymes). At least two groups (52, 148) also have reported difficulty in retaining CPB2 activity (measured by cytotoxicity or mouse lethality) during purification, making it a rather difficult toxin to study.

Our interest in CPB2 stemmed from the differences in clinical symptoms of patients presenting with CPE-associated food poisoning (typically caused by isolates carrying a chromosomal *cpe* gene) versus the clinical symptoms of CPE-associated antibiotic associated diarrhea and sporadic diarrhea (typically caused by isolates carrying a plasmid *cpe* gene) (29). It should be noted that the association between gene location and disease is not 100%, as at least one report has linked plasmid-*cpe* isolates to a single food-poisoning outbreak in Japan (143). While these two CPE-associated GI diseases (FP and AAD) are caused by isolates with some basic biological differences, including increased resistance to environmental stress (FP isolates are more resistant to heat, cold, and chemical preservatives (85, 120)) and different growth rates (FP isolates grow faster and have a wider temperature growth range (85)), these different environmental sensitivities would appear to be more important in allowing FP isolates to survive in their “disease” niche and help to explain the source of infection, but do not explain the different clinical symptoms. This is especially true when one considers that the more environmentally resistant FP isolates cause less severe disease than AAD/SD isolates. Furthermore, the different location of the *cpe* gene in FP vs. AAD/SD isolates has not been shown to result in different levels of production of CPE or altered timing of CPE production, and the toxin genes do not vary in sequence regardless of their location, i.e. CPE is equally cytotoxic regardless of the gene location (39). These results in particular suggest that a second factor, such as an accessory toxin, may contribute to the pathogenesis of AAD/SD infections.

More recently, the *cpe*/IS*1470*-like plasmid, pCPF4969, from AAD isolate F4969 was shown to be conjugative when transfer of an antibiotic resistance-tagged version of this plasmid was demonstrated between *C. perfringens* isolates (25). Conjugation, which would allow

transfer of the *cpe*/IS*1470*-like plasmid to *cpe*-negative *C. perfringens* normal intestinal flora, was postulated as a potential mechanism to explain why AAD/SD cases require a lower initial inoculum of bacteria than FP cases (which require >10^6 bacteria) (25, 29). Transfer of the *cpe*-plasmid to resident flora that are able to successfully colonize the GI tract also could provide an explanation for the multiple bouts of diarrhea associated with AAD compared to FP (which typically resolves after one diarrheic episode). Even if a few chromosomal *cpe* isolates were able to colonize the intestine after or during the initial infection, they may not be able to reach sufficient numbers to cause a second diarrheic episode without transfer of the *cpe* gene to other *C. perfringens* isolates. In contrast, AAD isolates colonizing the intestine would be able to transfer the *cpe*-plasmid to resident flora, possibly allowing *cpe*-plasmid isolates to reach numbers sufficient to cause multiple episodes of diarrhea.

As stated above, the production of an accessory toxin by AAD isolates also would provide an explanation for the more severe and longer lasting symptoms of AAD vs. FP. The presence of two toxins together in the GI tract would be expected to cause increased pathology, and thus more severe clinical symptoms. Furthermore, the production of a vegetatively-produced toxin (CPE is only produced during sporulation) would allow bacteria to cause disease during both vegetative and sporulation life-cycles.

Due to the discovery of CPB2 and its potential association with GI disease in animals (51, 52), we sought to determine whether CPB2 could be an accessory toxin responsible for the different clinical symptoms between cases of FP and AAD/SD. As reported in Chapter 4.0, we determined that most AAD/SD isolates, in opposition to FP, isolates produce two CPB2 toxin variants that are both active on Caco-2 cells (49). The implications of these findings will now be discussed.

Consistent with previous findings (125, 153) for animal disease isolates, the *cpb2* gene in human GI disease isolates also was mapped to large plasmids (49). Unlike animal GI disease isolates, the *cpb2* gene in human isolates was found on two different sized plasmids of either ~50 kb or ~80 kb in size. Sequencing and PFGE/SB results identified two *cpb2* variants, h1 and h2, which are found on either the *cpe*/IS*1151* plasmid (all *cpb2*-positive) or on a separate plasmid in *cpe*/IS*1470*-like isolates (70% *cpb2*-positive), respectively. During the publishing of these human CPB2 findings, a report on *cpb2* genes in animal disease isolates identified two variants known as typical *cpb2* (*cpb2h2*-like) or atypical *cpb2* (*cpb2h1*-like) (73). While generally similar to the human variants (Table 4.1), the animal *cpb2* sequences are slightly different than the human sequences. A phyolgenetic analysis of the *cpb2* genes available in GenBank and this study reveals that typical and atypical *cpb2* genes cluster into 4 different groups in a

phylogram (Figure 5.1). While typical and atypical *cpb2* sequences are present on different branches, as expected, within those branches type E and type D atypical *cpb2* genes form subclusters that are distinct from most of the other atypical *cpb2* genes. Furthermore, the human *cpb2h1* and *cpb2h2* genes are grouped on separate branches from each other and from most of the animal *cpb2* genes. This phylogram suggests that *cpb2* exists as a number of subgenotypes with a continuum of diversity extending between and across atypical/h1 and typical/h2 *cpb2* genes. Since atypical, typical, h2, and h1 *cpb2* genes mainly cluster on separate branches, I would propose that these genes retain their own nomenclature for identification rather than, for example, referring to all h1/atypical *cpb2* genes as atypical *cpb2* genes.

While both *cpb2h2* and typical *cpb2* genes appear to be located on similar sized plasmids, the plasmid size on which atypical *cpb2* genes are located is not known. Based on sequence similarity with *cpb2h1*, I would hypothesize that the atypical *cpb2* genes may be located on larger-sized plasmids than the typical *cpb2* genes, and possibly are present on the typing toxin plasmids (similar to results for *cpe*/IS*1151* plasmids). PFGE/SB studies should be pursued with those isolates to determine if atypical or typical *cpb2* genes are located on the typing toxin virulence plasmids.

Figure 5.1 Phylogram showing the relationship of *cpb2* genes. DNA sequences comprising nucleotides 323-581 (~1/3 of the ORF) from the *cpb2* genes of 42 isolates were used to generate this tree. The numbers in parentheses indicate branches containing rough clusters of related genes.

The evolution of a toxin with two clearly distinct, yet related, nucleotide and amino acid sequences is unusual within *C. perfringens*. With the possible exception of a single α toxin sequence (76), most *C. perfingens* toxin sequences are highly conserved (131). In stark contrast to CPB2, which shows variations across ~1/3 of the amino acid sequence, the CPE amino acid sequence (excluding type E isolates which carry a silent *cpe* gene (15)) is identical

regardless of its genome location or type ((39) and D.J. Fisher and S. Sayeed [non-type A *cpe* genes], unpublished). Sequencing and translation of the *cpb* gene (D.J. Fisher, unpublished), *etx* gene (S. Sayeed, unpublished), and *pfoA* gene (this study and (48)) in different *C. perfringens* types have revealed limited sequence differences (<5 across and within types). *In vitro* Caco-2 cell assays suggest that the CPB2 amino acid differences do affect activity since CPB2h1 is more cytotoxic for Caco-2 cells than CPB2h2 (49). Whether this is due to differences in binding or post binding steps is not known because structure/function relationships for CPB2 are not understood, making it difficult to speculate how these amino acid differences affect activity. It should be noted that the amino acid sequence similarities between CPB2h1 and CPB2h2 are not randomly distributed across the protein (and similarly the conserved regions in typical and atypical CPB2 sequences), but roughly clusters into 6 groups of 16-30 amino acids (excluding the signal peptide [Figure 4.2]). The relative conservation of these clusters of amino acids suggests these regions may be important for activity. Future studies involving domain swapping between CPB2h1 and CPB2h2 or alanine-scanning mutagenesis could shed light on the roles of these conserved regions in cytotoxicity.

It is interesting that *cpb2* gene divergence also is reflected by plasmid types and species of origin. The *cpb2h1* gene is typically located in isolates 1) that carry IS*1151* sequences (for human *cpe*-positive isolates (49, 98)) and 2) that are isolated from non-porcine species ((73) and D.J. Fisher, unpublished). In contrast, the typical *cpb2* gene is usually 1) on a smaller plasmid (125, 152, 153) (includes *cbp2h2* (49)) and 2) is isolated almost exclusively from porcine species (73). There are a number of possibilities that could explain this *cpb2* gene/plasmid divergence. The degree of differences between the two *cpb2* genes could suggest that 1) the *cpb2* gene is ancient and diverged soon after its creation or 2) that a divergent event was accompanied by divergence in speciation that increased pressure on the gene to change, accelerating the mutation rate. For example, the original *cpb2* plasmid (possibly a pCP13-like plasmid) may have been modified by the insertion of various elements to create the *cpb2*/*cpe*/IS*1151* plasmid. These two different plasmids may result in the ability of isolates to colonize different hosts (swine vs. humans, i.e. divergence in speciation) and thus apply selective pressure on *cpb2* to adapt to its new host. Such changes could include a modified binding domain that allows CPB2 to interact with different receptors present in the GI tract of human vs. swine.

Results linking *cpb2h1* (and hypothetically other atypical *cpb2* genes) to conjugative plasmids could explain why the atypical/h1 *cpb2* genes are found in all types (A-E) (11, 98). In contrast, if the typical *cpb2* and *cpb2h2* genes are present on non-mobile pCP13-like plasmids

(125), as their sizes suggest, these genes would not be anticipated to transfer between isolates. Supporting this idea, typical *cpb2* and *cpb2h2* genes have only been reported in type A and type C isolates (49, 73, 152, 153). In terms of gene mobility, unlike most other plasmid-encoded *C. perfringens* toxins (113), *cpb2* genes are not flanked by IS elements (98, 125) and are probably not mobile (unless transferred via conjugation).

It is not clear why isolates carrying atypical *cpb2* or *cpb2h1* genes are not isolated from porcine species, but it is possible that 1) those isolates are not present in a location where they could colonize swine or 2) those isolates lack other genes that are required to colonize and cause disease in swine. Those genes relevant to point (2) may, 1) be present on the typical *cpb2* plasmids and/or 2) be present elsewhere within isolates carrying the typical *cpb2* plasmid. It has recently been hypothesized that the plasmid encoded *cna* gene (which is thought to encode a functional collagen adhesin) may play a role in colonization and disease by allowing bacteria to attach to damaged portions of the intestine where the underlying collagen layer has been exposed. The *cna* and *cpb2* genes are almost always present together within isolates (74) (these genes are present on the same plasmid (98, 125)). However, the presence of the *cna* gene in isolates carrying *cpb2h1* genes suggests that *cna* is not a swine-specific colonization factor.

In regard to the bigger picture of plasmid evolution in *C. perfringens*, studies reported in this thesis have demonstrated that at least two distinct *cpe* plasmids exist (98). Nonetheless, these divergent plasmids still carry a conserved backbone that encodes a cluster of genes known to be involved in conjugation (11). These genes appear to be present on most *C. perfringens* virulence plasmids, with the possible exception of the typical *cpb2* and *cpb2h2* virulence plasmids ((98) and TIGR, unpublished). However, the ability of the virulence plasmids (with the exception of the *cpe*/IS*1470*-like plasmid (25)) to transfer via conjugation still awaits experimental verification. Nonetheless, RFLP analysis of *cpe*/IS*1151*–positive AAD isolates suggest that those isolates are not clonal (39), strengthening the case for horizontal gene transfer. The ability of the virulence plasmids to transfer via conjugation provides an explanation for the toxin biodiversity in *C. perfringens* and is particularly interesting given the recent identification of a binary toxin in *C. difficile* (115) (a growing health threat in the U.S. and other countries (105)). This binary toxin is very similar to the ι toxin from type E isolates (~81% amino acid identity) and the *C. spiroforme* toxin (~82%), all of which contain B subunits that are more distantly related to the *Bacillus anthracis* protective antigen (~28%) (12). It would be interesting to determine whether the binary toxin in *C. difficile* is carried on plasmids with similarity to the type E plasmid from *C. perfringens*, which appear to be related to the *cpe*/IS*1151* plasmid (15).

Furthermore, it would be interesting to determine whether other clostridia carry any of the *C. perfringens* plasmid-borne toxins (including CPB2), which has not yet been reported.

Finally, it has been reported by others that the atypical *cpb2* gene is not expressed *in vitro* based on the failure to detect *cpb2* transcript or CPB2 via Western blot (27, 75). It should be noted that those studies employed different culture conditions, media, and time intervals for detecting CPB2 from the conditions used in this study. Using our conditions, we were able to detect atypical CPB2 and CPB2h1 in culture supernatants using two independently produced rabbit anti-CPB2 polyclonal antibodies (our PRF&L antibodies (48, 121) plus rabbit polyclonal antibodies supplied by Dr. Michel Popoff, Pasteur Institute (49)). Recently, a second group has reported the production of CPB2 in culture supernatants from a human disease isolate carrying the *cpb2h1* gene (58). Furthermore, cloning of the *cpb2h1* gene, which is very similar to the atypical *cpb2* gene, into a *C. perfringens* shuttle vector allows for the production of CPB2 from a naturally cpb2-negative *C. perfringens* isolate (49). Lastly, the *cpb2h1* promoter has successfully been used to drive the production of a foreign gene in *C. perfringens* (34). More speculatively, given the small amount of "extraneous" DNA sequences (non-ORF sequences) present on the sequenced virulence plasmids (which are >74% coding, not including promoter regions (98)), it seems strange that a non-expressed *cpb2* gene would be maintained without accumulating nonsense mutations. For example, in type E isolates it has been hypothesized that the *iap/ibp* toxin gene inserted into a *cpe*/IS*1151*-like plasmid, disrupting the *cpe* promoter region (15). Regardless of the validity of this hypothesis (which is difficult to prove), in all type E isolates tested the *cpe* gene is silent. Sequencing of those silent *cpe* genes has revealed numerous nonsense mutations throughout the ORF, i.e. unlike the "silent" atypical cpb2 gene, the silent *cpe* gene is not maintained as a translationally active sequence.

Regardless of the conflicting CPB2-production findings, it is critical to determine if both typical CPB2/CPB2h2 and atypical CPB2/CPB2h1 are produced *in vivo*. Some reports have detected typical CPB2 using immunohistochemistry in the GI tract of horses (9, 148) and an elephant (10) that died from GI disease, and using similar methods we have detected atypical CPB2 in the GI tract of a goat suffering from GI disease associated with *cpb2*-positive type D isolates (this study, Figure 4.18). However, those studies were performed with naturally diseased animals and it is not clear whether CPB2 was produced during disease or after the animal had succumbed to the infection (time after death until tissue processing was not reported). More controlled *in vivo* experiments need to be performed to address this issue. It also would be beneficial to develop an ELISA that could detect CPB2 in the feces of infected humans and animals. This would facilitate studies assessing the importance of CPB2 in human

GI disease, an important issue since the causes of up to 80% (72) of nosocomial GI disease cases are unknown. Unfortunately, due to the proteolytic sensitivity of this toxin, detection of CPB2 in feces may not be practical (similar problems exist for detecting the trypsin-sensitive β toxin in fecal and intestinal contents).

5.2 UNRAVELING THE MECHANISM OF ACTION OF CPB2

C. perfringens makes a large number of well-characterized, lethal, membrane-active toxins including PFO, CPE, α, ε, β, and ι toxins (107). All of these toxins, except α toxin, contain at least one component (statement modified to include the binding portion of the binary ι toxin) that can polymerize and form pores in the membranes of sensitive cells. Excluding ι toxin, which ultimately damages cells through the modification of actin, pore-formation directly leads to the death of sensitive cells through the disruption of the colloid-osmotic equilibrium. Cells treated with membrane active/pore-forming toxins typically die much more quickly than cells treated with toxins that act intracellularly; however, there is a trade off between these lifestyles. Toxins that act within cells typically possess enzymatic properties and are capable of repetively performing their "job", which allows smaller doses of the toxin to intoxicate a cell. For example, a single molecule of diphtheria toxin is sufficient to kill a cell (155). In contrast, pore-forming toxins must polymerize to form a pore, which requires multiple molecules (and often multiple pores) to kill a single cell. These different mechanisms are often directly reflected by the LD_{50} of the toxin, where toxins with enzymatic properties such as botulinum toxin, tetanus toxin, and shiga toxin have low LD_{50} compared to CPE and PFO (53). However, the toxin's target (for example, the cells within the nervous system versus epithelial cells) and binding affinity also are important for establishing the LD_{50} of a toxin. A good example of this is ε toxin, which despite functioning as a pore-forming toxin, is highly potent, possibly due to preferential effects on the nervous system (131).

To determine the mechanism of action of CPB2, *in silico* studies were first performed to determine if CPB2 had any previously characterized homologues. Unfortunately, no nucleotide or amino acid sequences showing similarity to CPB2 are found in GenBank. Analysis of the predicted CPB2 secondary structure only identified a single predicted transmembrane region,

which coincides with the experimentally-verified signal peptide. However, results for primary sequence analyses do not always agree with the eventual experimentally determined function of proteins and subdomains. Further complicating the CPB2 story is the presence of two quite different CPB2 variants (~60% identity).

As a basic follow-up to the *in silico* analysis, the identification of a cell line sensitive to CPB2 was sought. Since we were interested in determining if CPB2 had the potential to act as a toxin within the GI tract, and specifically within the human GI tract, we initially explored the sensitivity of Caco-2 cells to CPB2. Caco-2 cells are a well-characterized model of the human intestinal epithelium and are currently used to study CPE (128), which would allow us to compare the effects of CPB2 treatment with that of CPE.

Studies with Caco-2 cells demonstrated that CPB2h1 is more active (~10 fold) than CPB2h2 (49), possibly due to the different amino acid sequences between these toxin variants, which could, for example, affect toxin binding to sensitive cells. However, both toxins caused similar cytopathic effects, including cell rounding, membrane bleb formation, and cell detachment, within a short time after treatment (~1 hr with a 10 μg/ml dose). Those results were consistent with CPB2 being another membrane active *C. perfringens* toxin, so further studies were performed to address this hypothesis. CPB2 was determined to cause ^{86}Rb release from Caco-2 cells and Caco-2 cells treated with CPB2 in the presence of dextran (1500 Da) could be protected from the cytotoxic mechanism of this toxin. While not conclusive, these results are consistent with CPB2 acting via pore-formation. It is interesting to note that CPB2 putatively forms larger pores than CPE, since Caco-2 cells treated with CPB2 in the presence of sucrose were not protected against CPB2, but were protected against CPE. The putative CPB2 pore size also appears to be larger than the pore formed by ε toxin or β toxin (131), but smaller than the pore formed by PFO (which are too large to allow for protection by an osmoprotectant of ~1500 Da) (61). The larger pore size for CPB2, which would potentially require a greater number of monomers to polymerize, could partly explain the higher mouse LD_{50} for CPB2 (52) compared to CPE, ε toxin, and β toxin (53). Similarly, this line of reasoning could explain the larger dose of CPB2 required to cause the release of 50% ^{86}Rb from Caco-2 cells compared to CPE. However, it can not yet be ruled out that CPB2 simply has a lower binding affinity than CPE, which also could explain the observed difference in activity.

Since CPB2 appeared to act via pore formation, preliminary studies to measure the binding of CPB2 to Caco-2 cells and to detect CPB2-complex formation in Caco-2 cells were pursued. Pore-forming toxins typically bind to the surface of cells and then polymerize to form a higher molecular weight species. Under the proper extraction and protein gel conditions these

complexes can be detected. Attempts to detect ^{125}I-labeled CPB2 binding to cells and to observe a membrane associated CPB2-species that was greater in size than free CPB2 were unsuccessful. However, the results presented in section 4.2.3, which found that a large portion of the ^{125}I-CPB2 was degraded and more trypsin sensitive than native CPB2, suggested that the iodinated toxin may have been damaged during the iodination procedure. Thus, the negative results for binding and complex formation may have been due to inactive/unstable ^{125}I-CPB2h1. A CPB2 Western blot approach also was pursued to detect CPB2-complexes. While no complexes could be detected, it is important to note that CPB2/membrane fractions were extracted with SDS and then run on SDS-PAGE gels under reducing conditions. While some toxins do produce SDS-PAGE stable membrane complexes, many do not produce complexes that are stable under these relatively harsh conditions (128). It also is possible that the CPB2 complex could have been degraded by proteases released from the cells during processing, especially since CPB2 is very sensitive to proteolysis by trypsin (this study and (52)).

Ultimately, the continued study of the mechanism of action of CPB2 would benefit greatly from the development of a recombinant expression system. This system would allow 1) easy manipulation of the *cpb2* gene for structure/function studies, 2) higher purification yields, and 3) purification of CPB2 from a toxin negative background. To address the third point, which deals with possible contamination of our CPB2 preparation with other *C. perfringens* toxins we ran a number of controls including 1) testing mock purifications from the *cpb2*-negative parent *C. perfringens* isolate carrying an empty vector on Caco-2 cells and 2) testing supernatants from the empty vector parent constructs on Caco2 cells (49). Both control experiments were non-cytotoxic for Caco-2 cells. Thirdly, transformation of the parent strain with *cpb2* resulted in supernatants that were CPB2 positive and cytotoxic (49). Lastly, the dextran study suggests that the activity in the purified preparation was not due to PFO, since cells treated with PFO would not be protected by dextran polymers of 1500 Da because of the large PFO pore size. However, we can not rule out that CPB2 activity may require a cofactor that is produced by *C. perfringens* isolates. While the development of a neutralizing anti-CBP2 monoclonal antibody would further support our findings that CPB2 is responsible for the observed cytopathic effects with Caco-2 cells, this also would not rule out CPB2 interacting with a *C. perfringens* cofactor. Ultimately, ultra pure CPB2 preparations or the production of CPB2 in a toxin negative background (such as *E. coli*) will be required to address this issue.

As described in section 4.2.5, attempts were made to produce active, recombinant CPB2 using commercially available *E. coli*-based recombination systems. While these attempts were unsuccessful, this work was not rigorous enough to rule out pursuing this approach in the future.

Efforts were not made to remove the protein tags from the recombinant CPB2 (some of which, such as the MBP and GST tags, were quite large) nor were efforts made to produce a stable CPB2 molecule lacking its signal peptide and the recombinant tag (initial efforts to produce CPB2 without its signal peptide resulted in degraded products). Furthermore, due to the presence of multiple cysteines within CPB2 (indicating possible disulfide bond formation), CPB2 may have to be directed to the *E. coli* periplasm to allow for proper folding. Lastly, a Gram-positive expression system (for example, the *Bacillus megaterium* expression system from MoBiTec, which has been used to produce *C. difficile* TcdA (28)) may be a worthwhile alternative approach to consider for developing recombinant CPB2.

CPB2 mechanism of action studies also explored the lability of this toxin. CPB2 appears to be sensitive to oxygen, heat, and trypsin. Those results suggest that CPB2 may require ideal conditions within the host to be active and/or may require a unique method of delivery to target cells that prevent the degradation/inactivation of CPB2. One method of delivery that would minimize the environmental exposure of CPB2 involves the direct transfer of CPB2 from the bacterial membranes to the host cell membrane. Recently, it was reported that VacA from *Helicobacter pylori* can be surface associated and subsequently transferred to target cells by bacteria/cell contact (67). Interestingly, the bacterial-associated VacA was more active than VacA purified from *Helicobacter pylori* culture supernatants. There also are a limited number of reports describing bacteria producing contact-dependent hemolysins (44, 59). The lability of CPB2 reported in this study and two other studies, suggested that CPB2 may not be stable in the supernatant and could possibly utilize an alternative method of delivery. Basic studies with our recombinant *cpb2*-positive *C. perfringens* constructs revealed that CPB2 could survive repeated washings of bacteria and still be detected in bacterial lysates using CPB2 Western blotting. Consistent with this observation, studies ((9, 10, 148) and findings reported in Figure 4.18) using immunhistochemistry to detect CPB2 at lesion sites within animals that died from CPB2-associated GI disease, found that CPB2-staining localized to the surface of the bacteria. In at least one of these studies, bacterial lysate fractions also were found to contain CPB2 detectable by Western blot.

These findings have impacted our efforts to develop an animal model for studying CPB2 (discussed in 5.3) and could explain the lability and difficulty of obtaining consistently active CPB2 from supernatant purifications. Unfortunately, if CPB2 is transferred from the bacterial surface to target cells, this could complicate the development of an *E. coli* expression system. The surface localization of CPB2 was somewhat surprising, since both CPB2 variants have a classic Gram-positive secretion signal. However, since immunoreactive bands consistent with

the size of processed toxin were found on gels run with bacterial lysates, it is possible that the toxin is secreted and then attaches to the bacterial surface. Precedence for the secretion of a protein and subsequent attachment to the bacterial surface does exist in Gram-positives, though these proteins typically contain a C-termianl surface-sorting LPXTG motif (which are absent from the cpb2 variants) that is used by sortases to covalently attach the protein to the peptidoglycan cell wall matrix (89). However, “anchorless” surface proteins have been identified in *Streptococcus* (13, 20, 36, 64, 80), and if CPB2 is transferred from the bacterial surface to target cells, an anchorless attachment to the bacterial surface would be favored over a covalent linkage.

5.3 CPB2: *IN VIVO* PATHOGENESIS

5.3.1 The potential role of CPB2 in human GI disease

Ultimately, the importance of CPB2 in pathogenesis needs to be addressed using an *in vivo* animal disease model and isogenic toxin knockout mutants. Previously, groups have studied either purified CPB2 in guinea pig ileal loops (52) or CPB2-producing isolates in a calf intestinal loop model (88). While these models are sensitive to CPB2, we were primarily interested in trying to study the role of CPB2 in pathogenesis within the context of GI disease caused by *cpe/cpb2*-positive human AAD/SD GI disease isolates. Since the action of CPE and CPE-positive supernatants has been well characterized in the rabbit ileal loop model (119, 124), we sought to determine whether this model would respond to CPB2. If rabbit ileal loops proved sensitive to CPB2, we would then have a model in place for studying purified CPB2 and *cpb2* and/or *cpe* single and double isogenic toxin knockout mutants without having to characterize both CPB2 and CPE in a new *in vivo* model.

Initially we sought to assess the ability of purified CPB2h1 to damage rabbit ileal and colonic loops. Using a relatively high dose of toxin (300 μg/ml) we were able to detect damage to the intestinal epithelium. In ileal loops, this consisted of desquamation of the intestinal villi, mostly at the tips of the villi, that was not apparent in control loops. Colonic tissue samples were

very mucoid, blunted, and contained cellular debris compared to control loops that appeared healthy. Fluid accumulation was not present in either the colon or the ileum. In contrast, CPE at a dose of 300 μg/ml would be expected to cause desquamation, blunting of villi, and fluid accumulation in the ileum within a relatively short time period (124). These results would suggest that CPE is more potent than CPB2, at least in the rabbit ileum, and is consistent with CPE's lower LD_{50} and the lower amount of CPE required to cause a 50% ^{86}Rb release from Caco-2 cells. However, unlike CPB2, CPE appears to bind and cause an increase in mucus, but does not cause obvious cell damage in the rabbit colon (94). While these CPB2 ileal loop results support our hypothesis that CPB2 could be acting as an accessory toxin in disease, *in vivo* studies with CPB2 did not always give consistent results, i.e. in some experiments damage was not detected in CPB2-treated ileal loops. Therefore, this model requires more development.

It is possible that the lability of CPB2 makes it reliant on host conditions for pathogenesis, a factor that can be hard to control during *in vivo* experiments. It also is possible that the rabbit ileal loop model may not be as responsive to CPB2 as other animal models. Furthermore, all studies were performed with CPB2h1 leaving open the possibility that CPB2h2 (or possibly typical or atypical CPB2) may be more active in this model. To address this question, it would be interesting to perform the rabbit ileal loop studies with the other CPB2 variants.

As discussed in section 5.2, it is possible that CPB2 may be associated with the bacterial surface. This conclusion is supported by both *in vitro* CPB2 Western blotting results and CPB2 immunohistochemistry experiments performed with tissue samples from animals suffering from natural GI disease (9, 10, 148). Precedence for the delivery of a toxin from bacteria to the surface of the target cell does exist, and in at least one case (*Helicobacter pylori* VacA), the bacterial-associated fraction of toxin was more active than free toxin (67). If CPB2 acts in a similar manner, it would be important to study CPB2 *in vivo* using live bacteria. Preliminary experiments in which rabbit intestinal loops were treated with the CPB2-producing *C. perfringens* transformants using culture supernatants, washed bacteria, or whole cultures did not show signs of damage. However, those experiments were only performed once (in duplicate). To fully address the pathogenic potential of CPB2-producing *C. perfringens*, these experiments need to be repeated using longer time periods, and possibly different animal species/CPB2 variants. An ideal, though more costly model for studying CPB2, would be a piglet ileal loop model. This model is ideal because of the strong epidemiological association between typical CPB2 and enteritis in piglets and adult swine (153). Lastly, it also is possible that products from accessory genes encoded by the *cpb2* plasmids (not supplied by the *cpb2*-

positive *C. perfringens* transformants), such as CNA, are important for *in vivo* pathogenesis (74, 98, 125). To test this possibility, *in vivo* experiments also should be performed with naturally *cpb2*-positive isolates.

If an animal disease model can be established, isogenic *cpb2* and/or *cpe* single and double toxin knockout mutants should be constructed and tested in this model. Attempts were made to construct *cpe* and *cpb2* knockouts (using classical allelic exchange and Targetron® technology [Sigma] (34)) in the *cpe*/*cpb2*-positive AAD isolate, F5603, which contains the sequenced plasmid, pCPF5603. While a *cpe*::intron mutant was constructed using Targetron®, attempts to inactivate the *cpb2* gene were not successfully, possibly due to the low E-value (predicts intron insertion efficiency) for the *cpb2* intron-insertion site. It may be necessary to construct the *cpb2* knockouts using a classical allelic exchange approach.

5.3.2 CPB2 as a potential accessory toxin in non-type A GI diseases

The pathogenesis of GI diseases caused by non-type A isolates is poorly understood (134, 135, 146). While animals succumbing to non-type A GI disease are believed to die due to enterotoxaemias (from ε toxin or β toxin), currently it is not completely understood how ε toxin and β toxin cross the intestinal epithelium, especially since purified β toxin reportedly does not induce intestinal damage (134, 135). It has been proposed that accessory toxins, such as α toxin or CPB2, could facilitate or increase the absorption of other toxins into the bloodstream by damaging the intestinal epithelium. While CPB2 is certainly not required for the pathogenesis of non-type A isolates (due to its absence in some non-type A disease isolates (27, 51)), it is possible that the presence of CPB2 could result in more severe disease. It also is possible that if CPB2 alone can cause GI disease in domestic animals (a strong possibility in porcine species), even if specifically vaccinated against ε toxin, then an animal infected with CPB2-positive type D isolates could still suffer from GI disease due to CPB2.

As a means to start exploring the importance of accessory toxins produced by non-type A isolates for lethality, supernatants from non-type A disease isolates were used to challenge mice via the i.v. route. The mouse i.v. injection model was chosen because 1) it is the currently accepted model for assessing vaccine efficacy, 2) it allows for the testing of many isolates, 3) it provides a straight forward model for studying the effects of multiple toxins during the toxaemic phase of disease, and 4) there are no well-characterized small animal oral challenge models

available (48, 121). It also is important to note that while the actions of many *C. perfringens* toxins have been studied individually (107, 131), the lethality effects of different combinations of toxins that would occur together in a natural infection have not been studied.

By, 1) correlating lethality with various toxin concentrations present in vegetative supernatants and 2) performing toxin neutralization studies with type B, C, and D isolate supernatants, we determined that the plasmid-encoded typing toxins (ε and β toxins) are the major contributors to lethality in the mouse i.v. injection model ((48, 121) and this study). These results suggest that when vaccine production companies assess the efficacy of their vaccines using an i.v. injection model, they are looking at the ability of the vaccine to protect against either the ε and/or β toxins. However, the mouse i.v. model has an obvious flaw, in that it does not address the role of the major lethal toxins or the potential accessory toxins in a natural disease model where toxins would first interact with the intestinal epithelium before being absorbed into the circulation. So while the ε and β toxins are probably primarily responsible for the lethality of non-type A infections during the toxaemic phase of disease, other factors, including CPB2, may be required for, and/or contribute to, the overall lethality of the isolate by facilitating the absorption of the ε or β toxins. This idea is supported by reports that purified CPB administered orally or injected into ileal loops cannot reproduce the pathogenesis of a natural type C infection, which requires bacteria and supernatants (134, 135). To address whether accessory toxins can contribute to a more natural model of infection, toxin knockout mutants need to be generated in “simple” toxin backgrounds (containing just the typing toxins) and also in complex toxin backgrounds (isolates carrying their typing toxins plus CPB2 and/or CPE). The data generated from our type B-D mouse lethality studies have already facilitated the selection of a number of candidates in which to generate toxin knockout mutants.

5.4 SUMMATION

Overall, the results reported in this dissertation support our main hypothesis that the production of an accessory toxin by plasmid *cpe*-positive *C. perfringens* isolates causing human AAD/SD could explain the more severe and longer lasting clinical symptoms compared to human FP caused by chromosomal *cpe*-positive *C. perfringens* isolates. Namely, we have determined that

most AAD/SD isolates in contrast to FP isolates carry the *cpb2* gene and produce CPB2, which is present in both vegetative and sporulating culture supernatants. Purified CPB2 was shown to be cytotoxic for Caco-2 cells, using a mechanism of action consistent with pore-formation. While animal experiments indicate that CPB2 is not primarly responsible for the lethality during the toxaemic phase of non-type A infections, our *in vitro* data indicates that CPB2 has the potential to damage the intestinal epithelium of humans (and, by extension, animals).

In patients suffering from AAD/SD, the production of two intestinally-active toxins (CPB2 and CPE) during sporulation should result in increased severity of disease. Secondly, since CPB2 also is produced by vegetative cultures, there is potential for GI disease in the absence of sporulation. While CPB2 is quite labile, changes in the intestinal conditions due to diet changes and/or antibiotic treatment (which are probably common in hospitalized patients) could result in an environment that is conducive for CPB2 activity, i.e. an environment that allows *cpb2*-positive *C. perfringens* isolates to colonize the intestinal tract (due to antibiotic therapy) and be in close association with the epithelium (minimizing the exposure of CPB2 to the lumen) and an environment that is low in trypsin (due to a change in diet). A possible model for CPB2-associated disease in humans is shown in Figure 5.2. Using a similar line of reasoning, the lack of normal, adult trypsin production levels and/or GI flora in newborn animals could explain, in part, why *cpb2*-positive type A and *cpb2*-positive type C isolates are strongly associated with GI disease in piglets. Furthermore, the production of a toxin (CPB2) that damages the intestinal epithelium by non-type A isolates could facilitate increased absorption of ε toxin or β toxin resulting in a more lethal infection.

While epidemiological studies and *in vitro* studies have made a reasonable case for CPB2 playing a role in pathogenesis, future work should initially focus on defining an animal model for studying CPB2 to definitively determine whether it can contribute to, or cause, GI disease *in vivo*. The development of a suitable animal model and *cpb2*-knockout constructs would facilitate studies regarding the effects of CPB2 *in vivo* and, possibly more importantly, justify continued research on *cpb2* genetics and epidemiology, the CPB2 mechanism of action, and CPB2 vaccine development. It is appreciated that suitable animal models do not always exist (for instance, Gonorrheal research is limited by only having a human disease model); however, the widespread distribution of species in which the *cpb2* gene has been identified suggest that this should not be a major problem. Hopefully, this dissertation will facilitate research in the future to address the remaining issues necessary to settle the current debate in our field over the importance of CPB2 in pathogenesis.

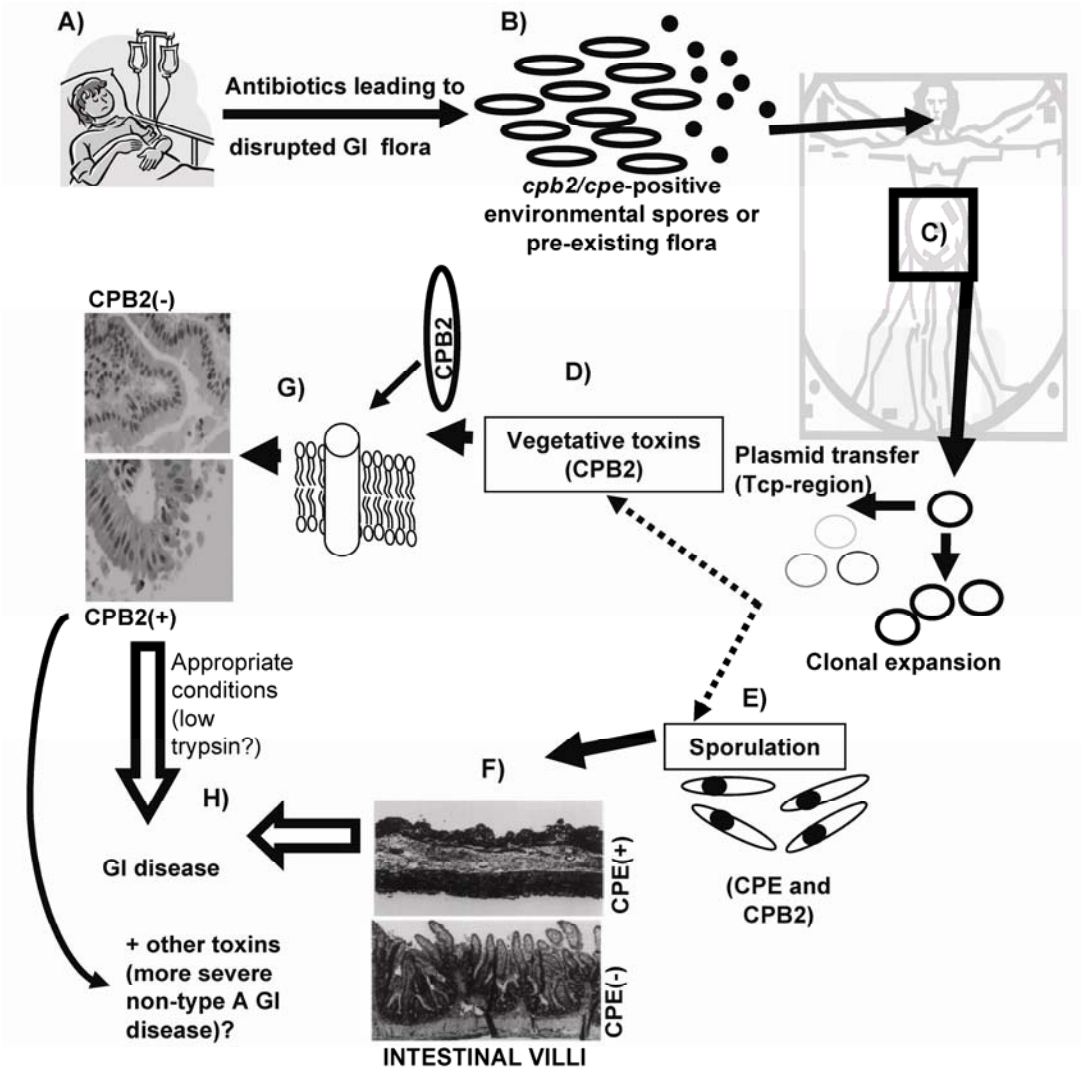

Figure 5.2 Model for CPB2-associated GI disease. A patient's normal flora is disrupted (A) and the patient is colonized by *cpe*/*cpb2*-positive *C. perfringens* (B). The bacteria pass into the intestine (C) where they can either 1) undergo clonal expansion or 2) transfer the *cpe*/cpb2-plasmid to normal *C. perfringens* flora which also would undergo clonal expansion. Under vegetative conditions CPB2 should be produced by these isolates (D), while under sporulating conditions CPE (E) and CPB2 would be present in the lumen of the intestine. The presence of CPE would cause damage to the intestinal epithelium (F, modified from (119)), and under appropriate conditions, CPB2 also could damage the intestinal epithelium alone (G) or in

combination with CPE (H). By extension, the production of CPB2 in combination with non-type A toxins also could cause more severe GI disease in domestic animals.

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7.0 APPENDIX

PRIMARY LITERATURE:

1) FISHER, D. J., K. MIYAMOTO, B. HARRISON, S. AKIMOTO, M. R. SARKER, AND B. A. MCCLANE**.** 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.

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Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene

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Summary

Clostridium perfringens **type A isolates carrying an enterotoxin (*cpe*) gene are an important cause of human gastrointestinal diseases, including food poisoning, antibiotic-associated diarrhoea (AAD) and sporadic diarrhoea (SD). Using polymerase chain reaction (PCR), the current study determined that the *cpb2* gene encoding the recently discovered beta2 toxin is present in <15% of food poisoning isolates, which typically carry a chromosomal *cpe* gene. However, >75% of AAD/SD isolates, which usually carry a plasmid *cpe* gene, tested *cpb2*$^+$ by PCR. Western blot analysis demonstrated that >97% of those *cpb2*$^+$/*cpe*$^+$ AAD/SD isolates can produce CPB2. Additional PCR analyses, sequencing studies and pulsed field gel electrophoresis experiments determined that AAD/SD isolates carry *cpb2* and *cpe* on the same plasmid when IS*1151* sequences are present downstream of *cpe*, but *cpb2* and *cpe* are located on different plasmids in AAD/SD isolates where IS*1470*-like sequences are present downstream of *cpe*. Those analyses also demonstrated that two different CPB2 variants (named CPB2h1 or CPB2h2) can be produced by AAD/SD isolates, dependent on whether IS*1470*-like or IS*1151* sequences are present downstream of their *cpe* gene. CPB2h1 is ~10-fold more cytotoxic for CaCo-2 cells than is CPB2h2. Collectively, these results suggest that CPB2 could be an accessory toxin in *C. perfringens* enterotoxin (CPE)-associated AAD/SD.**

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Introduction

The Gram-positive, spore-forming, anaerobe *Clostridium perfringens* is an important human and veterinary pathogen. This bacterium can produce at least 15 different toxins, helping to explain its ability to cause a broad array of enteric and histotoxic infections (Rood, 1998; Petit *et al*., 1999; Smedley III *et al*., 2004). However, individual *C. perfringens* isolates express only some of these 15 toxins, providing the basis for a classification scheme that assigns *C. perfringens* isolates to one of five toxinotypes (A–E), based on their production of four typing toxins, i.e. alpha, beta, epsilon and iota toxins. Type A isolates are the predominant toxinotype and produce alpha toxin, but not beta, epsilon or iota toxins.

About 1–5% of global *C. perfringens* isolates, mostly belonging to type A, also produce CPE, the *C. perfringens* enterotoxin (McClane, 1996). Those enterotoxigenic type A isolates are associated with several important human gastrointestinal (GI) diseases, including *C. perfringens* type A food poisoning, sporadic diarrhoea (SD) and antibiotic-associated diarrhoea (AAD). While the enterotoxin gene (*cpe*) can have either a chromosomal or a plasmid location, food poisoning isolates typically carry their *cpe* gene on the chromosome (Cornillot *et al*., 1995), where it is associated with IS*1470* elements (Brynestad *et al*., 1997). In contrast, SD and AAD isolates usually have a plasmid-borne *cpe* gene (Collie and McClane, 1998) that is closely associated with either downstream IS*1151* or defective IS*1470*-like sequences (Miyamoto *et al*., 2002; 2004).

Patients with CPE-associated AAD or SD often present with more severe and longer-lasting GI symptoms than patients suffering from *C. perfringens* type A food poisoning (Carman, 1997). Currently, these symptomatic differences between different CPE-associated GI diseases are not well understood.

Gibert *et al*. (1997) recently identified a new *C. perfringens* toxin, named beta2 toxin (CPB2), which is encoded by the *cpb2* gene. Epidemiological surveys have

implicated CPB2 in several animal GI diseases, including equine typhlocolitis, diarrhoeic disease in piglets and dogs, and bovine enterotoxaemia (Gibert *et al*., 1997; Garmory *et al*., 2000; Manteca *et al*., 2002; Bueschel *et al*., 2003; Engstrom *et al*., 2003). Immunohistochemical staining has also demonstrated the presence of CPB2 at necrotic intestinal lesions in horses and in an elephant suffering from natural GI disease (Bacciarini *et al*., 2001; 2003). Although CPB2's molecular mechanism of action is poorly understood, purified CPB2 toxin can cause haemorrhagic lesions in a guinea pig ligated ileal loop model (Jolivet-Renaud *et al*., 1986).

To date, CPB2 has not been associated with any human GI disease. However, the linkage of CPB2 with veterinary GI disorders, coupled with the incomplete understanding of the more severe symptoms of CPE-associated human AAD/SD versus *C. perfringens* type A food poisoning, led us to investigate whether *cpe*-positive type A human GI disease isolates might produce CPB2 as an accessory enterotoxin.

Results

Polymerase chain reaction (PCR) detection of the cpb2 *gene in* cpe*-positive type A human GI disease isolates*

Two polymerase chain reaction (PCR) assays were used to evaluate whether the *cpb2* gene is present in a collection of *cpe*-positive type A human GI disease isolates that had previously been genotyped for the location (plasmid or chromosomal) of their *cpe* gene (Collie and McClane, 1998; Sparks *et al*., 2001; Wen *et al*., 2003). Two different PCR assays (Garmory *et al*., 2000; Waters *et al*., 2003), which amplify (respectively) 567 or 318 bp products from the *cpb2* gene of veterinary isolate CWC245, were used to minimize possible false-negative results from improper primer annealing that might occur as a consequence of *cpb2* sequence variations identified in prior studies of *cpb2*$^+$ isolates of non-human origin (Gibert *et al*., 1997; Shimizu *et al*., 2002).

These PCR surveys revealed that *cpb2* sequences are present in 79% of 48 tested *C. perfringens* type A human SD/AAD isolates carrying a plasmid *cpe* gene (Table 1 summarizes the PCR results for all tested isolates). Interestingly, *cpb2* sequences were detected in 100% of the 16 examined AAD or SD isolates known to carry an IS*1151* sequence downstream of their plasmid *cpe* gene; *cpb2* sequences were also present in 68% of the 32 examined AAD or SD isolates carrying an IS*1470*-like sequence downstream from their plasmid *cpe* gene.

In contrast, similar PCR analyses of type A chromosomal *cpe* isolates only identified *cpb2* sequences in isolates originating from 2 of 13 (~15%) different *C. perfringens* type A food poisoning outbreaks. Previous studies from our laboratory have suggested that chromosomal *cpe*-positive isolates obtained from a single food poisoning outbreak are often clonal (Sparks *et al*., 2001), which is consistent with our current *cpb2* PCR results, i.e. all six *cpe*-positive isolates obtained from a single 1980s Vermont food poisoning outbreak tested positive for *cpb2* sequences, while all five isolates obtained from a 2001 Oklahoma food poisoning outbreak tested negative for *cpb2* sequences.

For every surveyed *cpe*$^+$ type A human GI disease isolate, both internal *cpb2* primer pairs gave identical PCR conclusions about the presence or absence of the *cpb2* gene. Furthermore, both *cpb2* PCR assays amplified (Table 1) the expected *cpb2* product from Strain 13 (data not shown), a known *cpb2*$^+$/*cpe*$^-$ type A isolate (Shimizu *et al*., 2002), but not from ATCC 3624, a *cpb2*$^-$/*cpe*$^-$ type A isolate.

Analysis of CPB2 production by vegetative and sporulating cultures of cpe$^+$/cpb2$^+$ *human GI disease isolates*

As previous reports had indicated that the *cpb2* gene is silent in some *cpb2*$^+$/*cpe*$^-$ *C. perfringens* veterinary isolates (Bueschel *et al*., 2003), CPB2 Western blotting was performed to evaluate whether (i) the *cpe*$^+$/*cpb2*$^+$ type A human GI disease isolates identified in Table 1 PCR surveys actually produce CPB2 and (ii) whether that toxin production, if any, is by vegetative or sporulating cultures. Those Western blot analyses detected no immunoreactive band in the vegetative culture supernatant from the *cpb2*-negative control isolate ATCC 3624 (Fig. 1A). However, they did reveal the presence of a single immunoreactive band matching the 28 kDa molecular weight of mature CPB2 in vegetative culture supernatants (Fig. 1A) from 97% of our *cpe*$^+$/*cpb2*$^+$ AAD or SD isolates (Table 1). One *cpb2*$^+$ isolate carrying a chromosomal *cpe* gene, 191-10, was found to produce CPB2 during vegetative growth (Fig. 1A). In addition, Fig. 1A results identify substantial variations in CPB2 amounts produced during vegetative growth of different *cpe*$^+$/*cpb2*$^+$ type A human GI disease isolates. Quantitative CPB2 Western blot analysis revealed that the range of vegetative culture CPB2 production by these isolates is 50–500 ng of CPB2 ml^{-1}. Finally, these Western blot analyses failed to detect CPB2 in supernatants from two *cpe*$^+$/*cpb2*$^+$ type A human GI disease isolates, i.e. C1841 (Fig. 1A) and F4129 (not shown), or in supernatants from the *cpe*$^-$/*cpb2*$^+$ type A Strain 13 (not shown).

As CPE is produced exclusively during sporulation (Czeczulin *et al*., 1996), CPB2 Western blot analyses were also performed to assess CPB2 production by sporulating cultures of two representative *cpe*$^+$/*cpb2*$^+$ human GI disease isolates. Those experiments demon-

Table 1. List of isolates and results.

Isolate group	Strain	Type	*cpe* location	Date and source	Reference	*cpb2*	CPB2	IS element[a]
Gas gangrene	Strain 13	A	*cpe* negative		Shimizu *et al.* (2002)	+	–	–
	ATCC3624	A	*cpe* negative		Kokai-Kun *et al.* (1994)	–	–	–
	ATCC3624(B2h1)	A	*cpe* negative	ATCC 3624 with pB2(h1)v751	This study	+	+	–
	ATCC3624(B2h2)	A	*cpe* negative	ATCC 3624 with pB2(h2)v750	This study	+	+	–
	ATCC3624(pJIR751)	A	*cpe* negative	ATCC 3624 with pJIR751	This study	–	–	–
	ATCC3624(pJIR750)	A	*cpe* negative	ATCC 3624 with pJIR750	This study	–	–	–
Food poisoning isolates	NCTC8239	A	Chromosome	1950s, Europe	Collie and McClane (1998)	–	–	A
	NCTC8798	A	Chromosome	Clonal		–	–	A
	NCTC10239	A	Chromosome			–	–	A
	NCTC8235	A	Chromosome			–	–	A
	NCTC8238	A	Chromosome			–	–	A
	NCTC8359	A	Chromosome			–	–	A
	NCTC8679	A	Chromosome			–	–	A
	NCTC8799	A	Chromosome			–	–	A
	191-10	A	Chromosome	1980s, Hawaii	Sparks *et al.* (2001)	+	+	A
	FD1041	A	Chromosome	1980s, North America	Sparks *et al.* (2001)	–	–	A
	C-1489	A	Chromosome	1980s, Vermont	Sparks *et al.* (2001)	+	–	A
	C-1851	A	Chromosome	Clonal		+	–	A
	C-1869	A	Chromosome			+	–	A
	C-1881	A	Chromosome			+	–	A
	C-1887	A	Chromosome			+	–	A
	C-1841	A	Chromosome			+	–	A
	Ohio #23	A	Chromosome	1990s, North America	Sparks *et al.* (2001)	–	–	A
	513	A	Chromosome	Non-clonal		–	–	A
	527	A	Chromosome			–	–	A
	528	A	Chromosome			–	–	A
	537-5	A	Chromosome			–	–	A
	538-1	A	Chromosome			–	–	A
	E13	A	Chromosome			–	–	A
	R42	A	Chromosome			–	–	A
	01E803YR	A	Chromosome	1999, Oklahoma	[b]	–	–	A
	01E809MM	A	Chromosome	Clonal		–	–	A
	01E809MH	A	Chromosome			–	–	A
	01E802MA	A	Chromosome			–	–	A
	01E810MH	A	Chromosome			–	–	A
Sporadic diarrhoea	F4969	A	Plasmid	1990s, Europe	Collie and McClane (1998)	–	–	C
	F4406	A	Plasmid			+	+	B
	F5603	A	Plasmid			+	+	B
	F4591	A	Plasmid			–	–	C
	F4013	A	Plasmid			+	+	B
	F5537	A	Plasmid			+	+	C
	F4393	A	Plasmid			+	+	B
	F4396	A	Plasmid			+	+	C
	F4859	A	Plasmid			+	+	C
	F4129	A	Plasmid			+	–	C
Antibiotic-associated diarrhoea	B11	A	Plasmid	1980s, Europe	Collie and McClane (1998)	+	+	B
	B38	A	Plasmid			+	+	B
	B40	A	Plasmid			+	+	B
	B41	A	Plasmid			+	+	B
	B2	A	Plasmid			+	+	B
	B45	A	Plasmid			+	+	B
	NB16	A	Plasmid			+	+	B
	F36081	A	Plasmid	Late 1990s, North America	Sparks *et al.* (2001)	+	+	B
	F38660	A	Plasmid			–	–	C
	H38094	A	Plasmid			+	+	B
	M18069	A	Plasmid			–	–	C
	M19874	A	Plasmid			–	–	C
	M22792	A	Plasmid			–	–	C
	M24326	A	Plasmid			–	–	C
	M26413	A	Plasmid			–	–	C
	M34401	A	Plasmid			–	–	C
	M35584	A	Plasmid			+	+	C
	M39558	A	Plasmid			+	+	C
	S43526	A	Plasmid			+	+	C
	T29447	A	Plasmid			–	–	C
	T34058	A	Plasmid			+	+	C
	T44123	A	Plasmid			–	–	B

Table 1. *cont.*

Isolate group	Strain	Type	*cpe* location	Date and source	Reference	*cpb2*	CPB2	IS element[a]
	T57603	A	Plasmid			+	+	B
	W24820	A	Plasmid			+	+	C
	W30554	A	Plasmid			+	+	C
	W43181	A	Plasmid			+	+	C
	F16865	A	Plasmid			+	+	C
	S10653	A	Plasmid			+	+	B
	S10748	A	Plasmid			+	+	C
	T285546	A	Plasmid			+	+	C
	T32214	A	Plasmid			+	+	C
	T39814	A	Plasmid			+	+	C
	W2624	A	Plasmid			+	+	C
	W30554	A	Plasmid			+	+	C
	W32500	A	Plasmid			+	+	C
	X5722	A	Plasmid			+	+	C
Normal human	MR2-4	A	Plasmid	1990s, Japan	Miyamoto *et al.* (2002)	+	+	C

a. IS elements downstream of the *cpe* gene = A (IS*1470*), B (IS*1151*), C (IS*1470*-like) (Miyamoto *et al.*, 2004)
b. Isolates kindly provided by John E. Bos, Oklahoma State Department of Health, Communicable Disease Division.

strated that sporulating cultures of both representative isolates produce a 28 kDa protein that is immunoreactive with CPB2 antibodies (Fig. 1B). CPE production in those two sporulating cultures was also verified by CPE Western blot analysis (Fig. 1C), thus demonstrating that a sporulating culture of a *cpb2*$^+$/*cpe*$^+$ isolate can simultaneously contain both CPB2 and CPE.

Nucleotide sequence analysis of the cpb2 *open reading frame in* cpe$^+$/cpb2$^+$ *human GI disease isolates*

Previous studies identified nucleotide variations, which result in both silent and missense mutations, between the *cpb2* open reading frames (ORFs) present in two *cpe*-negative isolates, i.e. Strain 13 (type A) (Shimizu *et al.*, 2002) versus CWC245 (type C) (Gibert *et al.*, 1997). Therefore, the current study sequenced the *cpb2* ORF of seven *cpe*$^+$/*cpb2*$^+$ type A human GI disease isolates, which revealed significant nucleotide differences between the *cpb2* ORF of those isolates and the previously published *cpb2* ORF sequence of Strain 13 or CWC245 (data not shown). Translation of the *cpb2* ORF sequences obtained for seven *cpe*$^+$/*cpb2*$^+$ type A human GI disease isolates demonstrated that considerable amino acid variations exist throughout the CPB2 protein encoded by *cpe*$^+$/*cpb2*$^+$ human GI disease isolates (Fig. 2, rows 3 and 4) versus veterinary disease isolates (Fig. 2, rows 1 and 2).

With respect to the *cpe*$^+$/*cpb2*$^+$ non-foodborne human GI disease isolates themselves, CPB2 amino acid sequence variations clustered into two groups, which were dependent on whether IS*1470*-like sequences (Fig. 2, row 3) or IS*1151* (Fig. 2, row 4) are present downstream of the isolate's plasmid *cpe* gene. To distinguish between these two IS-associated CPB2 variants, we propose use of the designations CPB2h1 (for the CPB2 variant made by isolates with IS*1151* sequences downstream

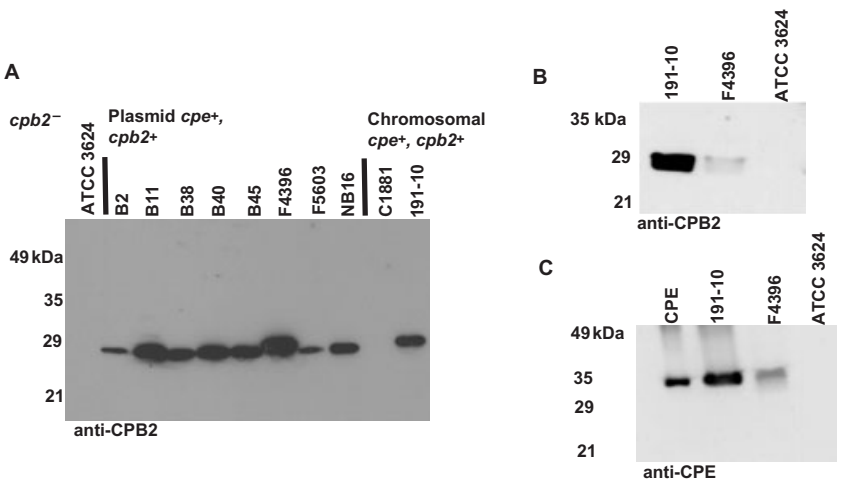

Fig. 1. CPB2/CPE Western blot analysis of vegetative and sporulating culture supernatants from representative *cpb2*$^+$/*cpe*$^+$ *C. perfringens* human GI disease isolates. Results shown in (A) are for concentrated vegetative *C. perfringens* culture supernatants from *cpb2*$^+$/*cpe*$^+$ human GI disease isolates immunoblotted with CPB2 antibodies. (B) and (C) show, respectively, CPB2 or CPE Western blot analysis of sporulating culture supernatants from *cpb2*$^+$/*cpe*$^+$ human GI disease isolates 191-10 and F4396. ATCC 3624 (a *cpb2*-negative isolate) served as a negative control for CPB2 and CPE detection. Purified CPE is included in (C) as a positive control.

Fig. 2. Translated CPB2 amino acid sequence alignment. Translated CPB2 sequences shown are from representative isolates carrying an IS*1470*-like element (row 3) or isolates carrying an IS*1151* element (row 4) near their *cpe* plasmid gene. Lanes 1 and 2 show previously reported CPB2 sequences from veterinary disease isolates (Gibert *et al*., 1997; Shimizu *et al*., 2002). The secretion signal present in the immature protoxin is marked with a black line.

of their plasmid *cpe* gene) and CPB2h2 (for the CPB2 variant made by isolates with IS*1470*-like isolates downstream of their plasmid *cpe* gene).

Consistent with Fig. 1A Western blot results, Fig. 2 indicates that both human CPB2 variants typically maintain an overall length of 265 amino acids, similar to the veterinary CPB2 variant (Gibert *et al*., 1997). With respect to amino acids substitutions in mature CPB2h1 versus CPB2h2, these human CPB2 variants exhibit 64% identity and 81% similarity; CPB2h2 also has 91% identity and 97% similarity to veterinary CPB2, while CPB2h1 shares only 63% identity and 80% similarity with veterinary CPB2.

Sequence analysis of the *cpb2* ORF from the two *cpe*$^+$ (chromosomal)/*cpb2*$^+$ food poisoning isolates demonstrated these isolates carry the CPB2h1 variant. In addition, the food poisoning isolate, C1841, which tested positive for *cpb2* by PCR but produced no detectable CPB2 by Western blot analysis, was found to carry a complete *cpb2* ORF. Therefore, premature stop codons do not account for the lack of CPB2 expression by C1841 (data not shown). F4129, the single *cpe*$^+$/*cpb2*$^+$ type A non-foodborne human GI disease isolate that failed to produce CPB2, was also found to encode a complete *cpb2* ORF (data not shown). Interestingly, sequencing of the *cpb2* ORF present in our laboratory's culture of Strain 13 (which fails to produce CPB2) detected a missing adenosine residue 10 nucleotides downstream from the first nucleotide in the start codon (data not shown). This nucleotide deletion results in the formation of a premature stop codon (after nine amino acids) that could explain the lack of CPB2 toxin production by this isolate.

Evaluating whether the cpb2 *gene is plasmid-borne in* cpe$^+$/cpb2$^+$ *AAD or SD isolates*

Previous studies reported that the *cpb2* gene in animal isolates can be located on plasmids varying in size from 54 kb to ~100 kb (Gibert *et al*., 1997; Shimizu *et al*., 2002; Waters *et al*., 2003). To determine the location (plasmid versus chromosomal) of the *cpb2* gene in our newly identified *cpe*$^+$/*cpb2*$^+$ type A human GI disease isolates, pulsed-field gel electrophoresis (PFGE) was performed under conditions allowing plasmid DNA, but not chromosomal DNA, to enter the pulsed-field gels. Those pulsed-field gels were then Southern blotted with DIG-labelled probes specific for *cpe* or *cpb2* sequences.

These PFGE experiments localized the *cpb2* gene to an ~75 kb plasmid in all tested *cpb2*-positive AAD or SD isolates carrying an IS*1151* sequence downstream of their plasmid *cpe* gene (Fig. 3A, lanes 3–5). This ~75 kb *cpb2*-carrying plasmid exactly matches the size of the *cpe* plasmid in those isolates (Fig. 3B), as confirmed by overlaying autoradiography films from blots hybridized with *cpe* or *cpb2* probes (Fig. 3C). In this PFGE experiment, the *cpe*$^-$/*cpb2*$^-$ type A isolate ATCC 3624 served as a negative control for hybridization of the *cpe* and *cpb2* genes probes (Fig. 3D).

When *cpb2/cpe*-positive AAD or SD isolates carrying an IS*1470*-like sequence downstream from their plasmid *cpe* gene were similarly analysed by PFGE and Southern blotting, the *cpb2* gene localized to a plasmid of ~50 kb, whereas the *cpe* gene was apparently present on a second plasmid of ~75 kb (Fig. 3A and B, lanes 1, 6 and 7). A ~50 kb *cpb2* plasmid was also detected in the two *cpb2*-positive food poisoning isolates carrying a chromosomal *cpe* gene (lane 2 and data not shown).

PCR evaluation of the relationship between plasmid-borne cpb2 *and* cpe *genes in AAD or SD isolates*

The Fig. 3 PFGE Southern blot results suggested that the *cpb2* and *cpe* genes reside on the same plasmid in AAD/SD isolates carrying IS*1151* sequences downstream of

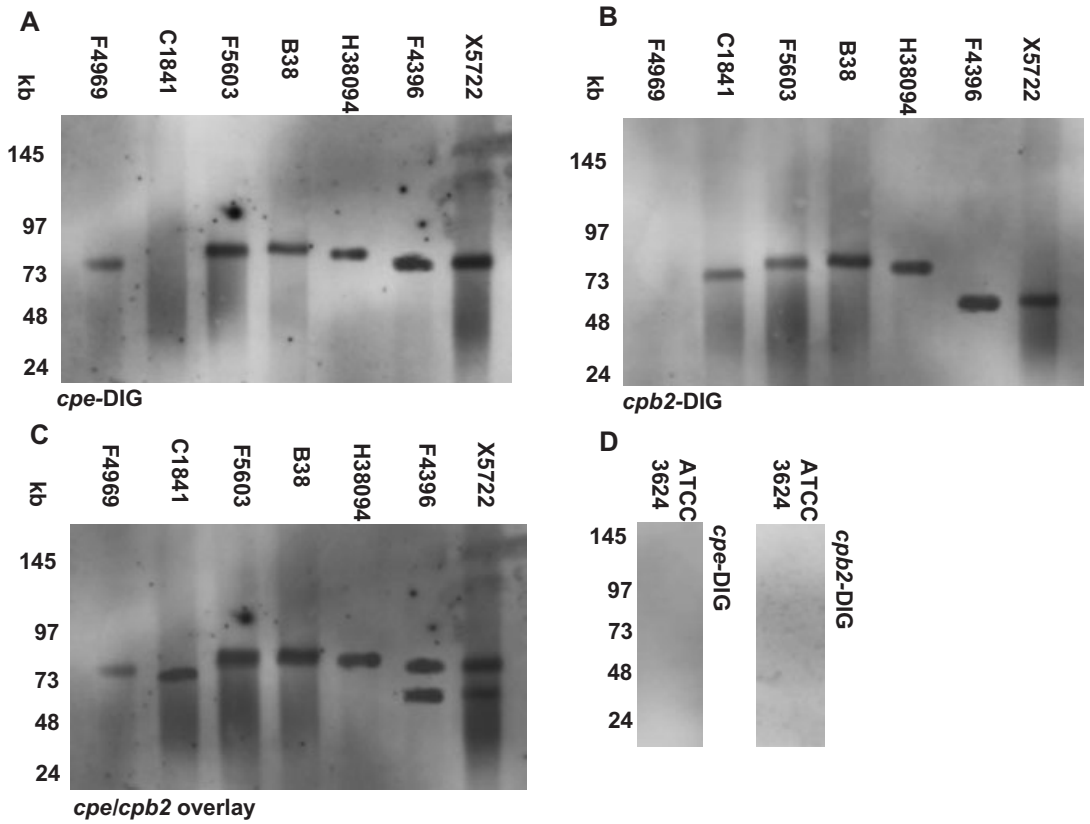

Fig. 3. PFGE/Southern blot analyses of the presence of *cpb2* and *cpe* genes in representative AAD/SD and food poisoning isolates.
A and B. PFGE conditions used for these blots allowed only plasmid DNA to enter into the gel matrix. Those PFGE gels were then subjected to Southern blot analysis with either *cpe* (A) or *cpb2* (B) DIG-labelled probes.
C. An overlay of the blots from (A) and (B), based on aligning the wells.
D. ATCC 3624, a *cpb2*/*cpe*-negative isolate, served as a negative control for the *cpe* and *cpb2* Southern blots. Molecular weight markers are shown on the left of each blot.

their plasmid *cpe* gene, but lie on different plasmids in AAD/SD isolates carrying an IS*1470*-like sequence downstream of their plasmid *cpe* gene. To conclusively determine whether the *cpb2* and *cpe* genes are present on the same plasmid in AAD or SD isolates carrying *cpe*-IS*1151* sequences, the current study partially sequenced pF5603, the *cpe* plasmid of AAD isolate F5603. This sequencing approach successfully linked the two toxin genes to pF5603 by demonstrating that the *cpb2* and *cpe* genes reside 19 175 nucleotides apart on this plasmid. Twenty ORFs, including *cpe* and *cpb2*, are present in this pF5603 region (Fig. 4A); these ORFs average 733 nucleotides in length, with an average coding percentage GC content of 25.2% (similar to the overall 28.6% GC content of *C. perfringens* DNA) (Shimizu *et al*., 2002).

The partial sequence information obtained for pF5603 was used to design a battery of 16 overlapping PCR reactions spanning the entire pF5603 region between *cpe* and *cpb2* (Fig. 4B). This PCR battery was then performed on representative AAD and SD isolates carrying IS*1151* sequences downstream of their plasmid *cpe* gene in order to confirm the Fig. 4 PFGE results suggesting that, like F5603, those isolates also carry their *cpb2* and *cpe* genes on the same plasmid. With this PCR battery assay, all but two tested *cpe*-IS*1151* isolates gave similar amplification patterns as F5603 (Fig. 4C). DNA from F4406 and F4013 failed to amplify products from PCR reactions 2–10 (Table S1 in *Supplementary material*), suggesting a DNA deletion may have occurred in the *cpb2-cpe* region of those two isolates. To confirm that the failure of PCR reactions 2–10 to amplify products from F4406 and F4013 was not simply a result of minor sequence variations affecting primer annealing, a DIG-labelled DNA probe was made from PCR product 4 after amplification from isolate F5603. This probe failed to hybridize with *Xba*I-digested DNA from either F4406 or F4013, but did hybridize strongly with the expected 5.9 kb *Xba*I fragment of F5603 DNA (data not shown).

The same battery of *cpb2-cpe* PCR reactions was also performed (Fig. 4; Table S1 in *Supplementary material*) on AAD or SD isolates carrying IS*1470*-like sequences downstream of their *cpe* gene in order to (i) further confirm

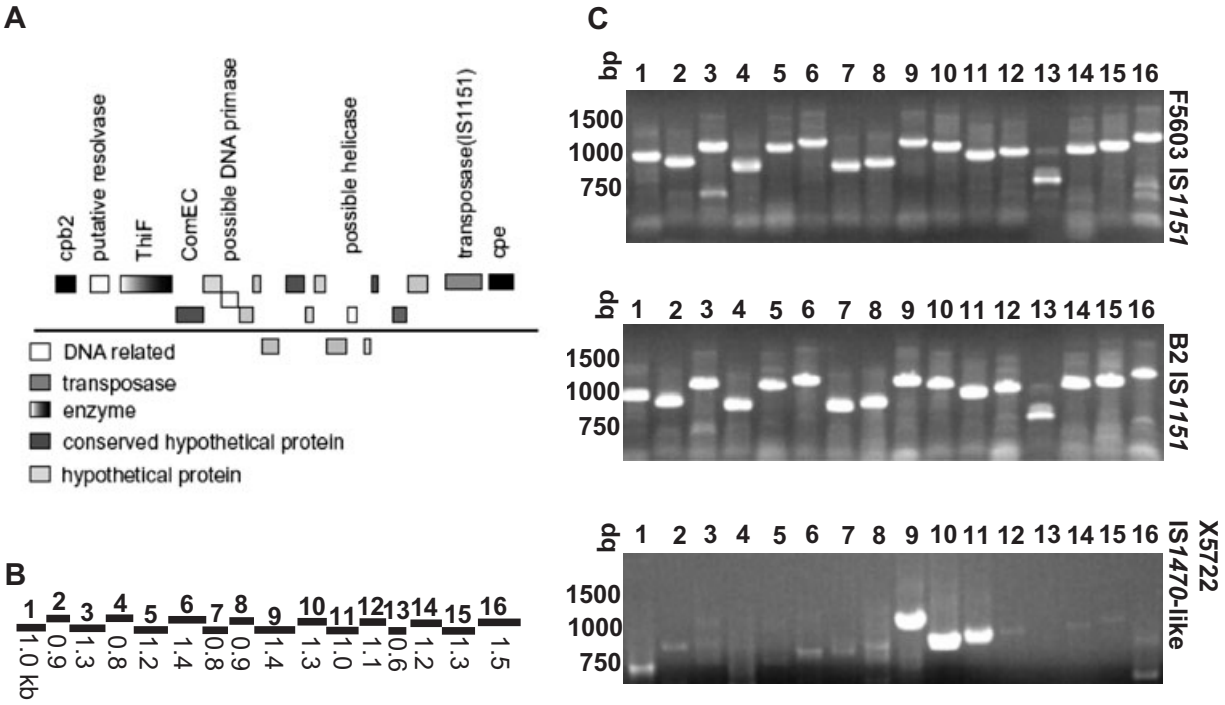

Fig. 4. Sequencing and PCR analyses of the *cpb2-cpe* region in AAD/SD isolates. Sequencing the *cpb2-cpe* region of the *cpe*$^+$/*cpb2*$^+$ plasmid (pF5603) in AAD isolate F5603 revealed multiple ORFs and their possible functions (colour-coded), as shown in (A). This pF5603 *cpb2-cpe* sequence was used to construct a battery of 16 overlapping PCR reactions (B) extending from the *cpb2* gene to the *cpe* gene (the size of each product is indicated by numbers under each bar), which was used to assess the diversity of the *cpb2-cpe* region in *cpb2/cpe*-positive human GI disease isolates. Representative results for this PCR assay are shown in (C) for two AAD/SD isolates carrying an IS*1151* sequence downstream from their *cpe* gene and for one AAD/SD isolate with an IS*1470*-like element downstream from its *cpe* gene. DNA marker sizes are shown to the left of the gels. The numbers (1–16) shown at the top of the gels indicates the PCR reactions. A summary of the PCR results from other *cpb2/cpe*-positive isolates is shown in Table S2 in *Supplementary material*.

PFGE results indicating that *cpb2* and *cpe* are not located on the same plasmid in these isolates and (ii) assess the similarity in sequences located downstream of the *cpe* gene in plasmids carrying *cpe*-IS*1151* sequences versus plasmids carrying *cpe*-IS*1470*-like sequences. Using DNA from representative isolates with *cpe*-IS*1470*-like sequences, this PCR battery only consistently amplified products from reactions 9–11; however, those PCR products differed in size from the reaction 9–11 products amplified from *cpe*-IS*1151* isolates. Collectively, these PCR results are consistent with Fig. 3 PFGE results indicating that *cpb2* and *cpe* are located on different plasmids in *cpe*-IS*1470*-like isolates.

Assessment of the CaCo-2 cell cytotoxicity of CPB2-containing culture supernatants from human AAD or SD isolates

The strong association noted in Table 1 between CPB2 toxin production and *cpe*$^+$/*cpb2*$^+$ type A non-foodborne human GI disease isolates suggests that CPB2 might be an accessory toxin contributing to the pathogenesis of CPE-associated human AAD/SD. If so, CPB2 should be active on human enterocytes or enterocyte-like cell culture lines, such as the human colon carcinoma CaCo-2 cell line. CaCo-2 cells are often used to study the effects of enteropathogens because they form polarized monolayers with tight junctions and brush border membranes.

To explore this hypothesis, initial experiments compared the consequences of treating CaCo-2 cells with concentrated culture supernatants from CPB2$^+$/CPE$^+$ versus CPB2$^-$/CPE$^+$ AAD/SD isolates. These experiments used supernatants harvested from cultures grown under either sporulating (to allow CPE production) or vegetative (no CPE production) conditions. The presence of CPB2 and/or CPE (as appropriate) in these supernatants was then confirmed by Western blotting before cytotoxicity testing. Those analyses detected ~2 μg ml^{-1} CPB2 and no CPE in the final concentrated vegetative supernatants. Non-concentrated sporulating supernatants contained ~1 μg ml^{-1} CPE and 0.25 to <0.05 μg ml^{-1} CPB2 (data not shown).

When CaCo-2 cells were treated for 5 h with concentrated vegetative culture supernatants prepared from several different CPB2$^+$/CPE$^+$ isolates, development of cytotoxicity was consistently observed, including nuclei

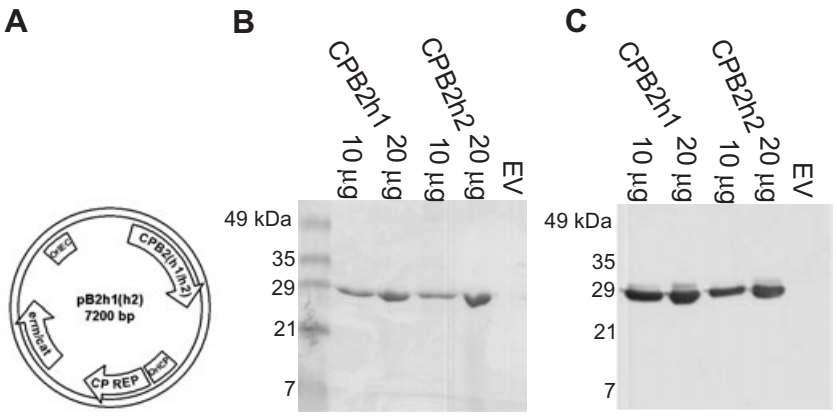

Fig. 5. Purification of CPB2 from recombinant *C. perfringens* ATCC 3624(B2h1) and ATCC 3624(B2h2) transformants. Vectors pB2h1 and pB2h2 were constructed to carry the entire CPB2 ORF, including both the promoter and terminator regions (A). These plasmids were then electroporated into ATCC 3624 to create either ATCC 3624(B2h1) or ATCC 3624(B2h2) to allow for overproduction of CPB2 to assist purification. Coomassie brilliant blue staining and Western blot analysis of the final purified CPB2 prepared from transformants are shown in (B) and (C) respectively. Molecular weight markers are shown to the left of each gel.

condensation and membrane bleb formation (Fig. S1 in *Supplementary material*). Cell detachment occurred if incubation at 37°C was continued beyond the initial appearance of cytopathic effects. In contrast, CaCo-2 cells treated with concentrated vegetative supernatants prepared from several CPB2$^-$/CPE$^+$ isolates (Fig. S1 in *Supplementary material*) or the CPB2$^-$/CPE$^-$ isolate ATCC 3624 (data not shown) exhibited no detectable cell damage.

Sporulating culture supernatants from several CPB2$^+$/CPE$^+$ or CPB2$^-$/CPE$^+$ human GI disease isolates were equally cytotoxic for CaCo-2 cells (Fig. S2 in *Supplementary material*). Although the morphologic damage observed was qualitatively similar, these alterations developed faster (starting at 30 min) using sporulating versus vegetative culture supernatants of CPB2$^+$/CPE$^+$ isolates. Sporulating culture lysates of CPB2$^-$/CPE$^-$ isolate ATCC 3624 failed to cause any cytotoxic effects (data not shown).

As vegetative culture supernatants of our CPB2$^+$/CPE$^+$ human GI disease isolates probably contain additional *C. perfringens* toxins (although not CPE; data not shown), it could be argued that other toxins besides CPB2 are actually responsible for the observed cytotoxic activity. To demonstrate more conclusively the involvement of CPB2 in the cytotoxic effects of CPB2$^+$/CPE$^+$ human GI disease isolates, *C. perfringens* transformants were constructed to overproduce either CPB2h1 or CPB2h2. These transformants, ATCC 3624(B2h1) or ATCC 3624(B2h2), were prepared (Fig. 5A) by cloning the entire *cpb2* gene (Ohtani *et al*., 2003) from either F5603 (which produces CPB2h1) or F4396 (which produces CPB2h2) onto pJIR751 or pJIR750, which are multicopy *C. perfringens–Escherichia coli* shuttle plasmids (Bannam and Rood, 1993) (Fig. 5A). When those recombinant plasmids were transformed into ATCC 3624 (a *cpb2*$^-$/*cpe*$^-$ type A isolate), Western blotting demonstrated that the resultant transformants produced high levels (~5 μg ml^{-1}) of CPB2h1 or CPB2h2 during vegetative growth (data not shown). Testing on CaCo-2 monolayers then confirmed that concentrated vegetative culture supernatants (containing ~50 μg ml^{-1} CPB2 determined by Western blotting) from both ATCC 3624(B2h1) and ATCC 3624(B2h2) killed CaCo-2 cells within 30 min treatment (Fig. 6C and D). In contrast, similarly concentrated supernatants from transformants ATCC 3624(pJIR751) and ATCC 3624(pJIR750), which carry the empty shuttle vectors, were not cytotoxic for CaCo-2 cells, even after a 15 h treatment (Fig. 6B and data not shown).

Cytotoxicity of purified CPB2 variants for CaCo-2 cells

To definitively evaluate whether the CPB2 toxin variants produced by CPB2$^+$/CPE$^+$ type A human GI disease variants are cytotoxic for CaCo-2 cells in the absence of other *C. perfringens* toxins, and to directly compare the relative cytotoxicity of CPB2h1 versus CPB2h2, each CPB2 human variant was purified from the overexpressing ATCC 3624(B2h1) or ATCC 3624(B2h2) transformant. The resultant preparations of purified CPB2h1 or CPBh2 were homogenous upon SDS-PAGE (Fig. 5B) and Western blotting confirmed the identity of these purified proteins as CPB2 (Fig. 5C).

When 10 μg ml^{-1} of purified toxin was added to confluent CaCo-2 monolayers grown in six-well dishes, cell damage developed within 1.5 h using either purified CPB2h1 or CPB2h2 (Fig. 6E and F). Morphological changes observed in CaCo-2 cells treated with pure CPB2h1 or CPB2h2 resembled those seen in CaCo-2 cells treated with concentrated vegetative culture supernatants from *cpb2*$^+$/*cpe*$^+$ isolates, which is consistent with CPB2 being important for the cell damage caused by those vegetative culture supernatants. A mock purification was also performed with our *C. perfringens* transformant carrying pJIR751 (empty vector). Similar to the supernatants from the ATCC 3624(pJIR751) and ATCC 3624(pJIR750) transformants, this mock preparation was not cytotoxic to CaCo-2 cells, even after a 5.5 h treatment (data not shown).

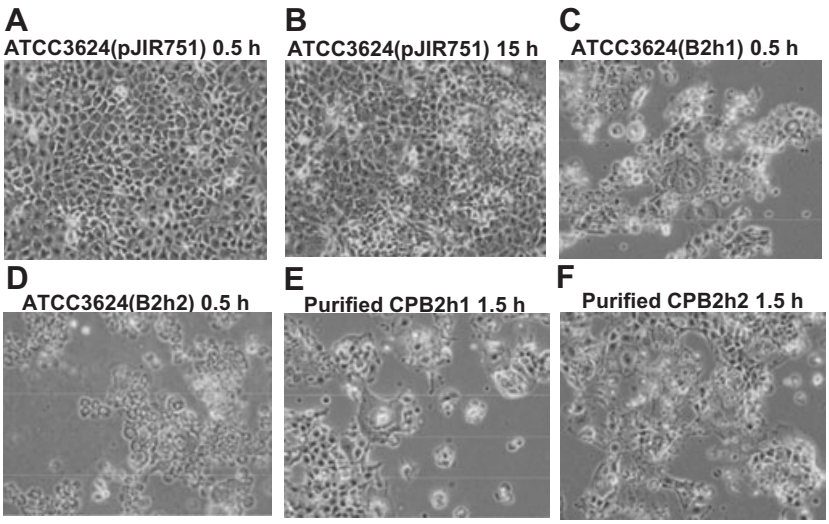

Fig. 6. CaCo-2 cell cytotoxicity of purified CPB2 or CPB2-containing concentrated culture supernatants from recombinant *C. perfringens* ATCC 3624(B2h1 or B2h2). Five-day-old, confluent CaCo-2 monolayers treated at 37°C for 30 min or 15 h with culture supernatants from ATCC 3624(pJIR751) (which carries the empty vector) are shown in (A) or (B) respectively. Concentrated vegetative culture supernatants from the CPB2-producing ATCC 3624(B2h1 or B2h2) transformants were added to CaCo-2 cells for 30 min (shown in C and D). CaCo-2 cells treated with 10 μg ml^{-1} purified CPB2h1 or CPB2h2 for 1.5 h are shown in (E) and (F). Pictures were taken at a final magnification of 210×.

To quantify the relative cytotoxicity of the purified CPB2h1 versus CPB2h2 variants, an assay was performed where CaCo-2 cells were treated with varying doses of each CPB2 variant for 2 h before determining cytotoxicity using a neutral red viability assay (Fig. 7). This assay determined that the amount of toxin required to reduce CaCo-2 cell viability by 50% (TCD50) is 0.3 μg ml^{-1} for CPB2h1, but is 4 μg ml^{-1} for CB2h2. These toxin concentrations fall within the 0.2–20 μg ml^{-1} concentration range previously determined (Gibert *et al.*, 1997) as being sufficient for pure CPB2 from veterinary isolates to cause death of I407 cells (which ATCC now considers as HeLa cells).

Discussion

Cases of CPE-associated AAD and SD typically involve *C. perfringens* type A isolates carrying a plasmid-borne *cpe* gene, while cases of *C. perfringens* type A food poisoning usually involve type A isolates carrying a chromosomal *cpe* gene (Cornillot *et al.*, 1995; Collie and McClane, 1998). Recent studies identified several factors contributing to these strong *cpe* genotype:GI disease relationships. The strong involvement of chromosomal *cpe* isolates in food poisoning involves, at least in part, those isolates being (i) generally more heat-resistant than plasmid *cpe* isolates, which facilitates their survival in improperly cooked or stored foods (Sarker *et al.*, 2000) and (ii) more abundant than plasmid *cpe* isolates in the food vehicles commonly causing *C. perfringens* type A food poisoning (Wen and McClane, 2004). With respect to the strong involvement of plasmid *cpe* isolates in CPE-associated non-foodborne human GI diseases, it is noteworthy that conjugative transfer of the *cpe* plasmid has been demonstrated *in vitro* (Brynestad *et al.*, 2001). This becomes relevant as it is believed that the inoculum of *cpe*-positive *C. perfringens* initiating CPE-associated human AAD or SD is considerably smaller than the infecting dose for *C. perfringens* type A food poisoning (Carman, 1997). Therefore, small numbers of plasmid *cpe* isolates might be able to establish human AAD/SD because they can conjugatively transfer their *cpe* plasmid to the *cpe*-negative *C. perfringens* isolates found in the normal human intestinal flora, which should convert those normal flora *C. perfringens* recipients to enteric virulence. As normal flora *C. perfringens* isolates are presumably well-adapted for persisting in the intestinal environment, this putative *in vivo* plasmid transfer could also help explain why CPE-associated non-foodborne human GI diseases

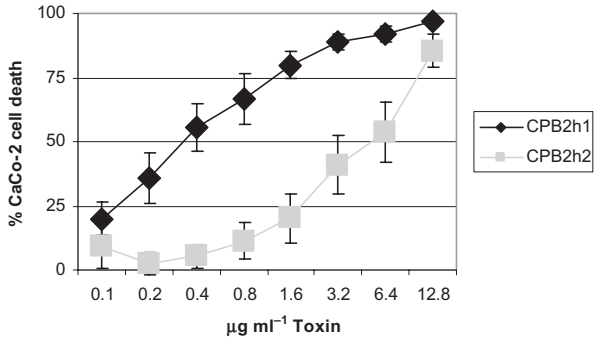

Fig. 7. Determination of TCD_{50} for CaCo-2 cells treated with CPB2h1 or CPB2h2. Cells were treated at 37°C with varying amounts of purified CPB2h1 or CPB2h2 dissolved in HBSS containing 0.01% neutral red dye for 2 h. After treatment, cells were washed with HBSS to remove excess dye and cell viability was determined using a microplate reader that measured dye retention (i.e. absorbance at 570 nm). Values are expressed as the per cent loss of CaCo-2 cell viability compared with a control well treated with HBSS/neutral red without toxin. Data points represent three independent toxin purifications and error bars show the standard error of the mean.

tend to be more long-lasting than those of *C. perfringens* type A food poisoning.

However, conjugative transfer of the *cpe* gene does not readily explain why the symptoms of CPE-associated non-foodborne human GI diseases are often more severe than those of *C. perfringens* type A food poisoning (McClane, 1996; Carman, 1997). Nor are those symptomatic differences attributable to variations in the amount or toxicity of CPE produced by AAD/SD isolates carrying a plasmid *cpe* gene versus food poisoning isolates carrying a chromosomal *cpe* gene (McClane, 1996; 1998). Similarly, differences in CPE regulation between plasmid *cpe* versus chromosomal *cpe* isolates fail to explain clinical observations, as all *cpe*-positive type A isolates produce the enterotoxin only during sporulation (Czeczulin *et al*., 1996; Zhao and Melville, 1998). Furthermore, isogenic *cpe*-knockout studies demonstrated that, in a rabbit ileal loop model, CPE is required for the enteric virulence of both AAD isolate F4969 and food poisoning isolate derivative SM101 (Sarker *et al*., 1999). Intestinal pathology was observed in those isogenic *cpe* mutant studies when rabbit ileal loops were treated with sporulating culture lysates (containing CPE), but not with vegetative culture lysates (lacking CPE), prepared from either the F4969 or SM101 parent strains. Those observations have been somewhat difficult to reconcile with clinical reports indicating that GI symptoms of CPE-associated AAD/SD sometimes continue for days or weeks, an observation that might appear more consistent with toxin expression by vegetative cultures playing a major role in the pathogenesis of CPE-associated non-foodborne GI diseases. In this regard, it becomes notable that the current study now identifies F4969 as an atypical human AAD isolate that is *cpb2*-negative, i.e. our previous isogenic *cpe* mutant studies were not addressing the possible contribution of CPB2 to enteric virulence during many cases of human *C. perfringens* AAD or SD.

The possibility of an accessory enterotoxin, such as CPB2, contributing to the pathogenesis of CPE-associated AAD/SD could help resolve the apparent dilemma regarding the greater severity of symptoms between CPE-associated AAD/SD versus *C. perfringens* type A food poisoning. The current study directly supports this possibility by reporting that a large percentage of our collection of human AAD/SD isolates, which had been gathered from diverse geographic sources over a 20-year period, produce the CPB2 toxin. It is notable that this collection of *cpe*-positive type A human non-foodborne GI disease isolates contains the highest percentage of CPB2-positive *C. perfringens* isolates found, to date, outside of type A and C isolates implicated in porcine GI diseases (Waters *et al*., 2003).

Two other important observations in the current study further support the potential involvement of CPB2 in human GI diseases, particularly AAD/SD. First, our *cpb2*$^+$/*cpe*$^+$ human GI disease isolates were found to produce CPB2 during vegetative growth, which is consistent with CPB2 production by vegetative bacteria growing *in vivo* possibly explaining, at least in part, the more severe and longer-lasting symptoms often associated with CPE-associated AAD/SD compared with *C. perfringens* type A food poisoning. Second, pure CPB2 was shown to be active on CaCo-2 cells, which supports the possibility of this toxin being an accessory enterotoxin during AAD/SD as CaCo-2 cells are a commonly used *in vitro* model for the human intestinal epithelium. Furthermore, as the causative agents of approximately 70% of human AAD cases remain unknown (Hogenauer *et al*., 1998), this observation also raises the possibility of *cpb2*$^+$/*cpe*$^-$ isolates, as well as *cpb2*$^+$/*cpe*$^+$ isolates, sometimes causing non-foodborne human GI diseases. Collectively, these observations suggest a need for developing methods to epidemiologically assess the possible presence of CPB2 in the stool of patients suffering from either CPE-associated AAD/SD or AAD/SD of unknown cause.

Western blot analyses performed in this study also indicated that sporulating cultures can simultaneously contain both CPB2 and CPE, opening the possibility that these two enterically active toxins could both be present in the GI tract during some human GI disease episodes. The simultaneous presence of two enteric toxins in the GI tract could further explain the more severe and longer-lasting symptoms often associated with CPE-associated AAD/SD compared with *C. perfringens* type A food poisoning. The role of CPB2 in the pathogenesis of CPE-associated AAD/SD needs to be further addressed *in vivo* through the construction and testing of *cpb2/cpe* isogenic knockout mutants prepared from *cpb2*$^+$/*cpe*$^+$ human AAD/SD isolates.

Another important discovery of the current study is that *cpb2*$^+$/*cpe*$^+$ human AAD/SD isolates produce two different CPB2 sequence variants, CPB2h1 (associated with IS*1151*) and CPB2h2 (associated with the IS*1470*-like element). CPB2h2 is much more similar in sequence to the veterinary CPB2 than is CPB2h1. Structure–function analysis of CPB2 has not yet been performed, but cytotoxicity results from this study indicate that CPB2h1 is more active than CPB2h2 on CaCo-2 cells. This observation opens the possibility of evolutionary CPB2 host adaptation.

The toxin TCD50 of CPB2h1 and CPB2h2 (0.3 and 4 $\mu g\ ml^{-1}$ respectively) is similar to the TCD_{50} of CPE (0.3 $\mu g\ ml^{-1}$; data not shown) for CaCo-2 cells and several isolates produced this level of CPB2 *in vitro* during vegetative growth. The higher concentration of CPB2h2 required for cytotoxicity may be achievable *in vivo* by concentrated secretion of CPB2 at the enterocyte cell surface by adherent bacteria. Furthermore, it is also possible that

more CPB2 toxin is produced *in vivo* versus *in vitro*. Such comparisons are not currently possible due to methodology limitations for measuring CPB2 levels in intestinal contents or disease-associated faeces. Sporulating isolates produce fivefold more CPE than CPB2, which may explain the absence of apparent differences between the Caco-2 cell cytotoxicity caused by sporulating supernatants from CPB2$^+$/CPE$^+$ isolates versus CPB2$^-$/CPE$^+$ isolates.

The variability in human CPB2 sequences detected in this study coupled with previous reports of CPB2 sequence difference in veterinary disease isolates indicates that CPB2 is an unusual *C. perfringens* toxin. The sequences of other *C. perfringens* toxins, such as CPE, epsilon, alpha, beta and iota toxins are generally conserved. Among most of those other toxins, only relatively minor amino acid sequence differences have been identified, with variant toxins typically differing by only two or three amino acids (Hunter *et al*., 1992; 1993; Sheedy *et al*., 2004). In fact, CPE apparently maintains identical amino acid sequences in most or all isolates carrying functional *cpe* genes (Collie and McClane, 1998; S. Sayeed, D. Fisher and B. McClane, unpublished).

The current study also provides substantial new insights into the virulence plasmids that play a major role in the enteric virulence of *C. perfringens*, where genes encoding beta toxin, epsilon toxin, iota toxin, CPB2 and CPE can all be plasmid-borne (Petit *et al*., 1999). Despite their pathogenic importance, the virulence plasmids of *C. perfringens* have received surprisingly little study to date, with most previous analyses of plasmid-borne *C. perfringens* virulence factors restricted to the sequences present immediately upstream and downstream of toxin gene loci (Hunter *et al*., 1992; Katayama *et al*., 1996; Brynestad *et al*., 1997; Billington *et al*., 1998; Miyamoto *et al*., 2002). Our study now demonstrates that the *cpb2* and *cpe* genes are encoded on the same plasmid (~75 kb) in type A human AAD/SD isolates carrying an IS*1511* element downstream of their plasmid *cpe* gene. To date, the *cpb2* gene has been identified via PCR in all *C. perfringens* types, making it one of the most widely distributed non-chromosomal toxin genes of *C. perfringens* (Gibert *et al*., 1997; Bueschel *et al*., 2003; Waters *et al*., 2003). As *cpe* plasmids can be conjugative (Brynestad *et al*., 2001), determining that *cpb2* can be present on *cpe* plasmids offers conjugative transfer as one potential contributing explanation for the widespread distribution of *cpb2*. Further supporting the possible transmissibility of the plasmid carrying *cpb2/cpe*/IS*1151* sequences are PFGE results from previous studies (Sparks *et al*., 2001) indicating that isolates carrying this particular plasmid are often non-clonal. Finally, the current identification of a plasmid carrying and expressing *cpb2* and *cpe* genes represents, to our knowledge, the first example of a *C. perfringens* extrachromosomal genetic element carrying two functional toxin genes. One previously identified type D isolate was found to possess a plasmid carrying both the *cpe* gene and an epsilon toxin gene, although it was not determined whether that type D isolate actually produces CPE and epsilon toxin (Dupuy *et al*., 1997).

PFGE and PCR results from this study also indicate that, when present, *cpb2* and *cpe* genes are located on different plasmids in type A human AAD/SD isolates carrying an IS*1470*-like element downstream from their plasmid *cpe* gene. The *cpe* plasmid present in these non-foodborne human GI disease isolates is ~75 kb, while their *cpb2* plasmid has a similar 50 kb size as pCP13, the sequenced *cpb2* plasmid from *cpe*-negative Strain 13 (Shimizu *et al*., 2002). Strain 13 also encodes a *cpb2* variant that is more like the *cpb2* gene from veterinary disease isolates. Localization of *cpb2* and *cpe* to different plasmids in type A isolates carrying IS*1470*-like sequences downstream of their plasmid *cpe* gene provides the best documentation to date for a single *C. perfringens* isolate harbouring multiple large virulence plasmids. Given this finding, it becomes interesting that some plasmid-borne toxin genes have never been found together in a single isolate, opening the possibility of multiple *C. perfringens* plasmid incompatibility groups.

Like the *cpb2*$^+$/*cpe*$^+$ human AAD/SD isolates examined in the current study, *cpb2*-positive porcine GI disease isolates also generally produce CPB2 (Bueschel *et al*., 2003; Waters *et al*., 2003). In contrast, *cpb2*-positive animal disease isolates from non-porcine origins often fail, for still unknown reasons, to produce CPB2 (at least *in vitro*). The current study and results from Jost *et al.* (2005) shed some light on this situation by independently determining that Strain 13 fails to produce CPB2 due to a single nucleotide deletion that results in a premature stop codon in the *cpb2* ORF. It also deserves mention that the nucleotide deletion identified in the *cpb2* gene of our laboratory's cultures of Strain 13 and those of Jost *et al.* was not found by Shimizu *et al*. (2002) during sequencing of the Strain 13 genome (Shimizu *et al*., 2002), i.e. this mutation may only be present in Strain 13 stocks maintained by certain laboratories. However, it is also notable that the Strain 13 literature reports *cpb2* RNA levels (which would not be affected by this *cpb2* deletion) rather than documenting actual production of the CPB2 protein (Ohtani *et al*., 2003).

There must be additional reasons why some *cpb2*$^+$ isolates fail to produce CPB2 as no mutations introducing a premature stop codon into the *cpb2* ORF were identified by sequence analysis of our two *cpb2*$^+$/*cpe*$^+$ human GI disease isolates that failed to produce CPB2. Sequence analysis of the upstream *cpb2* promoter sequences in those two *cpb2* human GI disease isolates failing to pro-

duce CPB2 toxin did not identify any base changes in the −10 to −35 region of the previously mapped *cpb2* promoter (data not shown) (Ohtani *et al*., 2003). This finding opens the possibility that these two isolates could be deficient in VirR/VirS-mediated regulation, which has been shown to regulate CPB2 expression levels (Ohtani *et al*., 2003).

In summary, this study offers several new contributions to our understanding of *C. perfringens* virulence plasmids, paving the way for additional studies of the plasmids carrying *cpb2* and/or *cpe* in human AAD/SD and veterinary isolates. The discovery that human AAD/SD isolates produce either of two CPB2 variants with different activities will impact understanding of CPB2 structure–function relationships. Finally, by demonstrating the presence of *cpb2* genes in a high percentage of AAD/SD isolates and by showing that the CPB2 variants made by those isolates are cytotoxic for a cell culture model of the human GI tract, this study potentially helps to explain the symptomatic differences between cases of CPE-associated AAD/SD versus cases of food poisoning. Additional epidemiologic and pathogenesis studies are also now underway to further explore the possible role of CPB2 in human AAD/SD.

Experimental procedures

Growth conditions for bacterial isolates

Clostridium perfringens type A isolates used in this study are listed in Table 1. Unless stated otherwise, all *C. perfringens* isolates were grown overnight at 37°C in TGY [3% trypticase (Difco), 2% glucose (Sigma), 1% yeast extract (Difco), 0.1% cysteine (Difco)] or FTG (Difco). For PCR analysis and cloning, *C. perfringens* isolates were grown on Brain Heart Infusion (BHI, Difco) agar plates that were incubated for 16 h at 37°C in anaerobic jars. *E. coli* strains were grown overnight at 37°C in LB broth or on LB agar plates. For growth of recombinant *C. perfringens* or *E. coli*, either erythromycin (50 μg ml^{-1} for *C. perfringens* and 150 μg ml^{-1} for *E. coli*; Sigma), chloramphenical (20 μg ml^{-1} for *C. perfringens* and 100 μg ml^{-1} for *E. coli*, Sigma) or ampicillin (100 μg ml^{-1}, Sigma), respectively, were added to the appropriate media.

PCR detection of the cpb2 *gene*

Polymerase chain reaction analysis of the *cpb2* gene in *C. perfringens* isolates was performed using colony lysates, as previously described (Wen *et al*., 2003). This *cpb2*-specific PCR used primers 1F and 2R (Table S2 in *Supplementary material*) at a concentration of 1 μM to amplify a 318 bp internal PCR product from the *cpb2* gene. A multiplex PCR assay using six primer pairs was also performed to amplify internal gene regions of *etx*, *cpb*, *plc*, *iab* and *cpe* (see Garmory *et al*., 2000 for primer sequences). This multiplex PCR also amplifies 567 bp internal region from the *cpb2* gene using primers *cpb2*MPF and *cpb2*MPR. TaqComplete 1.5 mM $MgCl_2$® (Gene Choice) was used for both PCRs, which were performed in a Techne Techgene® thermocycler. Cycling conditions for amplifying the internal *cpb2* gene products of 318 bp and 567 bp were as previously described (Garmory *et al*., 2000; Waters *et al*., 2003). All PCR products were separated on a 2% agarose gel and then visualized using ethidium bromide staining.

Western blot analyses of CBP2 and CPE production

To assess CPB2 toxin production by *cpe*$^+$/*cpb2*$^+$ *C. perfringens* type A isolates during vegetative growth conditions, 0.1 ml of an overnight FTG (Difco) culture was inoculated into 10 ml of TGY. That TGY culture was then grown at 37°C until an OD_{600} of 1.5 was reached, as determined using a Bio-Rad Smart Spec®. To analyse CPB2 production during sporulating conditions, 0.1 ml of an overnight FTG culture was inoculated into 10 ml of Duncan Strong media [1.5% proteose peptone (Difco), 0.4% yeast extract (Difco), 0.1% sodium thioglycolate (Sigma), 1% Na_2HPO_4-$7H_2O$ (Sigma) and 0.4% soluble starch (Difco)], and that culture was grown for 16 h at 37°C. Both vegetative and sporulating cultures were then centrifuged and the resultant culture supernatants were retained for CPE and CPB2 Western blot analysis. To analyse CPB2 levels in vegetative and sporulating cultures, supernatants were first concentrated 20-fold at 4°C using Amicon® Ultra centrifugal filter devices (Millipore). After concentration, those supernatants were added to SDS loading buffer containing 5% β-mercaptoethanol (Bio-Rad) and then boiled for 10 min. The processed samples were immediately loaded onto a 12% SDS-PAGE gel and electrophoresed at 30 mA. The proteins were then electrotransferred onto nitrocellulose paper (Bio-Rad). Those blots were blocked with Blotto before probing with a rabbit polyclonal anti-CPB2 antibody (kindly supplied by Michel R. Popoff) diluted 1:500 in Blotto. After probing, the blot was washed three times in 0.1% Tween 20, Tris-buffered saline (TTBS) before the addition of an anti-rabbit IgG antibody horseradish peroxidase conjugate (Sigma), which was diluted 1:1000 in Blotto. Blots were then washed with TTBS before visualization of immunoreactivity with Westpico Substrate® (Pierce) and autoradiography.

For CPE Western blot detection, sporulating cultures were prepared as described above for CPB2, except that supernatants were not concentrated or boiled. CPE was then detected using a rabbit anti-CPE polyclonal antibody (1:500). All other CPE Western blotting steps were performed as described for CPB2 Western blot analysis.

cpb2 *sequencing analysis*

The B2seqF/B2seqR primer pair (Table S2 in *Supplementary material*) was employed to PCR amplify a product from the *cpb2* ORF using the following cycling conditions: 94°C for 3 min, 30 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, followed by a single extension of 72°C for 10 min. Those PCR products were then sequenced at the University of Pittsburgh core sequencing facility (http://www.genetics.pitt.edu/services.html). Primers 1F, 4R, *cpb2*MPF and *cpb2*MPR were used to sequence both strands of each *cpb2* PCR product (Table S2 in *Supplementary material*). DNA sequence assembly, amino acid translations and the amino acid alignment were performed using

BIOEDIT (Hall, 1999). These *cpb2* sequences have been submitted to GenBank database under Accession No. AY730630-6. Deduced CPB2 sequences not determined during this study were obtained from GenBank (http://www.ncbi.nlm.nih.gov) (Gibert *et al*., 1997; Shimizu *et al*., 2002).

Identification of cpe and cpb2 *plasmids by PFGE and Southern blotting*

DNA plugs for PFGE were prepared by resuspending *C. perfringens* bacterial pellets isolated from overnight TGY cultures in 2% PFGE-certified agarose (Bio-Rad), as described previously (Collie and McClane, 1998). PFGE electrophoresis was then carried out on those plugs in a CHEF-DR® II PFGE system (from Bio-Rad) using 1% PFGE-certified agarose (Bio-Rad) and 0.5x TBE with the PFGE chamber maintained at 14°C using a Mini-Chiller® (Bio-Rad). The PFGE electrophoretic conditions were 6 V for 24 h with a 1–25 s switch time. After electrophoresis, the gels were stained with ethidium bromide to detect DNA. Separated DNA in the PFGE gels was then transferred to positively charged nylon membranes (Roche) and probed, as previously described (Collie and McClane, 1998), with a DIG-labelled DNA probe constructed using the PCR DIG Probe synthesis kit (Roche). The *cpb2* primers 1F/2R and the *cpe* primers $2F^{cpe}/5R^{cpe}$ (*Supplementary material*) were used to amplify the respective toxin genes for probe synthesis, utilizing PCR conditions described previously (Collie and McClane, 1998). PFGE blots were probed and developed using reagents from the DIG DNA labelling and Detection kit (Roche). DNA hybridization was visualized with CSPD, ready-to-use substrate from Roche.

Nucleotide sequencing of the cpb2-cpe *region of pF5603*

Clostridium perfringens isolate F5603, a $cpb2^+/cpe^+$ isolate with IS*1151* sequences downstream of its plasmid *cpe* gene (Miyamoto *et al*., 2004), was grown in TGY and crude plasmid fractions were prepared, as described previously (Miyamoto *et al*., 2002). The crude plasmid preparation from F5603 was digested to completion with *Xba*I (Roche), and then ligated into an *Xba*I-digested, pSK+ pBlueScript vector (Invitrogen) using T4 DNA ligase (Invitrogen). After transformation of the resultant recombinant plasmids into *E. coli* HB101 (Invitrogen), transformants were screened by Southern blot analysis to identify transformants carrying an *Xba*I insert of F5603 plasmid DNA. This Southern blot screening was performed with four DIG-labelled probes corresponding to fragments of the *C. perfringens* plasmid pCW3 (pJIR15, pJIR16, pJIR18 and pJIR32) that have been shown previously to hybridize with the *cpe* plasmid (Brynestad *et al*., 2001). Positive transformants were grown in LB broth with ampicillin (100 μg ml^{-1}) to amplify the insert-carrying plasmid, which was then extracted using the QIA Prep Mini Kit from Qiagen.

Initial sequencing was performed on both ends of plasmid DNA inserts putatively identified by Southern blotting. That sequencing was performed with T3 or T7 promoter region primers (T7UP: 5′-GCCAGGGTTTTCCCAGTCACGAC-3′, and SPT3: 5′-CAGCTATGACCATGATTACGCC-3′) using the thermocycler conditions recommended by the manufacturer for those primers. Sequences obtained for those inserts were compared against the previously determined genome sequence of *C. perfringens* Strain 13 (Shimizu *et al*., 2002) by BLAST analysis to eliminate chromosomal DNA inserts from further analysis. Apparent plasmid inserts were then completely sequenced by PCR walking. The organization and arrangement of pF5603 DNA inserts was further analysed by RFLP analysis. Crude pF5603 plasmid preparations digested with *Cla*I, *Pst*I, *Hin*dIII, *Eco*RI, *Eco*RV (NEB) were subjected to Southern blotting using DIG-labelled probes derived from sequencing both ends of inserts present in the *Xba*I fragment containing transformants. The organization and arrangement of the plasmid DNA inserts was then further confirmed by direct sequencing of PCR products amplified from each insert. These procedures yielded a single contig including the region between the *cpb2* and *cpe* genes.

Bioinformatics

Once sequencing of the pF5603 region between the *cpe* and *cpb2* genes had been completed, the putative ORFs in this region were identified using ORF Finder from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), followed by manual detection of putative SD sequences. The selected putative ORFs were then used for database searches with FASTA (http://www.fasta.genome.jp/) and BLAST (http://www.ncbi.nlm.nih.gov/blast/). Results from this analysis including ORFs and the nucleotide sequences are located in GenBank under Accession No. AY737555.

PCR analyses of cpb2-cpe *gene linkage in other* $cpb2^+/cpe^+$ *type A human GI disease isolates*

To obtain template for these PCR analyses, DNA was purified from isolates grown overnight in TGY, as described previously (Wen *et al*., 2003). The purified DNA was then subjected to PCR amplification using the battery of primers listed (Table S3 in *Supplementary material*), which were employed at a final concentration of 1 μM. This PCR reaction included TaqComplete 1.5 mM $MgCl_2$® from Gene Choice and involved the following cycling conditions: 5 min at 94°C and 30 cycles of three segments at 94°C for 60 s, 55°C for 60 s, 72°C for 1.5 min and a final extension at 72°C for 5 min. PCR products were separated on a 1% agarose gel and visualized using ethidium bromide staining.

Preparation of a recombinant C. perfringens *construct that overproduces CPB2*

To construct a recombinant *C. perfringens* isolate carrying a multicopy plasmid for overproduction of CPB2, the *cpb2* gene was cloned from F5603, a *cpb2/cpe*-positive *C. perfringens* type A AAD isolate. The F5603 *cpb2* gene was first amplified by PCR using primers B2CPF and B2CPR (Table S2 in *Supplementary material*). That PCR reaction involved the following thermocycler conditions: 94°C for 3 min, 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1.50 min, followed by a single extension of 72°C for 10 min. The resultant PCR product was then cloned into pCR2.1 Topo (Invitrogen), as

directed by the manufacturer, to create pB2h1TOPO. The pB2h1TOPO plasmid was then purified and transformed into XL-1 blue cells using the QiaPrep Mini Kit from Qiagen. The *cpb2* insert was excised from 10 μg of pB2TOPO DNA using *Eco*RI and *Bam*HI (New England Biolabs) and isolated by electrophoresis on a 1% agarose gel and then purified using the Squeeze-N-Freeze® kit from Bio-Rad. The purified *cpb2* insert was then ligated with T4 DNA ligase (Invitrogen) into the *Eco*RI/*Bam*HI-digested pJIR751 (Bannam and Rood, 1993). The resultant plasmid, pB2h1, was used to transform chemically competent *E. coli* DH5α (Invitrogen) and transformants were selected on LB plates containing erythromycin (150 μg ml^{-1}). A single clone was selected and grown in LB broth to amplify pB2h1 for purification. *C. perfringens* ATCC 3624 (a *cpb2/cpe*-negative type A isolate) was transformed with pB2h1 using previously described electroporation techniques (Sarker *et al*., 1999). A clone designated ATCC 3624(B2h1) was then selected by growth on BHI agar plates containing erythromycin (50 μg ml^{-1}). The identity of ATCC 3624(B2h1) was confirmed by PCR screening using the 1F/2R primers. All plasmid constructs were verified both by restriction analysis (*Eco*RI/*Bam*HI) and by automated nucleotide sequencing of the PCR product amplified using primers B2CPF/B2CPR, as described earlier. The vector pB2h2 and transformant ATCC 3624(B2h2) were constructed in an identical manner to pB2h1/ATCC 3624(B2h1) except that the primers B2(13)F and B2(13)R (Table S2 in *Supplementary material*) were substituted for B2CPF/R to amplify the *cpb2* gene from F4396 (an IS*1470*-like AAD isolate) and the B2h2 ORF was subcloned into the *C. perfringens–E. coli* shuttle vector pJIR750 (Bannam and Rood, 1993) encoding chloramphenical resistance.

Purification of CPB2 from ATCC 3624(B2h1)/ATCC 3624(B2h2)

The CPB2 purification procedure was modified from Gibert *et al*. (1997). ATCC 3624(B2h1) or ATCC 3624(B2h2) was grown in 4.5 l of TGY media at 37°C until an OD_{600} of 1.5 was reached. The bacteria were then pelleted by centrifugation at 10 000 *g* for 20 min at 4°C. The supernatant was retained and incubated at 40% ammonium sulphate saturation under constant stirring for 1 h at 4°C. The solution was then centrifuged at 4°C for 20 min at 10 000 *g* and the resultant pellet was suspended in 10 mM Tris, pH 7.5 and dialysed overnight at 4°C versus 10 mM Tris, pH 7.5. After dialysis, the solution was loaded on a DEAE sepharose CL6B (Sigma) column that was pre-equilibrated in 10 mM Tris, pH 7.5. After loading, the DEAE-CL6B column was washed with two bed volumes of 10 mM Tris, pH 7.5 and bound protein was eluted using a gradient wash of 0–100% 10 mM Tris, 100 mM NaCl, pH 7.5. All chromatography steps were performed at 4°C.

The elution profile of CPB2 was determined by analysing 15 μl of each column fraction on a 12% SDS-PAGE gel stained with G250 Coomassie brilliant blue (Bio-Rad). Elution fractions yielding a single band upon Coomassie brilliant blue staining were then confirmed as containing CPB2 by Western blotting. Fractions containing pure CPB2 were then pooled and concentrated to ~2 mg ml^{-1} using Amicon™ Ultra centrifugal filter devices (Millipore). Final purity of the pooled CPB2 was assessed by SDS-PAGE and the concentration of the pooled CPB2 was determined by Coomassie brilliant blue staining and Lowry protein assay.

Determining the cytotoxicity of CPB2 on CaCo-2 cells

CaCo-2 cells were seeded in 60 mm cell culture Petri dishes (Corning) at 250 000 cells per dish. The cells were then grown to confluence (about 5 days) in DMEM supplemented with 5% FBS (Mediatech Incorporated), 1% non-essential amino acids (Sigma), 1% glutamine (Sigma) and 1% streptomycin/penicillin (Sigma) at 37°C with 5% CO_2.

To assess the effects of culture supernatants from *cpb2/cpe*-positive *C. perfringens* isolates on CaCo-2 cells, vegetative and sporulating cultures were prepared as discussed above. Supernatants from those vegetative or sporulating cultures were then concentrated 20-fold using Amicon® Ultra centrifugal devices (Millipore) and 1 ml of each concentrated supernatants (or concentrated TGY/DS media as a control) was added to 1 ml of DMEM growth media and the mixture was applied to confluent CaCo-2 cell cultures.

In other experiments, purified CPB2h1/h2 was added, at a final concentration of 10 μg ml^{-1}, to confluent CaCo-2 cell growing in 60 mm Petri dishes. Treated cells were then incubated at 37°C for specified periods. Pictures of the confluent cultures were then taken using a Canon Powershot G5 fitted to the Zeiss Axiovert 25 microscope and images were processed for publication using Adobe Photoshop 6.0.

Quantification of CPB2h1/h2 activity on CaCo-2 cells using the neutral red cell viability assay

The neutral red assay used in this study was modified from Mahoney *et al*. (1989). Briefly, CaCo-2 cells were seeded at 10 000 cells ml^{-1} into a 96-well plate and grown to 70% confluence with DMEM growth media. The cells were then treated with varying amounts of CPB2h1/h2 diluted in 100 μl of HBSS containing 0.01% neutral red (Sigma) for 2 h at 37°C. Cells were then washed three times with 100 μl of HBSS followed by treatment with 100 μl of 1% acetic acid, 50% ethanol. Plates were then incubated for 20 min at room temperature and viability was determined by measuring absorbance at 570 nm using a Dynex MRX Revelation microplate reader with Revelation software package 4.21.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4573/mmi4573sm.htm

Fig. S1. CaCo-2 cells treated with CPB2-positive vegetative culture supernatants.
Fig. S2. CaCo-2 cells treated with CPB2-positive sporulating culture supernatants.
Table S1. Results from *cpb2-cpe* PCR assay.
Table S2. DNA primers for *cpb2-cpe* PCR direct linkage assay.
Table S3. Miscellaneous DNA primers for PCR.

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SUPPLEMENTAL DATA FOR FISHER *ET AL*. 2005

Table 1 Supplement. Results from *cpb2-cpe* PCR assay.

Strain	IS element	Toxin gene(s)	PCR Reactions[b]														
		cpb2/cpe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
F5603	**IS*1151***		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F4013			+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+
F4406			+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
T57603			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H38094			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T44123			+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
B2			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B40			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B38			+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
		cpb2/cpe																
F4396[a]	**IS*1470-like***		-	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-
X5722[a]			-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
W43181[a]			-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
S43526[a]			-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-
MR2-4[a]			-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
		cpe																
M24326[a]	**IS*1470*-like**		-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
F4969[a]			-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
		cpb2/cpe																
C1841	**IS1470**		+	-	-	-	-	-	-	-	+[ta]	+[ta]	+[ta]	-	-	-	-	-
191-10			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]PCR products amplified in these isolates did not match the expected size

[b]+, presence of PCR product; -, absence of PCR product

Table 2 Supplement. DNA Primers for *cpb2-cpe* PCR direct linkage assay.

Primer	Sequence (5'-3')	Product Size (bp)[Ω]
B2 3F	GAACCTGTTATTTCATATGATTTAAG	1037
41870R	CATTTAGGTAGGCCAAGAGTAGAATATC	
41870F	GATATTCTACTCTTGGCCTACCTAAATG	896
42703R	CATCAACATATGTTACCCTACCAATAC	
42703F	GTATTGGTAGGGTAACATATGTTGATG	1286
43989R	ATAATCTTGTAAGGCATGCATTATTC	
43989F	GAATAATGCATGCCTTACAAGATTAT	814
44803R	GAATTAGATTTTGGTTTAGTGATGCTGCATG	
44803F	CATGCAGCATCACTAAACCAAAATCTAATTC	1245
46048R	GTTATGGATTGCTCCAGGAATATTTG	
46048F	CAAATATTCCTGGAGCAAACCATAAC	1407
47455R	CTTTTGGTGAACTTGGCGAATC	
47455F	GAATCGCCAAGTTCACCAAAAG	823
48278R	CATAGATCTTGTTGTGCTGCTAAGC	
48278F	GCTTAGCAGCACAACAAGATCTATG	880
49158R	GAGCATTAACGGATTTAGCATCTG	
49158F	CAGATGCTAAATCCGTTAATGCTC	1388
50546R	GACCTTTATATTACTGGAGTAAGATGTATTCAG	
50546F	CTGAATACATCTTACTCCAGTAATATAAGGTC	1277
51823R	GTTTTGGAGTGTCCTTATTGTTCTTAAG	
51823F	CTTAAGAACAATAAGGACACTCCAAAAC	1031
52854R	GCATAAACATTCCTGTTAAATAAGCTATTCCA	
52854F	TGGAATAGCTTATTTAACAGGAATGTTTATGC	1122
53976R	CTGGTATGTTCACAGAAATGGTTGTTAG	
53976F	CTAACAACCATTTCTGTGAACATACCAG	558
54534R	GAGCTCTGCTTAGATTTACTAAGTAGAG	
54534F	CTCTACTTAGTAAATCTAAGCAGAGCTC	1208
55742R	GACACTTAATAGCGGTCAGAGAAGC	
55742F	GCTTCTCTGACCGCTATTAAGTGTC	1298
57040R	GTTAAATTAGAGCGATTCATGTGC	
57040F	GCACATGAATCGCTCTAATTTAAC	1519
CPE-F	TTAGAACAGTCCTTAGGTGATGGAG	

[Ω]PCR product sizes are listed for each primer pair (i.e. B2 3F and 41870R = 1037 bp)

Table 3 Supplement. Miscellaneous DNA Primers for PCR.

Primer	Sequence (5'-3')	Product Size (bp)[Ω]
B2CPF[a]	ATCGAATTCGTAAAGGAATCCATAAAAATTTAGG	1229
B2CPR[b]	ATGGATCCGTTATAAATAAATATAATTCTCTAAAACC	
B2(13)F[b]	ATTAGGATCCGATTTAATTTTCCATTATAGTGCTAGT	1313
B2(13)R[a]	TATACTTAAGCTGGTAATTTTTTCTTTTATCGATG	
B2seqF	AAACTGAATTTTTAAATGGTGC	825
B2seqR	TCCACATCCAATGATCTACAA	
1F	GTTAAAAATTTGATATAATTGAATTG	318
2R	GACTAAGTCACTTCATATTTTTTTC	
cpb2 MPF	AGATTTTAAATATGATCCTAACC	567
cpb2 MPR	CAATACCCTTCACCAAATACTC	
2F[cpe]	GGTACCTTTAGCCAATCA	639
5R[cpe]	TCCATCACCTAAGGACTG	

[Ω]PCR product sizes are listed for each primer pair (i.e. B2CPF and B2CPR = 1229 bp)

[a]EcoRI sites are underlined

[b]BamHI sites are underlined

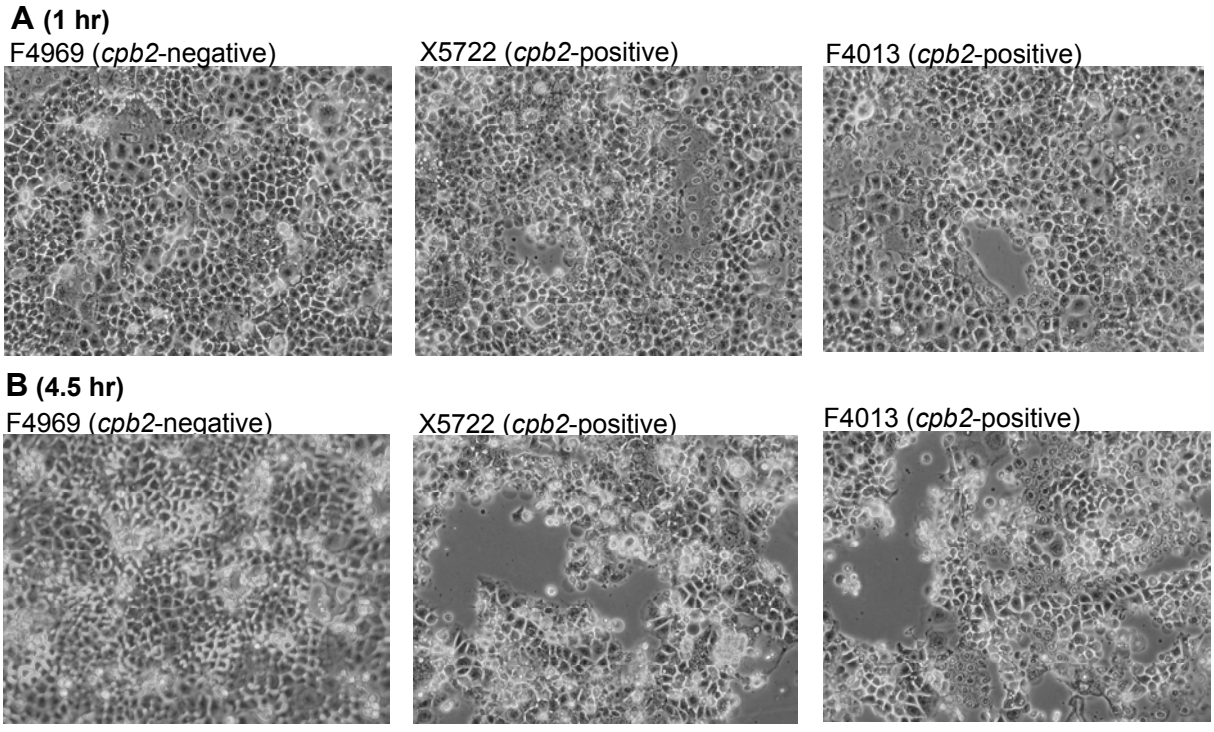

Fig. 1. Supplement. CaCo-2 cells treated with CPB2-positive vegetative culture supernatants. Confluent, 5-day old CaCo-2 cell monolayers were treated, as indicated, with concentrated vegetative culture supernatants from either *cpb2+/cpe+* or *cpb2-/cpe+* isolates. The presence of CPB2 in the culture supernatants used to treat these CaCo-2 cells was verified by CPB2-Western blot analysis (data not shown). Treated cells were incubated at 37°C and cytotoxicity was measured by monitoring these cells for morphological changes using phase contrast microscopy. CaCo-2 cells treated with CPB2-negative culture supernatants from isolate F4969 are shown in (A/B). CaCo-2 cells treated with CPB2-positive culture supernatants are shown after 1 hour (A) or 4.5 hours of treatment. Microscopy pictures were taken at 210x magnification.

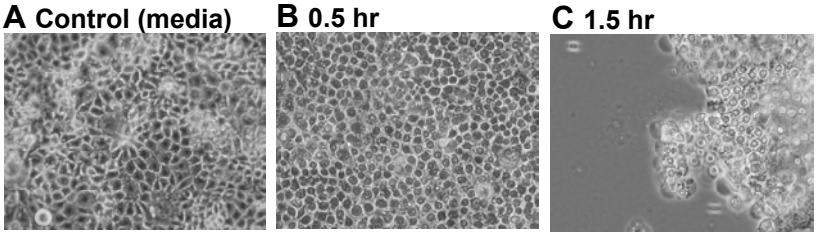

Fig. 2. Supplement. CaCo-2 cells treated with CPB2-positive sporulating culture supernatants. CaCo-2 cells were grown until confluency (5 days) and then treated with sporulating culture supernatants from either *cpb2+/cpe+* isolates or *cpb2-/cpe+* isolates and incubated at 37°C. The presence of CPB2 and/or CPE in the culture supernatants was assessed by Western blotting and the appropriate antibodies (data not shown). Control CaCo-2 cells treated with media alone are shown in A. Representative cells treated with CPE+/CPB2+ supernatants from F5603 are shown in B and C. Morphological changers were assessed using phase contrast microscopy. Cell damage was assessed after 30 minutes of treatment (B) or after 1.5 h of treatment (C). Pictures were taken at a final magnification of 210x.

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Complete Sequencing and Diversity Analysis of the Enterotoxin-Encoding Plasmids in *Clostridium perfringens* Type A Non-Food-Borne Human Gastrointestinal Disease Isolates†

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Enterotoxin-producing *Clostridium perfringens* type A isolates are an important cause of food poisoning and non-food-borne human gastrointestinal diseases, e.g., sporadic diarrhea (SPOR) and antibiotic-associated diarrhea (AAD). The enterotoxin gene (*cpe*) is usually chromosomal in food poisoning isolates but plasmid-borne in AAD/SPOR isolates. Previous studies determined that type A SPOR isolate F5603 has a plasmid (pCPF5603) carrying *cpe*, IS*1151*, and the beta2 toxin gene (*cpb2*), while type A SPOR isolate F4969 has a plasmid (pCPF4969) lacking *cpb2* and IS*1151* but carrying *cpe* and IS*1470*-like sequences. By completely sequencing these two *cpe* plasmids, the current study identified pCPF5603 as a 75.3-kb plasmid carrying 73 open reading frames (ORFs) and pCPF4969 as a 70.5-kb plasmid carrying 62 ORFs. These plasmids share an ~35-kb conserved region that potentially encodes virulence factors and carries ORFs found on the conjugative transposon Tn*916*. The 34.5-kb pCPF4969 variable region contains ORFs that putatively encode two bacteriocins and a two-component regulator similar to VirR/VirS, while the ~43.6-kb pCPF5603 variable region contains a functional *cpb2* gene and several metabolic genes. Diversity studies indicated that other type A plasmid *cpe*$^+$/IS*1151* SPOR/AAD isolates carry a pCPF5603-like plasmid, while other type A plasmid *cpe*$^+$/IS*1470*-like SPOR/AAD isolates carry a pCPF4969-like plasmid. Tn*916*-related ORFs similar to those in pCPF4969 (known to transfer conjugatively) were detected in the *cpe* plasmids of other type A SPOR/AAD isolates, as well as in representative *C. perfringens* type B to D isolates carrying other virulence plasmids, possibly suggesting that most or all *C. perfringens* virulence plasmids transfer conjugatively.

Clostridium perfringens is an important pathogen of humans and domestic animals (24, 25). The virulence of this gram-positive bacterium is largely attributable to prolific toxin production; ~15 different *C. perfringens* toxins have been reported in the literature (29). However, individual *C. perfringens* isolates do not produce this entire toxin arsenal, providing pathogenic versatility that allows this bacterium to cause a spectrum of enteric and histotoxic diseases. Isolate-to-isolate toxin production variability also provides the basis for a commonly used classification scheme that assigns *C. perfringens* isolates to one of five toxinotypes (types A to E), based upon their expression of four toxins (α, β, ε, and ι toxins) (24). Type A isolates produce α toxin but not β, ε, or ι toxin.

Toxin production diversity among *C. perfringens* isolates is probably attributable, at least in part, to the presence of many toxin genes on large plasmids (24). For example, plasmids carry the genes encoding all of the typing toxins except α toxin, as well as the gene (*cpb2*) encoding the recently identified beta2 toxin (CPB2) (24). Despite their obvious pathogenic importance, the large toxin-encoding plasmids of *C. perfringens* have received limited study (4). To date, only a single *C. perfringens* plasmid (i.e., pCP13) carrying toxin gene sequences has been completely sequenced (27). However, two laboratories recently reported that the pCP13 *cpb2* sequences, which are the only potentially toxin-encoding sequences present on this plasmid, are translationally silent due to the presence of a premature stop codon that prevents CPB2 production (11, 16).

Currently, the best-studied *C. perfringens* virulence plasmids are the enterotoxin-encoding plasmids of type A isolates. Although they represent <5% of all *C. perfringens* isolates, type A isolates producing *C. perfringens* enterotoxin (CPE) are responsible for a very common human food poisoning (17). These bacteria also cause non-food-borne human gastrointestinal (GI) diseases, such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SPOR), and (likely) enteric disease in domestic animals (18). In type A food poisoning isolates, the enterotoxin gene (*cpe*) is usually chromosomal (8, 9), whereas type A isolates causing nonfoodborne human GI disease and animal disease isolates typically carry their *cpe* gene on a large plasmid (30). Whether chromosomal or plasmid-borne, the *cpe* gene of type A isolates is immediately preceded by an upstream IS*1469* element (6, 22).

Beyond those conserved IS*1469-cpe* sequences, considerable variation exists between the chromosomal and plasmid *cpe* loci of type A isolates. When chromosomal, the IS*1469-cpe* se-

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. List of *C. perfringens* isolates used in this study

Isolate group	Strain	Type	*cpe* location	Date and source	Reference	IS element[a]
Dog	13	A	None		27	
Dysentery/lamb	NCTC8533	B	None	1950s, United Kingdom	http://www.hpa.org.uk/srmd/div_cdmssd_nctc/default.htm	
Struck/sheep	NCTC3182	C	None	1930, unknown		
Sheep	NCTC8346	D	None	1950s, United Kingdom		
Calf	NCTC8084	E	Plasmid[b]	1943, United Kingdom		
	JIR4468[c]	A	*cpe* Δ(plasmid)	JIR325(pMRS4969) Cm^r	5	
Sporadic diarrhea/human	F4969	A	Plasmid	1990's Europe	9	IS*1470*-like
	F5603	A	Plasmid	1990's Europe		IS*1151*
	F4013	A	Plasmid	1990's Europe		IS*1151*
	F4396	A	Plasmid	1990's Europe		IS*1470*-like
AAD/human	B38	A	Plasmid	1980's, Europe	9	IS*1151*
	B40	A	Plasmid	1980's, Europe		IS*1151*
	NB16	A	Plasmid	1980's, Europe		IS*1151*
	B2	A	Plasmid	1980's, Europe		IS*1151*
	S43526	A	Plasmid	Late 1990's, NA[d]	30	IS*1470*-like
	W43181	A	Plasmid	Late 1990's, NA		IS*1470*-like
	H38094	A	Plasmid	Late 1990's, NA	30	IS*1151*
	M26413	A	Plasmid	Late 1990's, NA		IS*1470*-like
	X5722	A	Plasmid	Late 1990's, NA		IS*1470*-like
	T34058	A	Plasmid	Late 1990's, NA		IS*1470*-like
Healthy human	MR2-4	A	Plasmid	1990's, Japan	22	IS*1470*-like

[a] Source: Miyamoto et al., 2003.
[b] The *cpe* gene associated with type E isolates is silent due to multiple DNA sequence changes.
[c] JIR325 is a Rif^r, Nal^r strain 13 derivative transformed with pMRS4969 (pCPR4969 with its *cpe* gene specifically inactivated by insertion of a chloramphenicol resistance determinant).
[d] NA, North America.

quences are flanked by two IS*1470* sequences, which may represent the boundaries of an integrated ~6.3-kb transposon (6). In contrast, upstream or downstream IS*1470* elements are not associated with the plasmid IS*1469-cpe* region of type A isolates; instead, those isolates carry either an IS*1151* sequence or a defective IS*1470*-like sequence downstream of their plasmid *cpe* gene (22, 23). The discovery of two different IS-related sequences downstream of the plasmid *cpe* gene recently led to the identification of at least two distinct *cpe* plasmids in type A isolates causing human non-food-borne GI disease. F5603 (where IS*1151* sequences are located downstream of the plasmid *cpe* gene, making this a plasmid cpe^+/IS*1151*$^+$ type A isolate) is a type A SPOR isolate previously reported to carry an ~75-kb plasmid (pCPF5603) with functional *cpe* and *cpb2* genes (22). In contrast, F4969 (where defective IS*1470*-like sequences are present downstream of the plasmid-borne *cpe* gene, making this a plasmid cpe^+/IS*1470*-like$^+$ type A isolate) is a type A SPOR isolate previously reported to carry an ~75-kb plasmid (pCPF4969) with a functional *cpe* gene but no *cpb2* sequences (22).

Differences between pCPF5603 and pCPF4969 clearly extend beyond the presence or absence of a *cpb2* gene and what IS-related sequence is located downstream of the *cpe* gene. Recent studies (11) indicated that the ~19-kb region between the *cpb2* and *cpe* genes on pCPF5603 is largely conserved among several other plasmid cpe^+/IS*1151*$^+$ type A SPOR or AAD isolates. However, that ~19-kb region is absent from several plasmid cpe^+/IS*1470*-like$^+$ type A SPOR or AAD isolates (including F4969). Currently, it is unclear whether the *cpe* plasmids of cpe^+/IS*1151*$^+$ type A isolates share any similarity beyond the sequenced ~19-kb *cpe-cpb2* pCPF5603 region or whether the *cpe* plasmids of cpe^+/IS*1151*$^+$ type A isolates and cpe^+/IS*1470*-like$^+$ type A isolates share any similarity outside the sequenced *cpe-cpb2* region of pCPF5603.

It has also been shown that pCPF4969 can transfer, via conjugation, between *C. perfringens* isolates (5). Unpublished results referred to in a recent review (24) suggest that at least some type D isolates may also conjugatively transfer the plasmid carrying the epsilon toxin gene (*etx*). Thus, conjugative transfer of *C. perfringens* virulence plasmids, including at least some *cpe*-carrying plasmids, probably contributes to toxin production diversity among *C. perfringens* isolates. Furthermore, Southern blot hybridization studies have detected some similarity between pCPF4969 and pCW3, a *C. perfringens* conjugative plasmid encoding tetracycline resistance (5, 26). However, the transfer mechanism of pCPF4969 or pCW3 is not currently known.

Although the *cpe*-carrying plasmids of type A SPOR/AAD isolates represent a paradigm for *C. perfringens* virulence plasmids, many important questions about these plasmids remain unanswered. For example, do pCPF5603 and pCPF4969 represent the full diversity of *cpe* plasmids among type A SPOR/AAD isolates? Do these two *cpe* plasmids share any common regions? Are other virulence genes present on the unsequenced portions of pCPF5603 and pCPF4969? What mechanism mediates pCPF4969 transfer? Do *cpe* plasmids of other type A AAD/SPOR isolates also conjugatively transfer between *C. perfringens* isolates? If so, do they use transfer mechanisms similar to those of pCPF4969? To gain insights into these important questions, the current study completely sequenced pCPF5603 and pCPF4969 and then used those sequencing results to evaluate, by PCR, the diversity of *cpe* plasmids among type A SPOR/AAD isolates.

MATERIALS AND METHODS

Bacterial culture conditions. *C. perfringens* isolates (Table 1) were grown overnight at 37°C in either TGY (3% tryptic soy broth [TSB; Becton, Dickinson and Company, MD], 2% glucose [Sigma Aldrich Co., MO], 1% yeast extract [Becton, Dickinson and Company, MD], 0.1% L-cysteine [Sigma Aldrich Co., MO]) or FTG (fluid thioglycolate; Difco Laboratories, MI) medium. For construction of plasmid DNA libraries, *C. perfringens* isolates F5603 and F4969 were grown for 6 to 8 h in TGY medium, while JIR4468 (5) was grown for 6 to 8 h in TGY medium with 10 μg/ml chloramphenicol (Sigma Aldrich Co., MO). *Escherichia coli* HB101 (Invitrogen, CA) was grown in TSB (Difco Laboratories, MI). For growth of recombinant *E. coli*, ampicillin (100 to 150 μg/ml; Wako Chemical Co, Japan) was added to either TSB broth or TSB agar plates.

Complete or partial sequencing of the *cpe* plasmids from *C. perfringens* type A SPOR isolates F4969, F5603, and F4013. Using a previously described method (22), crude plasmid extracts were prepared from 1.5-ml cultures of JIR4468 or type A SPOR isolate F4013 or F5603. Those crude plasmid preparations were digested overnight with XbaI or HindIII (Roche Applied Science, IN, or New England Biolabs, MA) and then electrophoresed on a 1% agarose gel. Fragments (4 to 8 kb) were extracted from each gel using the Prep-A Gene DNA purification kit (Bio-Rad, CA) or Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad, CA). The extracted fragments were then ligated into digested and dephosphorylated pBlueScript II SK+ vector (Invitrogen, CA), using T4 DNA ligase (Roche Applied Science, IN) for 16 to 18 h at 16°C. Those recombinant plasmids were transformed into chemically competent *E. coli* HB101 and selected on TSB agar plates containing 100 μg/ml ampicillin.

Preliminary studies (data not shown) revealed that F4969 carries multiple plasmids. Since the presence of several plasmids, of unknown sequence, would technically complicate the isolation and sequencing of pCPF4969 from F4969, we instead performed pCPF4969 sequencing using JIR4468. JIR4468 is a previously prepared (5) *C. perfringens* strain 13 derivative transformed with pMRS4969, which is pCPF4969 with its *cpe* gene specifically inactivated by insertion of a chloramphenicol resistance marker. The rationale for using JIR4468 as the DNA source for pCPF4969 sequencing was that (i) a previous genome sequencing project had identified pCP13 as the only plasmid naturally present in strain 13, (ii) the pCP13 sequence was known, and (iii) 8 kb of sequence containing the complete pCPF4969 *cpe* locus was already available. Using pMRS4969 sequences for primer design, we then completely sequenced pCPF4969.

Furthermore, previous studies had suggested that pCPF4969 carries some sequences homologous to pCW3 (5), so transformants carrying potential *cpe* plasmid sequences were initially identified on the basis of Southern blotting with pCW3 probes. Briefly, transformants carrying DNA from JIR4468 or F5063 were electrophoresed on 1% agarose gels for 16–18 h, plasmid DNA fragments were transferred onto nylon membranes (Roche Applied Science, CA). Those membranes were then subjected to Southern blotting using a randomly digoxigenin (DIG)-labeled plasmid carrying a ClaI fragment of pCW3 (either pJIR15, pJIR16, pJIR18, or pJIR32), which had been prepared as described previously (5). Hybridized probes were detected with CSPD ready-to-use substrate (Roche Applied Science, CA). Transformants carrying plasmid DNA inserts hybridizing with pCW3 probes were then selected and their plasmid inserts were sequenced; the resulting sequence was compared against the previously determined (27) genome sequence of *C. perfringens* strain 13 and (for pCPF4969 sequencing) the pCP13 sequence in order to eliminate transformants carrying non-*cpe* plasmid DNA inserts from the plasmid library.

After assembling large contigs carrying plasmid *cpe* sequences by the above approaches, long-range PCR analysis was performed to close the remaining gaps between the contigs using the Expand Long Template PCR System from Roche Applied Science (CA). Briefly, crude plasmid preparations were used as DNA template for this long-range PCR, and primers 4-21DCMUP/3-03FD0, M-09FD/4-01FD1, and Xb-D2/1-45FD (see Table SI in the supplemental material) were designed to amplify products from the ends of the contigs using the following PCR program: 94°C for 1 min, 35 cycles of 94°C for 20 s, 55°C for 30 s, and 68°C for 8 min, and a final extension of 68°C for 12 min. The obtained long-range PCR products were then used as a DNA sequencing template to close each *cpe* plasmid sequence.

For sequencing the variable region of the *cpe* plasmid (pCPF4013) in type A SPOR isolate F4013, long-range PCR analysis was performed between the conserved region and the *cpb2* gene using *cpb2* gene primers 1F or 2R (11). Direct sequencing was then carried out using these long-range PCR products as a template.

Bioinformatic analyses of *cpe* plasmids. ORFs in pCPF4969, pCPF5603, and the variable region of pCPF4013 were identified using ORF Finder, which is available from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), while putative Shine-Delgarno sequences were detected manually. Identified ORFs were then used for database searches with FASTA (http://fasta.genome.jp/) and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) to identify homology or similarity with known genes or proteins. Results from these analyses, including all nucleotide sequences and ORFs, are located in GenBank under accession numbers AB236336 (pCPF4969), AB236337 (pCPF5603), and AB236338 (pCPF4013 variable region).

PFGE Southern blotting analysis of *cpe* plasmids in representative *C. perfringens* AAD/SPOR type A isolates. Except for slightly modifying the electrophoresis conditions to improve resolution of *cpe* plasmid size, pulsed-field gel electrophogesis (PFGE) was carried out as previously described by Fisher et al. (11). Briefly, specified AAD or SPOR isolates were grown overnight in TGY medium for DNA plug preparation. Following bacteria lysis, processed plugs were loaded onto 1% PFGE certified agarose gels (Bio-Rad) and run under the following conditions: 6 V/cm using ramped pulse times from 1 to 25 s for 24 h at 14°C. MidRange II PFGE markers (Bio-Rad) were used to determine plasmid size. Following electrophoresis, gels were stained with ethidium bromide to detect DNA. After detection, the DNA was transferred to positively charged nylon membranes (Roche Applied Science), prepared for Southern blotting, and hybridized with a DIG-labeled *cpe* probe as previously described (8). DIG labeling and detection reagents were obtained from Roche Applied Science. CSPD substrate (Roche Applied Science) was used for detection as directed by the manufacturer.

PCR analyses to determine whether *cpe* plasmids of other type A AAD/SPOR isolates carry the conserved region (including putative transfer ORFs) and variable regions of pCPF5603 or pCPF4969. Template DNA for all subsequent PCRs was obtained from colony lysates, which were prepared as described previously (32). Briefly, *C. perfringens* type A SPOR or AAD isolates carrying *cpe* plasmids were grown overnight in FTG broth and then streaked onto brain heart infusion agar plates (Difco Laboratories, MI). Following overnight growth, several colonies were picked from each brain heart infusion agar plate and lysed in sterile water by using a microwave to heat the sample.

Each PCR mixture contained 5 μl of template DNA, 40 μl of TAQ Complete 1.1× Master Mix (Gene Choice, Frederick, Maryland), and 2.5 μl of each primer pair (1 μM final concentration). Primers used for investigating conserved regions are listed in Table SII in the supplemental material, while Tables SIII and SIV list primers for assessing the variable regions of pCPF4969 and pCPF5603, respectively. To amplify portions of the putative pCPF4969 transfer region, the primers listed in Table SV in the supplemental material were used. PCRs were performed in a Techne (Burkhardtsdorf, Germany) thermocycler using the following conditions: 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s, and a single extension of 72°C for 5 min for the conserved region; 94°C for 5 min, 40 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 100 s, and a single extension of 72°C for 10 min for the pCPF4969 and pCPF5603 variable regions; and 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 100 s, and a single extension of 72°C for 10 min for the transfer region. PCR products were separated on 1% agarose gels and visualized with ethidium bromide staining.

Long-range PCR analyses to determine whether the pCPF4969/pCPF5603 conserved region is present in other *C. perfringens* isolates. The presence and orientation of several genes residing in the conserved region of pCPF4969/pCPF5603 (see Results) was confirmed by long-range PCR analyses of eight type A isolates carrying *cpe* plasmids and four non-type A strains. These long-range PCR analyses used the Expand Long Template PCR system from Roche Applied Science (CA) and the following primers: met-up3FD1/met-up1 for the ORF16 homolog-*dcm* region, ORF13HF/met-up3-4 for the ORF13 homolog-ORF16-homolog region, damF/3-3FU4 for the ORF13 homolog-*dam* region, cna-F/dam-F for the *dam-cna* region, and 8-118E/C1-6T3D for the *cna*-pCP59 homolog region (primer sequences are listed in Table SVI in the supplemental material). PCR was performed using crude plasmid preparations as a template and the following amplification conditions: 94°C for 1 min, 35 cycles of 94°C for 20 s, 55°C for 30 s, and 68°C for 8 min, and a final extension of 68°C for 12 min.

RESULTS

PFGE Southern blotting analyses to compare *cpe* plasmid size among representative *C. perfringens* type A AAD or SPOR isolates. As mentioned in the introduction, previous studies (22) determined that pCPF4969 and pCPF5603 differ in terms of the IS element downstream of their *cpe* genes, the presence or absence of *cpb2*, and the presence or absence of an ~19-kb

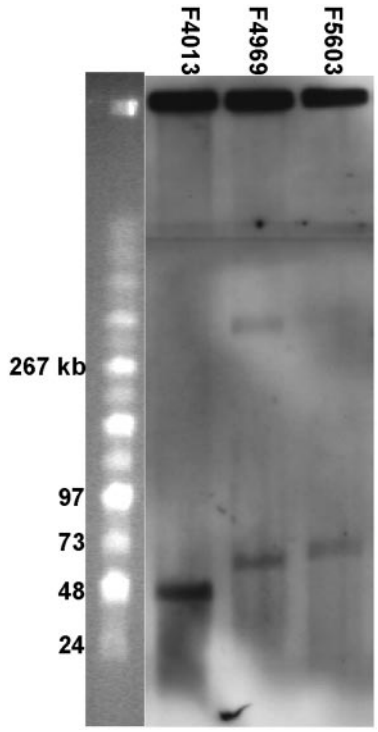

FIG. 1. PFGE/Southern blotting analysis of the *cpe* plasmid in select *C. perfringens* type A isolates. After PFGE, DNA was transferred to nylon membranes and probed with a DIG-labeled *cpe* probe. Southern blotting results are shown for *cpe*$^+$/IS*1470*-like$^+$ isolate F4969 (lane 3) and *cpe*$^+$/IS*1151*$^+$ isolates F5603 and F4013. Ethidium bromide-stained molecular weight markers are shown in the leftmost lane (sizes in kilobases are listed to the left of the markers).

cpe-cpb2 region (11), i.e., at least two distinct *cpe* plasmids exist among type A non-food-borne human GI disease isolates. In addition, overlapping PCR analyses found variations between the *cpe-cpb2* regions of F5603 and F4013, another plasmid *cpe*$^+$/IS*1151*$^+$ type A isolate (11). That preliminary finding indicated that some variation can occur even among *cpe* plasmids carrying the same IS element, although the extent of those variations was unknown.

Collectively, previous studies had only begun exploring the diversity of *cpe* plasmids among type A SPOR and AAD isolates. Even size variations between *cpe* plasmids from different type A SPOR/AAD isolates had not been clearly established due to the limited resolution of prior PFGE/Southern blotting studies, which suggested that pCPF5603 and pCPF4969 were both ~75 kb in size (11). To more accurately determine the size of *cpe* plasmids in type A non-food-borne human GI disease isolates, for the current study we performed PFGE Southern blotting using modified conditions that better resolve ~50- to 100-kb plasmids. When applied to five type A *cpe*$^+$/IS*1151*$^+$ SPOR or AAD isolates (F5603, F4013, B2, B38, and H38094) and five type A *cpe*$^+$/IS*1470*-like$^+$ SPOR or AAD isolates (F4969, X5722, W43181, S43526, and F4396), these new Southern blotting studies identified three different *cpe* plasmid sizes among these 10 isolates (Fig. 1 shows representative results for type A isolates whose *cpe* plasmids were completely or partially sequenced in this study) (results not shown for other surveyed isolates). All five surveyed plasmid *cpe*$^+$/IS*1470*-like$^+$ isolates were found to carry *cpe* plasmids of ~70 kb. In contrast, the *cpe* plasmid was ~75 kb in all surveyed plasmid *cpe*$^+$/IS*1151*$^+$ isolates except for F4013, which carries a *cpe* plasmid of only ~54 kb.

Sequencing of pCPF4969. Previous studies sequenced an ~8-kb region of the conjugative *cpe* plasmid pCPF4969 and found (in order) a putative DNA cytosine methylase (*dcm*), an IS*1469* element, the *cpe* gene, and a defective IS*1470*-like element (22). Coupling those previous findings with the PFGE/Southern blotting results shown in Fig. 1 suggested that ~62 kb of pCPF4969 sequence remained unknown. To determine whether this unknown pCPF4969 sequence could carry other virulence genes or genes potentially involved in conjugation or plasmid replication, pCPF4969 was completely sequenced.

This sequencing analysis indicated that the size of pCPF4969 is 70,480 bp (Fig. 2), in close agreement with the PFGE/Southern blotting results shown in Fig. 1. Using the NCBI ORF Finder, 62 ORFs with an average length of 835 nucleotides were identified, giving pCPF4969 a coding density of 73.5%. Of those 62 ORFs, ~25% classified as encoding hypothetical proteins with unknown functions. The GC content, 26.6%, is similar to that of the two previously sequenced *C. perfringens* plasmids (i.e., the 54.3-kb pCP13 carrying translationally silent *cpb2* sequences and the 10.2-kb, bacteriocin-encoding plasmid pIP404) but slightly lower than that of the *C. perfringens* type A strain 13 genome, 28.6% (27). Interestingly, several ORFs found on pCP13, including PCP56, PCP57 (*cna*), PCP58, PCP59, and PCP12, had similarly arranged homologs on pCPF4969. In addition, pCPF4969 carries homologs to pCP13 ORFs PCP60 and PCP61, which have low similarity at the nucleotide level but strong similarity at the amino acid level.

Known or potential virulence genes carried on pCPF4969 include *cpe*, *srt* (sortase), and *cna* (collagen adhesin). In addition, a putative bacterial two-component regulator, which shows high similarity at the amino acid level (although not at the nucleotide level) to the chromosomal *C. perfringens* VirR/VirS two-component regulator of virulence genes (1), was also identified on pCPF4969. Sequence scanning allowing for up to three base pair mismatches did not reveal the presence of any VirR binding boxes on pCPF4969. Moreover, the *C. perfringens* VirR DNA binding motif SKHR was not identified in the pCPF4969 VirR homolog (20). However, the conserved FxHxxKxS VirRc family motif (19) was found in the pCPF4969 VirR homolog.

pCPF4969 also putatively encodes two bacteriocins. The first bacteriocin shows amino acid similarity to propionicin SM1 (21) from *Propionibacterium jensenii* (e value, 1e−7) and is encoded by a gene cluster also containing a possible bacteriocin transporter. A second bacteriocin is apparently encoded by an operon comprised of nine ORFs whose products show amino acid similarity to SpaR, -K, -F, -E, -G, -C, -T, -B, and -S (e values ranging from 6e−4 to 3e−116), which is an operon involved in the synthesis and secretion of peptide-derived antimicrobial compounds known as lantibiotics (28).

Concerning putative replication origins, the *ori*-related repeat sequences found on pIP404 (13, 14) were not present on pCPF4969. GC skew analysis has successfully predicted the *ori* region from the *Bacillus thuringiensis* toxin plasmid pBtoxis (2), but similar GC skew analysis performed using Artemis soft-

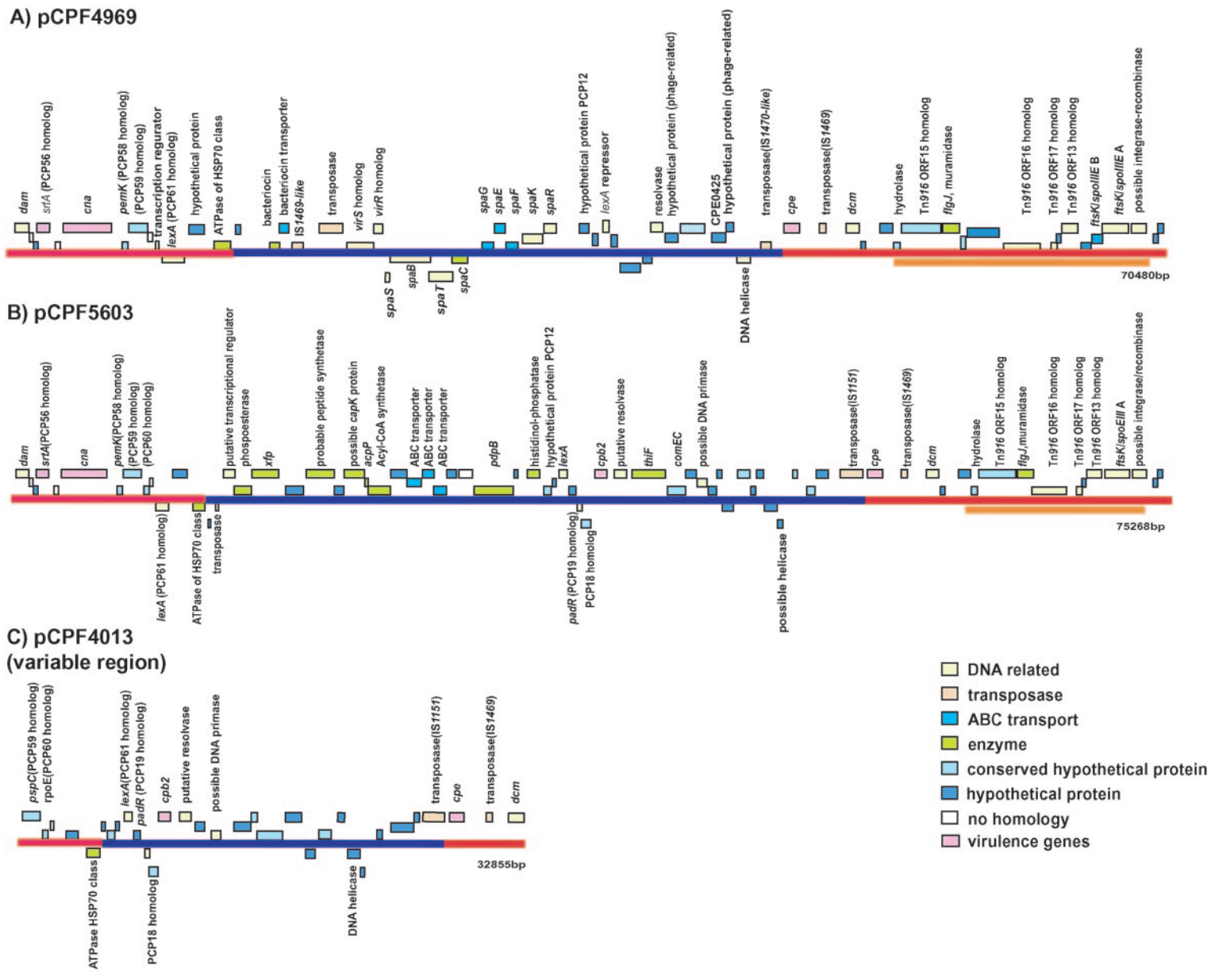

FIG. 2. Complete or partial maps of the *cpe* plasmids sequenced in this study. Maps display ORFs identified by ORF Finder using the *cpe* plasmid sequences obtained during this study. Complete maps of the *cpe* plasmid are shown for pCPF4969 (A) and pCPF5603 (B), and a partial map is shown for the variable region of pCPF4013 (C). For each map, a blue line highlights the putative variable region, red lines mark the putative conserved region, and an orange line delineates genes comprising the putative transfer region (if shown). Potential roles of each ORF are identified by the key in the lower right-hand corner of the figure.

ware failed to identify any putative *ori* region on pCPF4969. ORFs encoding putative primase, resolvase, and helicase were identified from the pCPF4969 sequence.

As mentioned earlier, past studies have shown that pCPF4969 can transfer via conjugation (5). Sequence analysis of pCPF4969 revealed the presence of an ORF cluster encoding proteins with amino acid similarity to those encoded by the conjugative transposon Tn*916* from *Enterococcus faecalis* (7). Since Tn*916* is capable of transferring in most gram-positive bacteria, the discovery of this Tn*916*-related conjugation machinery provides an attractive potential mechanism for pCPF4969 conjugative transfer. The putative pCPF4969 transfer region specifically carries ORFs encoding products showing similarity to a hydrolase (homologous to Tn*916* ORF14), a muramidase, and an integrase/recombinase as well as Tn*916* ORFs 13, 15, 16, 17, and 21. pCPF4969 also appears to encode an FtsK/SpoEIII homolog that shares amino acid similarity with the product of Tn*916* ORF21, which is thought to act as a DNA translocase. However, no Tn*916* regulatory genes, such as *xis* and *int*, were apparent on pCPF4969. Interestingly, a region downstream of the putative transfer region contains an ORF that putatively encodes a protein whose C-terminal region (amino acids 126 to 223) has homology to the N-terminal half of an integrase. No putative oriT sequence homologous with Tn*916* oriT (15) or with the RS_A sequence of Tn*4451* was identified on pCPF4969 (10).

Sequencing of pCPF5603. Previous PCR analyses (11) had determined that the 19-kb *cpb2-cpe* region of pCPF5603 is absent from pCPF4969, while the PFGE/Southern blotting results shown in Fig. 1 indicated that pCPF5603 is ~5 kb larger than pCPF4969. Since those data indicated that substantial size and sequence differences must exist between pCPF4969 and pCPF5603, pCPF5603 was also completely sequenced. Those analyses determined this *cpe* plasmid to be 75,280 bp, with a GC content of 25.3%, similar to the %GC content of pCPF4969 and the two other sequenced *C. perfringens* plasmids (13, 27). pCPF5603 carries 73 ORFs, each with an average length of 819 bp, giving this plasmid a coding density of ~79%. Of the 73 pCPF5603 ORFs, ~25% classified as encoding hypothetical proteins with unknown functions.

Comparison of pCPF5603 and pCPF4969 sequences identified strong similarity across an ~35-kb region extending from *cpe* to the ORF encoding an ATPase of Hsp70 (Fig. 2). This largely conserved sequence includes the putative Tn*916*-related transfer region and *cpe*, as well as *cna*, *srt*, and the other pCP13-related ORFs present on pCPF4969. The conserved region of pCPF4969 and pCPF5603 contains only two short stretches of divergent sequence (pCPF4969 carries an ~3-kb insertion between *flgJ* and ORF16 and a second 1.9-kb insertion between ORF13 and *ftskA* in the putative transfer region, both of which are missing from pCPF5603).

The conserved region shared by pCPF4969 and pCPF5603 comprises ≤50% of the total sequence for these two *cpe* plasmids, leaving an ~34.8-kb variable region for pCPF4969 and an ~43.6-kb variable region for pCPF5603 (Fig. 2). The size differences between these two variable regions, combined with the total determined sequences for pCPF4969 and pCPF5603, are consistent with PFGE/Southern blotting results shown in Fig. 1, indicating pCPF5603 to be ~5 kb larger than pCPF4969.

Similar to pCPF4969, no obvious replication region was identified in pCPF5603 by either sequence comparison against known *C. perfringens* replication regions or by using Artemis software. Regions of interest that are present on pCPF4969, but absent on pCPF5603, include the VirR/VirS homolog and the putative bacteriocins.

The unique region of pCPF5603 is flanked by two sequences that are, or are related to, insertion sequences, i.e., IS*1151* (which lies upstream of *cpe*) and a putative transposase showing similarity (e value, 1e−13) to a transposase for insertion sequence element ISRm3 from *Bacteroides thetaiotaomicron*. The pCPF5603-specific region carries the potential virulence gene *cpb2*, which is known to be expressed by F5603, as well as a large number of metabolism-related ORFs (11). Of those metabolism-related ORFs, a histidinol phosphatase ORF has been previously found in several *C. perfringens* strains (based on BLAST search results), although not in *C. perfringens* type A strain 13 (27). An ORF carrying the carbohydrate metabolism-related gene, *xrf*, has not been previously reported in *C. perfringens*. Several other potential metabolic ORFs, including those apparently encoding a predicted metal-dependent phosphoesterase, an acyl-CoA synthetase, and a putative acyl carrier protein could play a role in lipid metabolism. An ORF putatively encoding a peptide synthetase was also found. Three ORFs apparently encoding putative ABC type transport system components, two of which showed homology with systems found on the strain 13 chromosome (27), were also identified.

PCR survey to determine whether the pCPF4969/pCPF5603 conserved region ORFs are present in other plasmid *cpe*$^+$ *C. perfringens* type A isolates. To survey whether the largely conserved region of pCPF5603/pCPF4969 is present in other type A isolates carrying *cpe* plasmids, two PCR assays were used. The first PCR amplified internal portions of ORFs located in the largely conserved region of the pCPF4969/pCPF5603 plasmids, which allowed us to rapidly assess the presence of those ORFs in other type A isolates carrying *cpe* plasmids. ORFs selected for internal amplification were distributed throughout the common region of pCPF4969 and pCPF5603 and included the Tn*916*-related ORFs (ORF15, *flgJ*, ORF16, ORF13, and *ftsKA*), *dam*, *srt*, *cna*, and the homolog of pCP13 PCP59 (Fig. 3A and Table SVII in the supplemental material). Five plasmid *cpe*$^+$/IS*1470*-like$^+$ type A AAD or SPOR isolates (including F4969), one *cpe*$^+$/IS*1470*-like$^+$ type A isolate from a healthy human (MR2-4), six plasmid *cpe*$^+$/IS*1151*$^+$ type A AAD or SPOR isolates (including F5603), and two *cpe*-negative *C. perfringens* type A strains (strain 13, which carries pCP13, and ATCC 3624) were surveyed with this internal ORF PCR battery.

Consistent with previous sequencing results (27), this PCR battery only amplified the *cna* product from pCP13 (the primers used to amplify internal sequences from *srt* and the pCPF4969/pCPF5603 homolog of PCP59 from pCP13 shared <75% homology with their corresponding pCP13 sequences, explaining why these *srt* and PCP59 primer pairs did not amplify any products from strain 13). Furthermore, no PCR products were amplified from *cpe*-negative type A isolate ATCC 3624 (data not shown). However, this PCR battery produced positive results for all 12 surveyed type A isolates carrying *cpe* plasmids, with the exception that negative results were obtained with (i) isolate MR2-4 using primers for the *ftsKA* and

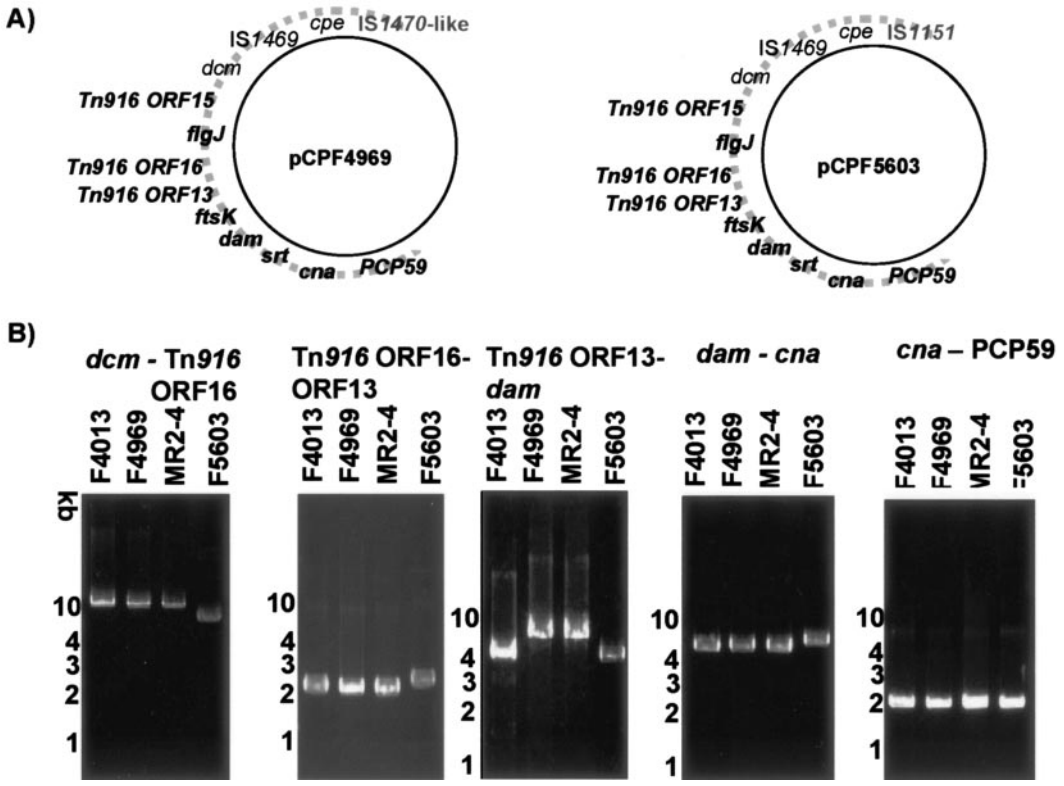

FIG. 3. PCR analyses of the putative conserved region in *cpe* plasmids of type A isolates. (A) The putative conserved regions of pCPF4969 and pCPF5603 are highlighted by gray dashed lines. An initial PCR assay used primers to amplify internal portions of representative genes (shown in bold) to assess the presence of these ORFs among plasmid *cpe*$^+$ *C. perfringens* type A isolates. Results for this internal assay were almost always positive for all tested isolates (see Table SVII in the supplemental material). (B) To assess the orientation of ORFs located in the conserved region, primers were designed to link ORFs located throughout the conserved region by long-range PCR. Representative results are shown for *cpe*$^+$/IS*1470*-like$^+$ isolates F4969 and MR2-4 and for *cpe*$^+$/IS*1151*$^+$ isolates F5603 and F4013. PCR products were run on 0.8% agarose gels and visualized with ethidium bromide. Molecular weight markers and sizes are shown to the left of each gel.

ORF15 homologs and (ii) isolate X5722 using primers for the *ftsKA* homolog (see Table SVII in the supplemental material). Based upon previous PCR results (22), Table SVII also indicates the presence of *dcm* and IS*1469* in the conserved region of all these type A plasmid *cpe*$^+$/IS*1470*-like$^+$ isolates and type A plasmid *cpe*$^+$/IS*1151*$^+$ isolates.

Since the surveyed ORFs in the largely conserved region of pCPF4969/pCPF5603 generally appear to be present in the 12 surveyed plasmid *cpe*$^+$ type A human isolates, a second, long-range, PCR assay was used as a rapid screen to determine whether these ORFs are arranged similarly as found in the common region of pCPF4969/pCPF5603, i.e., can these ORFs be connected together in a similar pattern of PCR products as produced from the pCPF5603/pCPF4969 conserved region? Common region ORFs selected for long-range PCR amplification linkage included *dcm*-ORF16, ORF13-ORF16, ORF13-*dam*, *dam-cna*, and *cna*-PCP59. Expected product sizes for these PCRs were 7.8, 3.1, 5.0, 5.9, and 2.8 kb from pCPF5603 and 10.4, 2.9, 6.8, 5.6, and 2.7 kb from pCPF4969. The slightly larger size of the *dcm*-ORF16 and ORF13-*dam* PCR products amplified from pCPF4969 versus pCPF5603 is due to the pCPF4969 conserved region carrying (respectively) an ~3-kb insertion between *flgJ* and ORF16 and a second ~2-kb insertion containing a 1-kb copy of *ftsKB* inserted between ORF13 and *dam* (Fig. 2). The slightly smaller ORF13-ORF16 PCR product amplified from pCPF4969 versus pCPF5603 is due to the presence of a 0.2-kb deletion of intervening sequence between these two ORFs in pCPF4969.

When F4969 and F5603 were subjected to this long-range PCR battery, products of the expected size were consistently obtained (Fig. 3B). Three additional plasmid *cpe*$^+$/IS*1470*-like$^+$ type A isolates (MR2-4, F4396, and X5722) were also tested with this PCR battery and yielded long-range PCR products consistent with their carrying ORFs in an arrangement similar to that found in the largely conserved region of pCPF4969 (Fig. 3B and data not shown). Likewise, three other plasmid *cpe*$^+$/IS*1151*$^+$ type A isolates (F4013, NB16, and H38094) supported amplification of similar-sized PCR products as those amplified from F5603. However, with plasmid *cpe*$^+$/IS*1151*$^+$ type A isolate F4013, primers that amplify the *dcm*-ORF16, ORF13-ORF16, and *dam-cna* regions amplified PCR products more closely matching the product size expected from the IS*1470*-like isolate F4969 than from the plasmid *cpe*$^+$/IS*1151*$^+$ type A isolate F5603. However, the ORF13-*dam* and *cna*-PCP59 products amplified from F4103 more closely matched the PCR products expected from F5603.

As expected from the pCP13 sequence (27), this long-range PCR battery failed to amplify any products from strain 13, except for the *cna*-PCP59 reaction.

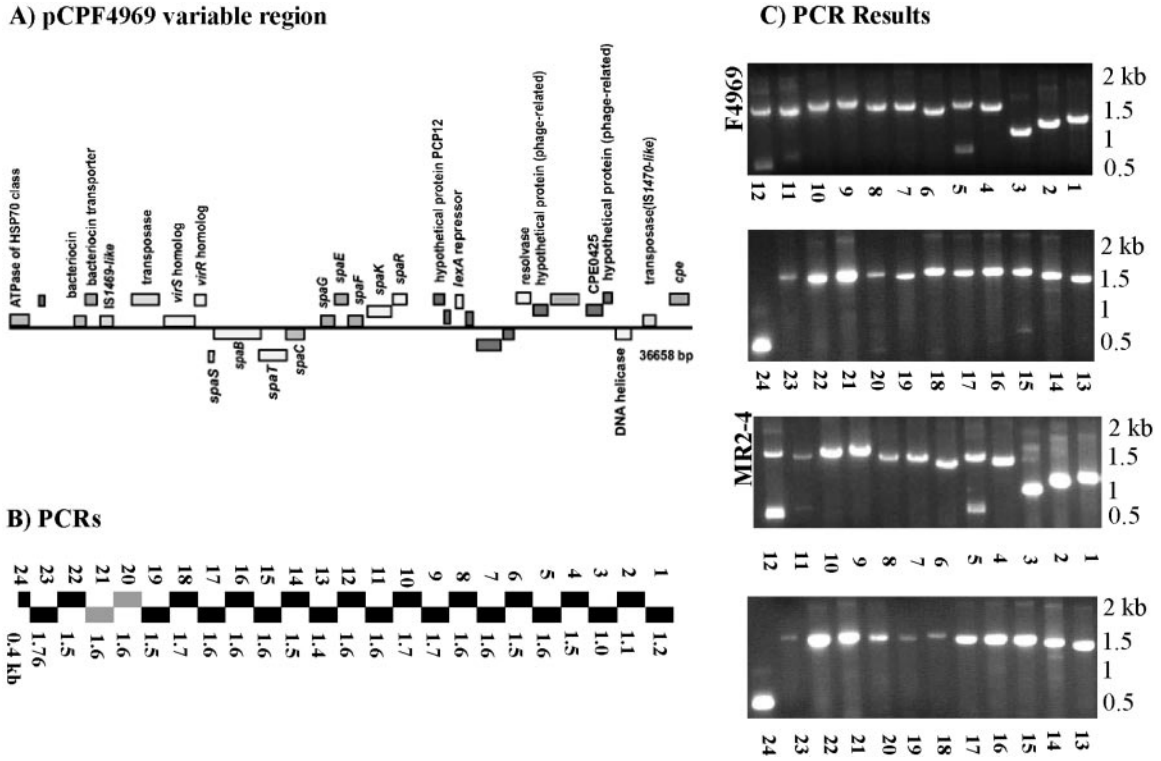

FIG. 4. Overlapping PCR survey of plasmid *cpe*$^+$ *C. perfringens* type A isolates using primers designed to amplify the pCPF4969 variable region. (A) Overlapping primers were designed to amplify the entire pCPF4969 variable region spanning from the ATPase of Hsp70 class homolog to *cpe*. (B) These 24 reactions were used to survey six plasmid *cpe*$^+$/IS*1470*-like$^+$ and six plasmid *cpe*$^+$/IS*1151*$^+$ *C. perfringens* type A isolates. (C) Results are shown for two representative *cpe*$^+$/IS*1470*-like$^+$ isolates, F4969 and MR2-4. Expected PCR product sizes are shown in panel B below the bars. PCR products were separated on 1% agarose gels and visualized with ethidium bromide staining. Molecular weight markers are indicated at the right of each gel. The gray bars in panel B indicate reactions that were not consistently amplified from all *cpe*$^+$/IS*1470*-like$^+$ isolates. Complete results from this PCR survey are shown in Table SVIII in the supplemental material.

Sequencing of the variable region in the F4013 *cpe* plasmid. In addition to the minor atypical features within its conserved region identified in Fig. 3, two other results indicated that the *cpe* plasmid of F4013 is unusual. A previous overlapping PCR assay covering the 19-kb *cpe-cpb2* variable region of pCPF5603 had identified the variable region of pCPF4013 as being different from other type A *cpe*$^+$/IS*1151*$^+$ plasmids (11). Second, the PFGE/Southern blotting results shown in Fig. 1 had demonstrated that pCPF4013 is much smaller than either pCPF4969 or pCPF5603. Collectively, these results suggested that the smaller pCPF4013 could be carrying a third kind of *cpe* plasmid variable region. To assess this possibility, the pCPF4013 variable region was completely sequenced.

This sequencing analysis confirmed significant differences between the variable region of pCPF4013 versus pCPF4969 or pCPF5603. The pCPF4013 variable region is significantly smaller (~24 kb) than the variable region of either pCPF4969 or pCPF5603, although it more closely resembles the variable region of pCPF5603 (Fig. 2). Most differences between pCPF4013 and pCPF5603 can be attributed to ~20 kb of the pCPF5603 variable region being absent from pCPF4013. Specifically, pCPF4013 lacks pCPF5603 variable region sequences present between the ORF putatively encoding an ATPase of Hsp70 and the *padR* ORF, i.e., nearly the entire ~20-kb metabolism-related gene cluster of pCPF5603 is absent from pCPF4013. Minor differences between pCPF5603 and pCPF4013, including the absence of *thiF* and *comEC* from pCPF4013, were also apparent in the region between *cpb2* and *cpe*.

Overlapping PCR analyses to determine whether the pCPF4969 variable region is present among other type A isolates carrying *cpe* plasmids. The presence of the pCPF4969 variable region among type A isolates carrying *cpe* plasmids was determined using an overlapping PCR battery that included 24 pairs of overlapping primers to directly connect ~36 kb of pCPF4969 sequence that spans from *cpe* to the ATPase of the Hsp70 class homolog (Fig. 4A and B). When used with F4969 as a positive control, this PCR battery produced products of the expected size for all 24 PCRs (Fig. 4C and Table SVIII in the supplemental material). When similarly applied to five other *cpe*$^+$/IS*1470-like*$^+$ type A isolates (four AAD or SPOR isolates and one isolate from a healthy human), this PCR battery produced results consistent with these five isolates all possessing the entire pCPF4969 variable region (Fig. 4C shows representative PCR results; also see Table SVIII in the supplemental material). The only consistent exceptions were that reactions 20 and 21 (Fig. 4B), which pass through a putative transposon gene in pCPF4969, were not amplified from four of the other five surveyed plasmid *cpe*$^+$/IS*1470*-like$^+$ type A isolates.

In contrast, six plasmid *cpe*$^+$/IS*1151*$^+$ *C. perfringens* type A

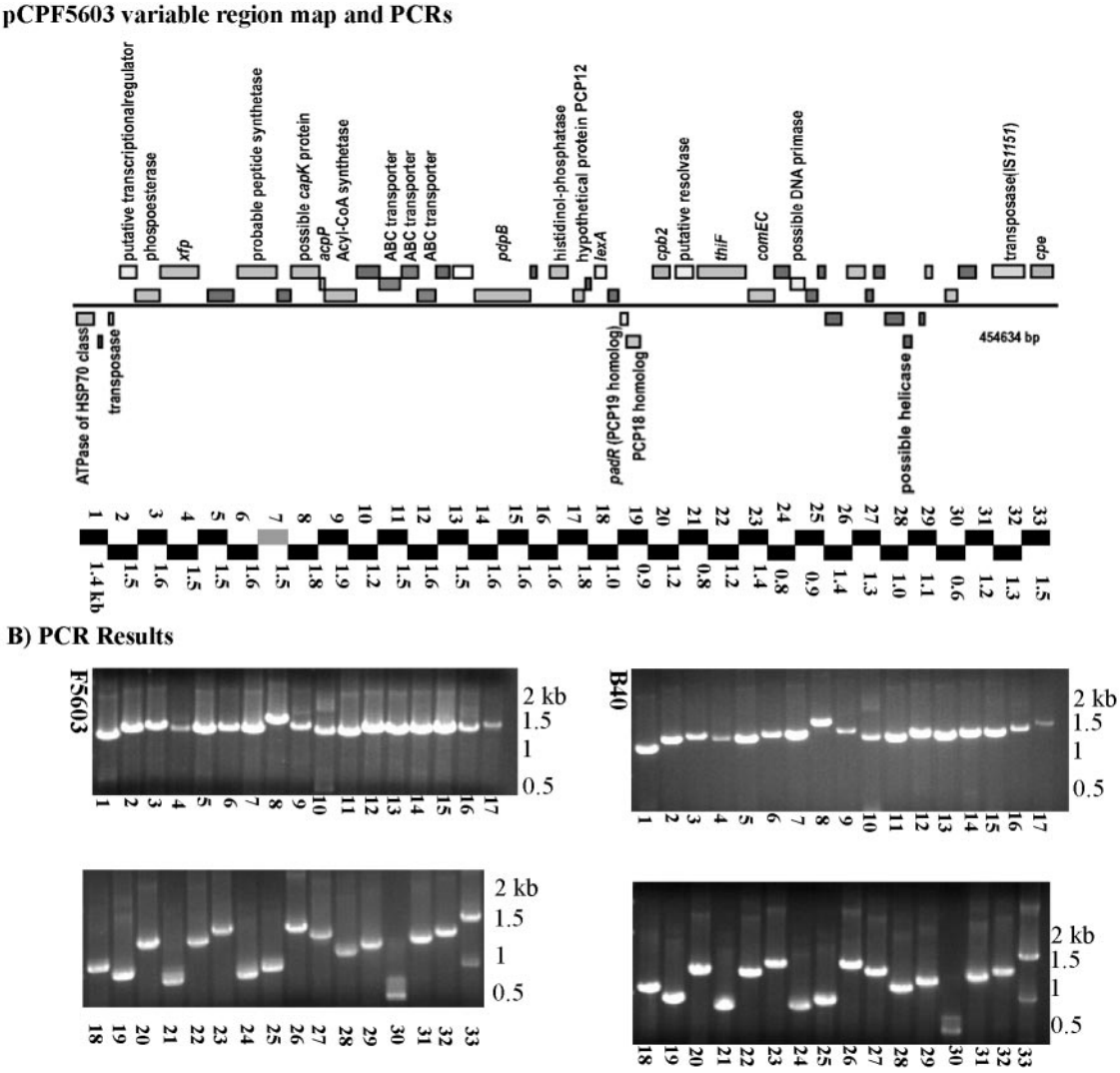

FIG. 5. Analysis of the pCPF5603 variable region in plasmid *cpe*$^+$ *C. perfringens* type A isolates using an overlapping PCR assay. (A) The pCPF5603 variable region (from the ATPase of Hsp70 class homolog to *cpe*) was amplified using 33 overlapping PCRs (black bars). PCR products were analyzed on 1% agarose gels and visualized with ethidium bromide staining. Using this PCR assay, the presence of the pCPF5603 variable region was assessed in six plasmid *cpe*$^+$/IS*1470*-like$^+$ and six plasmid *cpe*$^+$/IS*1151*$^+$ *C. perfringens* type A isolates. PCR reaction 7 (gray bar) was not consistently amplified by *cpe*$^+$/IS*1151*$^+$ isolates. (B) Representative PCR survey results are shown for *cpe*$^+$/IS*1151*$^+$ isolates F5603 and B40. The locations of molecular weight markers are indicated to the right of each gel. PCR results for all surveyed isolates are listed in Table SIX in the supplemental material.

isolates (all associated with AAD or SPOR) failed to support amplification of nearly all 24 reactions included in the pCPF4969 variable region PCR battery. The only consistent exceptions were for PCRs 4, 9, and 24, which amplified a region encoding two hypothetical proteins, a region encoding a homolog to PCP12 from pCP13 and a hypothetical protein, and a region encoding an internal portion of the Hsp70 ATPase homolog, respectively. All three of these DNA sequences should yield positive PCR results from *cpe*$^+$/IS*1151*$^+$ type A isolates since they are also found on pCPF5603.

Supporting the specificity of this PCR battery, strain 13 did not yield positive PCRs for the pCPF4969 variable region, consistent with the complete absence of this region from pCP13.

Overlapping PCR analyses to determine whether the pCPF5603 variable region is present among other type A isolates carrying *cpe* plasmids. A second overlapping PCR assay was designed to survey the presence of the pCPF5603 variable region among type A isolates carrying *cpe* plasmids. This PCR battery included 33 overlapping PCRs that span the entire ~45-kb pCPF5603 region from *cpe* to the Hsp70 ATPase homolog (Fig. 5A). When applied to F5603 as a positive control, this PCR battery amplified products of the expected size for all 33 reactions (Fig. 5B and Table SIX in the supplemental material). With minor exceptions, this PCR battery also consistently amplified products of the expected size from four of five other surveyed plasmid *cpe*$^+$/IS*1151*$^+$ type A AAD or SPOR isolates (representative results are shown in Fig. 5B, with all

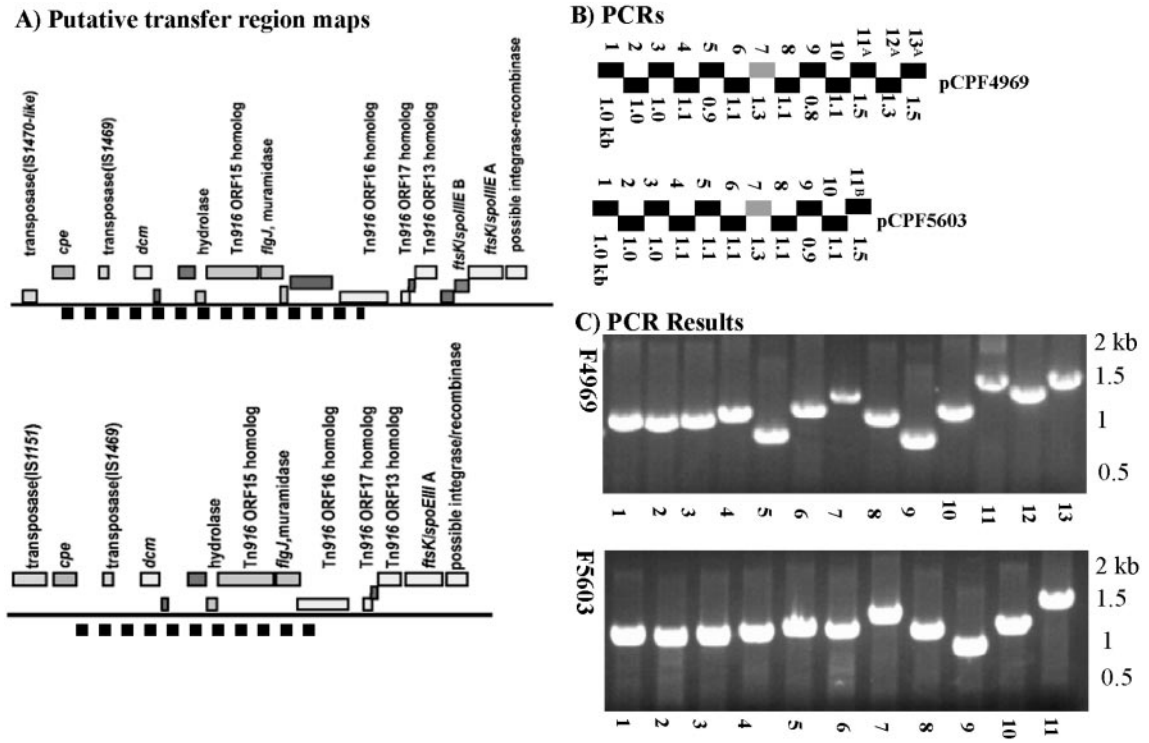

FIG. 6. PCR survey for the presence of the Tn*916*-related putative transfer region of pCPF4969 or pCPF5603 in other plasmid *cpe*$^+$ *C. perfringens* type A isolates. (A) Primers were designed to amplify a portion (dashed line) of the Tn*916*-related putative transfer regions from either pCPF4969 or pCPF5603. (B) Thirteen or eleven overlapping PCRs were used to survey six plasmid *cpe*$^+$/IS*1470*-like$^+$ and six plasmid *cpe*$^+$/IS*1151*$^+$ *C. perfringens* type A isolates for the presence of the putative transfer region. Reactions 1 to 10 were identical for both assays, whereas reactions 11A to 13A and reaction 11B were designed to amplify specific sequences in the pCPF4969 or pCPF5603 putative transfer region, respectively, as shown in panel B. Expected PCR product sizes are listed below the black bars in panel B. (C) Representative PCR results are shown for F5603 and F4969. Complete results from this survey are listed in Table SX in the supplemental material. PCR products were run on 1% agarose gels and stained with ethidium bromide. The positions of molecular weight markers are shown to the right of each gel.

results summarized in Table SIX in the supplemental material). The exception was *cpe*$^+$/IS*1151*$^+$ type A SPOR isolate F4013, which failed to amplify a substantial number of pCPF5603 variable region genes, consistent with the deletion of 20 kb of sequence from the pCPF4013 variable region detected by sequencing (Fig. 2).

In contrast, when this same PCR battery was applied to six plasmid *cpe*$^+$/IS*1470*-like$^+$ type A isolates (five associated with AAD or SPOR and one from a healthy human), only a few primer pairs consistently amplified products of the expected size. Those primers amplified the PCP12 homolog hypothetical protein (reaction 16) and regions flanking two hypothetical proteins (reactions 27 and 28), all of which involve sequences common to both the pCPF5603 and pCPF4969 sequence (Fig. 2).

As a negative control, this PCR battery did not amplify any products of the expected size from strain 13, which is consistent with the absence of this region from pCP13 (27).

Overlapping PCR analyses to determine whether the putative Tn*916*-related transfer region is present among other type A isolates carrying *cpe* plasmids. Initial long-range PCR testing for the presence of the pCPF4969 and pCPF5603 conserved regions (Fig. 3) indicated that all surveyed type A human isolates carrying *cpe* plasmids have the Tn*916*-related region found in the conjugative plasmid pCPF4969. However, those initial experiments sampled only a limited number of type A isolates carrying *cpe* plasmids and did not directly connect the Tn*916*-related region to the *cpe* gene present in those other type A isolates, i.e., conceivably, some or all of those isolates could carry their Tn*916*-related genes on either another, non-*cpe* carrying, plasmid or on their chromosome. Since establishing the presence of Tn*916*-related genes on *cpe* plasmids in other AAD/SPOR type A isolates has potential implications for predicting the conjugative transferability of those *cpe* plasmids, an overlapping PCR battery was constructed. This assay, which directly links the plasmid-borne *cpe* gene to the Tn*916*-related genes on pCPF4969 and pCPF5603, consisted of 10 common reactions and either 11 or 13 total PCRs (Fig. 6A and B), depending upon whether the primers were designed to amplify the transfer region of pCPF5603 or pCPF4969 (which contains a small insertion between the *flgJ* ORF and Tn*916* ORF16).

This transfer region PCR battery consistently amplified products of the expected size from all 12 surveyed type A isolates carrying *cpe* plasmids, 11 of which were associated with AAD or SPOR. The pattern of reactions amplifying products was highly consistent (see Table SX in the supplemental material; representative results are shown in Fig. 6C), depending upon whether the type A isolate was a plasmid *cpe*$^+$/IS*1151*$^+$ isolate (like F5603) or a plasmid *cpe*$^+$/IS*1470*-like$^+$ isolate

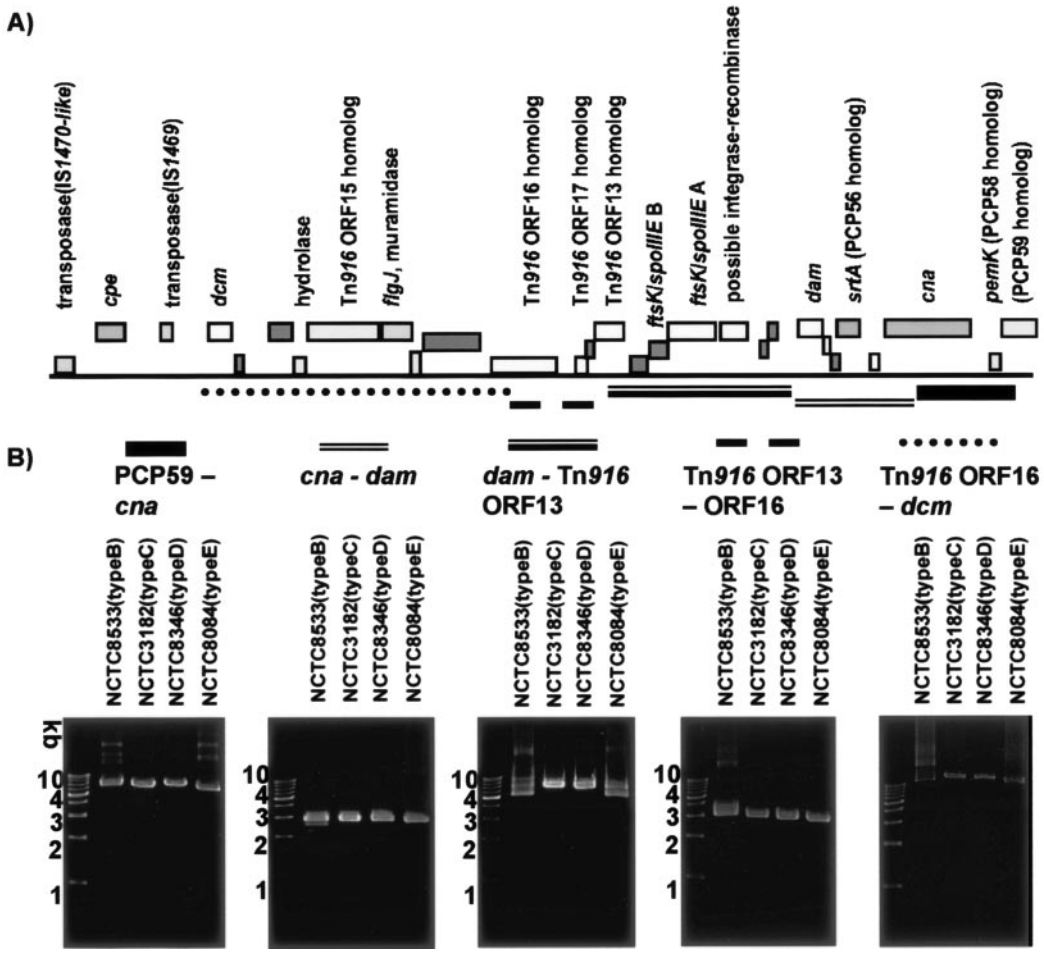

FIG. 7. PCR survey for the presence of the *cpe* plasmid conserved region in *C. perfringens* type B to D isolates. (A) Using the long-range PCR assay shown in Fig. 3, *C. perfringens* type B to D isolates were analyzed for the presence of the conserved region. (B) PCR products were separated on 0.8% agarose gels and stained with ethidium bromide. Results for each PCR are shown. Molecular weight markers are shown to the left of each gel. Isolate names and types are at the top of each gel.

(like F4969). The major exception was that reaction 7, which uses primers to connect the hydrolase homolog to Tn*916* ORF15, did not amplify a product in several isolates, regardless of their IS class (*cpe*$^+$/IS*1151*$^+$ or *cpe*$^+$/IS*1470*-like$^+$). Consistent with the long-range PCR results shown in Fig. 3, the plasmid *cpe*$^+$/IS*1151*$^+$ type A isolate F4013 was negative for pCPF5603-based PCR 11B but positive for pCPF4969-based reactions 11A to 13A.

Long-range PCR to determine whether the conserved region, including the putative pCPF4969 transfer ORFs, is present in other *C. perfringens* types. *C. perfringens* type B to E isolates all carry large virulence plasmids, which carry both the *etx* gene and the beta toxin gene (*cpb*), *cpb* only, *etx* only, or the iota toxin genes only, respectively (24). In addition, unpublished reports suggest that, like pCPF4969, the *etx* plasmid of type D isolates may transfer conjugatively. Collectively, those facts raised interest in testing whether the Tn*916*-related putative transfer ORFs of pCPF4969 and *cpe* plasmids in type A isolates are also present in representative *C. perfringens* type B to E isolates. To address that question, the long-range PCRs shown in Fig. 7A were used. Results obtained indicated the presence of the putative transfer region ORFs in each of the representative type B to E isolates (Fig. 7B). Each surveyed type B to E isolate supported PCR amplification of all five reactions that detect the presence of conserved region genes (including transfer ORFs). PCR product sizes generally matched those amplified from plasmid *cpe*$^+$ *C. perfringens* type A isolates. However, small variations in PCR product sizes for the *cna*-PCP59, Tn*916* ORF13-*dam*, and *dcm*-Tn*916* ORF16 PCRs were noted for particular isolates (Fig. 7B).

DISCUSSION

Toxin-encoding plasmids are important for the pathogenesis of many *C. perfringens* infections, but they have been poorly studied. The large size and low copy number of these plasmids complicates their isolation, analysis, and sequencing, as does the fact that many individual *C. perfringens* isolates (including both F4969 and F5603 [unpublished observations by Miyamoto et al.]) carry multiple plasmids. We overcame these technical challenges with a unique sequencing strategy that (i) employed JIR4468 (5), a strain 13 derivative transformed with

pMRS4969 (a slightly modified pCPF4969) to help with initial sequencing of pCPF4969, thereby eliminating other unsequenced plasmids from our screening library; and (ii) exploited the partial homology (5) between pCPF4969 and the *C. perfringens* tetracycline resistance-encoding plasmid pCW3 (26) by using pCW3-based probes for library screening, which substantially enriched for transformants carrying *cpe* plasmid inserts. Long-range PCR then closed gaps between contigs assembled from this pCW3 probe screening, allowing complete sequencing of the first two *C. perfringens* virulence plasmids carrying one or more functional toxin genes.

Our sequencing results revealed that pCPF4969 and pCPF5603 share an ~35-kb conserved region, along with (respectively) ~35- or ~40-kb variable regions. PCR surveys of other AAD or SPOR type A isolates from varied geographic origins strongly suggest that most *cpe*$^+$/IS*1151*$^+$ plasmids in AAD/SPOR type A isolates are similar to pCPF5603. The smaller F4013 *cpe* plasmid simply appears to be a pCPF5603 variant with an ~20-kb deletion in its variable region. Similarly, PCR surveys suggest that the *cpe*$^+$/IS*1470*-like$^+$ plasmids of most other AAD/SPOR type A isolates resemble pCPF4969. The existence of just two major *cpe* plasmid families in type A isolates is further supported by PCR results detecting a pCPF4969-like *cpe* plasmid in two type A plasmid *cpe*$^+$/IS*1470*-like animal disease isolates (22 and data not shown).

The two major *cpe* plasmid families of type A isolates apparently share a common evolutionary origin, based upon the strong similarity of the pCPF4969 and pCPF5603 conserved regions. This similarity includes the presence of nearly identical Tn*916*-related sequences, which suggests that an early step in *cpe* plasmid evolution involved Tn*916* integration onto a *C. perfringens* plasmid, perhaps one related to pCP13 (given the presence of some pCP13 ORF homologues in the pCPF5603/pCPF4969 conserved region). This integration likely created a progenitor conjugative plasmid that has subsequently transferred among type A isolates.

In one recipient, the progenitor plasmid probably acquired an IS*1469*-*cpe*-IS*1470* element to form the pCPF4969 *cpe* plasmid family. This progenitor plasmid may have simultaneously acquired both the IS*1469*-*cpe*-IS*1470*-like locus and the pCPF4969 variable region, consistent with the ability of IS*1469* and IS*1470* to mediate DNA excision (6). Alternatively, since phage-related genes are present immediately downstream of the IS*1470*-like sequences of pCPF4969, phage DNA carrying the pCPF4969 variable region may have integrated near the *dcm* region of the progenitor plasmid, followed by later insertion of an IS*1469*-*cpe*-IS*1470* element near *dcm*. This phage scenario offers interspecies DNA transfer as a potential explanation for the pCPF4969 variable region containing DNA sequences (e.g., the *spa* genes) also found in other gram-positive bacteria. After IS*1470* localized in pCPF4969, a mutation probably created the defective IS*1470*-like sequence found in the current pCPF4969.

Similarly, pCPF5603 may have resulted from (i) IS*1151*, IS*1469*, or the putative ISRM3-like transposase mediating simultaneous insertion of *cpe* and the pCPF5603 variable region into the progenitor conjugative plasmid or (ii) initial integration of some genetic element containing most pCPF5603 variable region sequences near *dcm*, followed by later insertion of an IS*1151*-*cpe*-IS*1469* element, also near *dcm*. The *C. perfringens* chromosome may have been one source for pCPF5603 variable region DNA, given the presence of many metabolic genes (including some found on the *C. perfringens* strain 13 chromosome) in this region.

The presence of both the IS*1469*-*cpe*-IS*1151* and the IS*1469*-*cpe*-IS*1470* regions near *dcm* on pCPF5603 and pCPF4969 is notable. This association is consistent with the *dcm* region of these plasmids representing a hot spot for IS element (and perhaps phage) insertion. Since *dcm* is also present in type B to D isolates (22), it will be interesting to determine whether toxin genes present on type B to D virulence plasmids are also located near *dcm*.

The same progenitor conjugative plasmid putatively involved in pCPF4969/pCPF5603 evolution may also have served as the backbone for other *C. perfringens* virulence plasmids. Consistent with this possibility are our PCR results demonstrating that the pCPF4969/pCPF5603 Tn*916*-related region (and some other conserved region ORFs) is present in four representative type B to E isolates carrying virulence plasmids encoding epsilon, beta, or iota toxins, while those sequences are absent from two type A strains (strain 13 and ATCC 3624) lacking plasmids that carry functional toxin genes. The Tn*916*-related region in the surveyed type B to D isolates is not present on a *cpe* plasmid since those isolates are *cpe* negative. Consistent with *C. perfringens* virulence plasmids sharing a common origin, it has already been proposed that type E plasmids arose from integration of a mobile genetic element carrying iota toxin genes onto an IS*1151*$^+$/*cpe*$^+$ plasmid in a type A isolate (3). Sequencing of type B to E virulence plasmids is under way to conclusively assess the similarity between other *C. perfringens* toxin-encoding plasmids and pCPF4969/pCPF5603.

Tn*916* can conjugatively transfer among most gram-positive bacteria and can mobilize plasmids into which it has inserted (7). Therefore, the presence of Tn*916*-related ORFs in pCPF4969 represents an attractive explanation for the conjugative transfer of this *cpe* plasmid (5). If Tn*916*-related ORFs mediate pCPF4969 conjugation, the presence of similar Tn*916* ORFs in all other surveyed type A AAD/SPOR isolates carrying *cpe* plasmids predicts that most or all *cpe* plasmids are transferable by conjugation. By further extension, detection of Tn*916* ORFs in *C. perfringens* type B to E isolates raises the possibility of other *C. perfringens* toxin-encoding plasmids also being conjugative, consistent with unpublished results purportedly demonstrating conjugative transfer of an epsilon toxin-encoding plasmid (24). If confirmed, conjugative transfer of type B to E virulence plasmids may (as previously proposed for pCPF4969 [5]) contribute to enteric disease by allowing a small initial infecting dose of a type B to E isolate to transfer its virulence plasmid to the *C. perfringens* type A isolates present in the normal flora, thereby amplifying the infection.

If the Tn*916*-related region mediates conjugative transfer of type A *cpe* plasmids and type B to E virulence plasmids, it is notable that some Tn*916* genes are missing from pCPF5603/pCPF4969 and those Tn*916* genes that are present have a significantly rearranged gene order (Fig. 8). Nevertheless, both *cpe* plasmid families carry the Tn*916* ORF 13–17 region involved in conjugative transfer (7). pCPF4969/pCPF5603 also carry an FtsK/SpoEIII homolog ORF with amino acid similar-

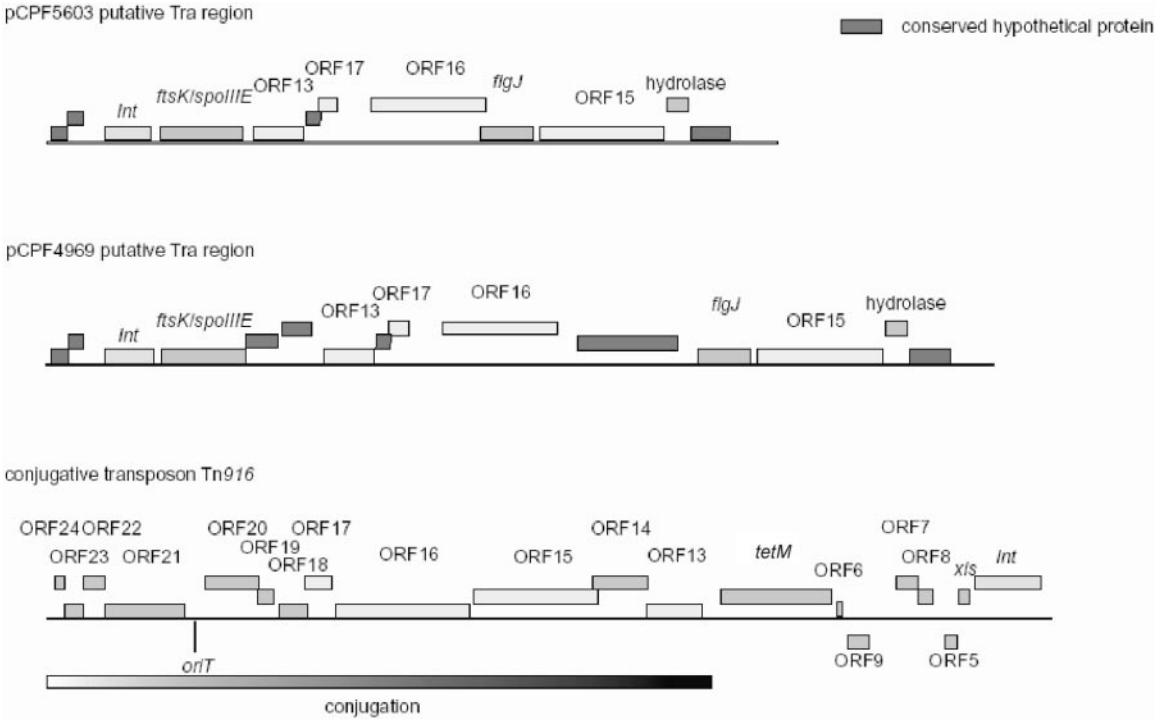

FIG. 8. Comparison of the pCPF5603/pCPF4969 putative transfer region gene order and the gene order of the conjugative transposon Tn*916*. For comparison, the putative transfer regions of pCPF5603 and pCPF4696 are shown schematically above the Tn*916* genome. ORFs important in Tn*916* conjugative transfer are highlighted by the shaded bar beneath the Tn*916* schematic. The bar increases in darkness to indicate ORFs known to be required for conjugative transfer of Tn*916*.

ity to Tn*916* ORF21, which encodes a DNA translocase. This FtsK/SpoEIII homolog may serve two functions for *cpe* plasmids in type A isolates, i.e., translocating these plasmids into the forespore during sporulation (thus ensuring stable inheritance of the *cpe* plasmid in germinating cells) and transferring DNA from donor to recipient cells during conjugation.

Both the pCPF4969 and pCPF5603 *cpe* plasmid families carry additional ORFs (besides *cpe*) that encode recognized or potential virulence factors. Like pCP13, these *cpe* plasmids carry a *cna* homolog that could encode a collagen adhesin (27). These *cpe* plasmids also carry a *srt* homolog that could encode a sortase; in several gram-positive pathogens, sortases anchor surface virulence factors (33). The pCPF4969 *cpe* plasmid family also carries ORFs with similarity, at the amino acid level, to the *C. perfringens* VirR/VirS two-component regulator that positively regulates chromosomal virulence genes encoding alpha toxin and perfringolysin O (1, 25). While lacking VirR/VirS homologs, pCPF5603 carries a functional *cpb2* gene that could be important for virulence since CPB2 can damage CaCo-2 cultured human colonic cells, i.e., CPB2 and CPE may cocontribute to intestinal disease caused by AAD/SPOR isolates carrying *cpe* plasmids of the pCPF5603 family (11).

The pCPF4969 *cpe* plasmid family also apparently encodes the bacteriocin propionicin SM1 and a lantibiotic bacteriocin encoded by the *spa* operon. Another sequenced *C. perfringens* plasmid, pIP404 (which carries no virulence genes or Tn*916*-related genes) also encodes a bacteriocin, Bcn5 (12). While sequencing F5603, we found a second large plasmid (besides pCPF5603) also encoding Bcn5 (Miyamoto et al., unpublished observation). The common presence of bacteriocin genes on sequenced *C. perfringens* plasmids suggests that bacteriocin production may be beneficial to *C. perfringens*, perhaps by reducing competition from other bacteria when *C. perfringens* is present in the soil or GI tract. The presence of pCPF4969

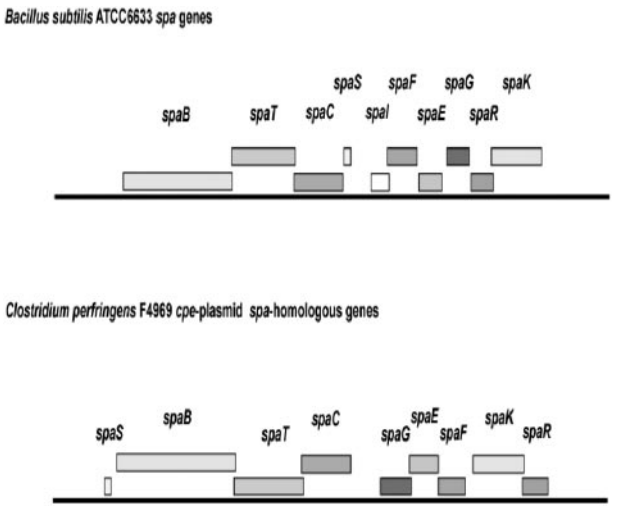

FIG. 9. Gene order comparison of the *spa* operon from *Bacillus subtilis* ATCC 6633 and the putative *spa* operon from *C. perfringens* pCPF4969. The putative *spa* operon found on pCPF4969 is shown below the *spa* operon identified in *B. subtilis* ATCC 6633 to compare the two gene arrangements.

bacteriocin-encoding ORFs also explains difficulties in using F4969 for mating experiments to demonstrate pCPF4969 conjugative transfer (5). Finally, it is notable that, relative to the *spa* operon of other gram-positive bacteria (31), the bacteriocin-related *spa* ORFs on pCPF4969 are atypically arranged and are missing some ORFs (Fig. 9).

While the current studies offer new insights into the sequences and diversity of the *cpe* plasmids in type A isolates, additional research is needed to identify sequences involved in plasmid replication and to confirm the putative role of Tn*916*-related genes in conjugative transfer. Other studies should evaluate whether the putative virulence genes identified on these *cpe* plasmids actually contribute to enteric pathogenesis. Finally, sequence comparisons of the *cpe* plasmids of type A isolates against the virulence plasmids of *C. perfringens* type B to E isolates will reveal whether these virulence plasmids share a common origin.

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SUPPLEMENTAL DATA FOR MIYAMOTO *ET AL.* 2006

Table SI. c*pe* plasmid primers used to connect contigs

Primer name	Sequence
4-21DCMUP	ATAGGCAACTGTATAGATTTGTAGTC
3-03FDO	GTTAATAGAAAAGGAGATAAGGTTGAGG
M-09FD	CTTACCCTTGGAGATGCTTCAGTTAACC
4-01FD1	CTTCATAAATTTAAAATTAAATAAAGGAG
Xb-D21	GGTGATGCTAATGCTATAGCACGAG
1-45FD	CTAATGTATAAATAGTATTACTAGC
1F	GTTAAAAATTTGATATAATTGAATTG
2R	GACTAAGTCACTTCATATTTTTTTC

Table SII. *cpe* plasmid conserved region PCR primers

Primer name	Sequence
ORF15-HR	CGCTGGATTTACTACATAGTCCTCTG
ORF15-HF	GACTATAGGAACTAGTGCTATAGTTGC
FLGJ-R	GCTCTATATAGTTAGTTGCGTCCAGG
FLGJ-F	CTAATCCTAATTTAACGGAAGAGCAG
ORF16-HR	GTCTCTATTATAATTAGAGTTAGCAGG
ORF16-HF	GTTAATCCAGGATATGAATATTGGTGC
ORF13-HR	ATTGAAAGAGAACTTTTGACCTGCTC
ORF13-HF	GTTTCAGTCTTTAAAGGTAATCAATCTG
FTSK-R	GTAAGCTTGAAACTCAATAGTGTTTGC
FTSK-F	GTTTAGACTATGAAAAAATCGGTAAGG
DAM-F	GAGATATCTGGAAGGAGTATATTCGATG
DAM-R2	CTTCTGATTGTCTTGAAACTGAATAATG
SRT-F	GTATTCCTGAAACACCTGAAAATACAG
SRT-R	TAATCTTTGTTTAGCATGATTAATACAAG
CNA-F	GTAGGGGAATTGATAGAACAAGACTTC
CNA-R	CTTTTATTTGAGTATCAACCATTTCAGC
PCP59-F	GAAACTATAGGAGTTGCAGTTGC
PCP59-R	CTTCACAGTGACTCTTTGTCCATCCAGC

Table SIII. pCPF4969 variable region PCR primers.

Reaction #	Primer name	Sequence	Binding Site	Product Size (bp)
1	CPEMF	TTAGAACAGTCCTTAGGTGATGGA	33568	1234
	4969P1R	CAGAAACACATAGTCTGCGGAGC	34802	
2	4969P1	GCTCCGCAGACTATGTGTTTCTG		1125
	IS1470-likeR	GTTCTTCTGGAGTTTTATAGTTTAGTATTCTCCTTG	35927	
3	IS1470-like	CAAGGAGAATACTAAACTATAAAACTCCAGAAGAAC		979
	4969P2R	CATATAATCAAACTTATGGCCAAG	36906	
4	4969P2	CTTGGCCATAAGTTTGATTATATG		1543
	4969P3R	CAACTAAGAACTCTAACCATTTTTCTAGAG	38449	
5	4969P3	CTCTAGAAAAATGGTTAGAGTTCTTAGTTG		1646
	4969P4R	CTTCTGCTAAATCATCTACAGATTTG	40095	
6	4969P4	CAAATCTGTAGATGATTTAGCAGAAG		1479
	4969P5R	CATTTATATCCAGCATATCTCCAAG	41574	
7	4969P5	CTTGGAGATATGCTGGATATAAATG		1625
	4969P6R	CATTATGGTGATAAGAGTTGGTATG	43199	
8	4969P6	CATACCAACTCTTATCACCATAATG		1601
	4969P7R	GTCTAGGAGCTTCTCTTTCTAAATCATC	44800	
9	4969P7	GATGATTTAGAAAGAGAAGCTCCTAGAC		1720
	4969P8R	CTTATTGCTACTTGACCACCATTC	46520	
10	4969P8	GAATGGTGGTCAAGTAGCAATAAG		1671
	4969P9R	CAGCATACGGCTACTCATAATAAC	48191	
11	4969P9	GTTATTATGAGTAGCCGTATGCTG		1565
	4969P10R	GTTTTTAAGTCATGTGCAAGAGATTC	49756	
12	4969P10	GAATCTCTTGCACATGACTTAAAAAC		1610
	4969P11R	GGTGAGTTTTCTGAATAGTGAGTGC	51366	
13	4969P11	GCACTCACTATTCAGAAAACTCACC		1412
	4969P12R	GATTTTTCCTTCCCATCTATTGAAC	52778	
14	4969P12	GTTCAATAGATGGGAAGGAAAAATC		1489
	4969P13R	CGAATAACTCTAGAATGCGATATG	54267	
15	4969P13	CATATCGCATTCTAGAGTTATTCG		1581
	4969P14R	GATACTAATAATACTATCAGTGAATGCTGG	55848	
16	4969P14	CCAGCATTCACTGATAGTATTATTAGTATC		1610
	4969P15R	CTTTGTTTATCCCATTGCAAGAATTAC	57458	
17	4969P15	GTAATTCTTGCAATGGGATAAACAAAG		1597
	4969P16R	CTCTGATCTACGACCACCATTG	59055	
18	4969P16	CAATGGTGGTCGTAGATCAGAG		1665
	4969P17R	CTACATCCATTCCTGATAATTCATTC	60720	
19	4969P17	GAATGAATTATCAGGAATGGATGTAG		1545
	4969P18R	GCAGGCCTCTATTGAGTTATCTAG	62265	
20	4969P18	CTAGATAACTCAATAGAGGCCTGC		1599
	4969P19R	CTGCTGAAGATGCTCAAAATGC	63864	
21	4969P19	GCATTTTGAGCATCTTCAGCAG		1635
	4969P20R	GATGAAAATTTCACTACTGTATTGGA	65499	
22	4969P20	TCCAATACAGTAGTGAAATTTTCATC		1555
	4969P21R	TGTTATTGAGTCTAATTGTTTTGCTC	67054	
23	4969P21	GAGCAAAACAATTAGACTCAATAACA		1599
	4969P22R	GAGCTGGTGGTGGTAGTTTAGATTTAC	68653	
24	4969P22	GTAAATCTAAACTACCACCACCAGCTC		470
	4969P23R	GATTTGGTATTAGGAGTACCAGCTTC	69123	

Table SIV. pCPF5603 variable region PCR primers

Reaction #	Primer name	Sequence	Binding Site	Product Size (bp)
1	4969P23	GATTTGGTATTAGGAGTACCAGCTTC	14215	1358
	5603P1R	AATTGCTAGTGCAGTTTCGATTC	15573	
2	5603P1	GAATCGAAACTGCACTAGCAATT		1510
	5603P2R	CAAATGCATAGTGAGAAGAGACTTG	17083	
3	5603P2	CAAGTCTCTTCTCACTATGCATTTG		1579
	5603P3R	CAAGGTTGGATGCAAGGTTATC	18662	
4	5603P3	GATAACCTTGCATCCAACCTTG		1502
	5603P4R	GATAAAAGTTTATGTTGTGATGAGTATG	20164	
5	5603P4	CATACTCATCACAACATAAACTTTTATC		1491
	5603P5R	GCTGTACTAAGTAAAGAACTACCATCATA	21655	
6	5603P5	TATGATGGTAGTTCTTTACTTAGTACAGC		1570
	5603P6R	CTGGAAAAGTCTTTGGTTTAATGAG	23225	
7	5603P6	CTCATTAAACCAAAGACTTTTCCAG		1549
	5603P7R	GTGTATGTACCGTAATCGTATTTC	24774	
8	5603P7	GAAATACGATTACGGTACATACAC		1829
	5603P8R	TCCAATTTATACACATATAGGACGAG	26603	
9	5603P8	CTCGTCCTATATGTGTATAAATTGGA		1881
	5603P9R	GATTTATCTGATGTGGAGGAACTATG	28484	
10	5603P9	CATAGTTCCTCCACATCAGATAAATC		1201
	5603P10R	GTATCTTTTGGAGGCTAATATGGAG	29685	
11	5603P10	CTCCATATTAGCCTCCAAAAGATAC		1471
	5603P11R	GATTATAATGAACAGGAATACTAGGTAAAC	31156	
12	5603P11	GTTTACCTAGTATTCCTGTTCATTATAATC		1559
	5603P12R	ATGGGATATAATCCGAATGTTAATG	32715	
13	5603P12	CATTAACATTCGGATTATATCCCAT		1506
	5603P13R	CATCACACCAACTTATGTATTAATCTAG	34221	
14	5603P13	CTAGATTAATACATAAGTTGGTGTGATG		1567
	5603P14R	GATAAAAAAATGGACGAAGCTTATTC	35788	
15	5603P14	GAATAAGCTTCGTCCATTTTTTTATC		1571
	5603P15R	GTAGCCAATGAATTAGATTTAACCAG	37359	
16	5603P15	CTGGTTAAATCTAATTCATTGGCTAC		1641
	5603P16R	GATGATTTAGAAAGAGAAGCTCCTAGAC	39000	
17	5603P16	GTCTAGGAGCTTCTCTTTCTAAATCATC		1770
	CPB23FR	CTTAAATCATATGAAATAACAGGTTC	40770	
18	CPB23F	GAACCTGTTATTTCATATGATTTAAG		1037
	41870R	CATTTAGGTAGGCCAAGAGTAGAATATC	41807	
19	41870	GATATTCTACTCTTGGCCTACCTAAATG		906
	42703R	CATCAACATATGTTACCCTACCAATAC	42713	
20	42703	GTATTGGTAGGGTAACATATGTTGATG		1280
	43989R	ATAATCTTGTAAGGCATGCATTATTC	43993	
21	43989	GAATAATGCATGCCTTACAAGATTAT		811
	44803R	GAATTAGATTTTGGTTTAGTGATGCTGCATG	44804	
22	44803	CATGCAGCATCACTAAACCAAAATCTAATTC		1244
	46048R	GTTATGGTTTGCTCCAGGAATATTTG	46048	
23	46048	CAAATATTCCTGGAGCAAACCATAAC		1407
	47455R	CTTTTGGTGAACTTGGCGATTC	47455	
24	47455	GAATCGCCAAGTTCACCAAAAG		823
	48278R	CATAGATCTTGTTGTGCTGCTAAGC	48278	
25	48278	GCTTAGCAGCACAACAAGATCTATG		880
	49158R	GAGCATTAACGGATTTAGCATCTG	49158	
26	49158	CAGATGCTAAATCCGTTAATGCTC		1391
	50546R	GACCTTATATTACTGGAGTAAGATGTATTCAG	50549	
27	50546	CTGAATACATCTTACTCCAGTAATATAAGGTC		1277
	51823R	GTTTTGGAGTGTCCTTATTGTTCTTAAG	51826	
28	51823	CTTAAGAACAATAAGGACACTCCAAAAC		1028
	52854R	GCATAAACATTCCTGTTAAATAAGCTATTCCA	52854	
29	52854	TGGAATAGCTTATTTAACAGGAATGTTTATGC		1125
	53976R	CTGGTATGTTCACAGAAATGGTTGTTAG	53979	
30	53976	CTAACAACCATTTCTGTGAACATACCAG		568
	54534R	GAGCTCTGCTTAGATTTACTAAGTAGAG	54547	
31	54534	CTCTACTTAGTAAATCTAAGCAGAGCTC		1195
	55742R	GACACTTAATAGCGGTCAGAGAAGC	55742	
32	55742	GCTTCTCTGACCGCTATTAAGTGTC		1292
	57040R	GTTAAATTAGAGCGATTCATGTGC	57034	
33	57040	GCACATGAATCGCTCTAATTTAAC		1543
	CPEMR	TTAGAACAGTCCTTAGGTGATGGA	58577	

Table SV. Transfer region PCR primers

			pCPF4969		pCPF5603	
Reaction #	Primer name	Sequence	Binding Site	Product Size (bp)	Binding Site	Product Size (bp)
1	CPEMR	TTAGAACAGTCCTTAGGTGATGGA	33568	1013	58577	1037
	59620R	GAGATATCCGTTAAACAGATCAAGTTG	32555		59614	
2	59620	CAACTTGATCTGTTTAACGGATATCTC		1037		999
	60619R	CATACTACCTACGTTGCATCTTAAGACGCTTAAATTAG	31518		60613	
3	60619	CTAATTTAAGCGTCTTAAGATGCAACGTAGGTAGTATG		1014		1025
	61644R	CTACGTGGAAATGTTAAATCTAAGAAC	30504		61638	
4	61644	GTTCTTAGATTTAACATTTCCACGTAG		1083		1067
	62711R	GTAATGCTCTCTAGTGTAACTAGATTATCG	29421		62705	
5	62711	CGATAATCTAGTTACACTAGAGAGCATTAC		895		1126
	63843R	CAACACATTAGTGATTAGTATTTCACC	28526		63831	
6	63843	GGTGAAATACTAATCACTAATGTGTTG		1118		1089
	64939R	GTAGACCTTCTATAGTTACTTCAGTTGGTTC	27408		64920	
7	64939	GAACCAACTGAAGTAACTATAGAAGGTCTAC		1270		1270
	66202R	CAGGAACTTCAGTAACACTTATATTACTATC	26138		66190	
8	66202	GATAGTAATATAAGTGTTACTGAAGTTCCTG		1068		1050
	67252R	GAATTGACATAAGCTGAAATCCTATTTG	25070		67240	
9	67252	CAAATAGGATTTCAGCTTATGTCAATTC		857		860
	68112R	TTATAAGGATCATCACTATTACCATAGC	24213		68100	
10	68112	GCTATGGTAATAGTGATGATCCTTATAA		1080		1109
	69219R	GAGCGAAGCAATCCTATGA	23133		69209	
11[A]	69219	CTTCATAGGATTGCTTCGCTC				1451
	70672R	CTTATAAATCCACATACAGACCAATACAG			70660	
11[B]	69219	CTTCATAGGATTGCTTCGCTC		1461		
	4969TNP2R	GTTACGAATCCGTTTTATTTGCTC	21672			
12[B]	4969TNP2	GAGCAAATAAAACGGATTCGTAAC		1301		
	4969TNP1R	CTTCATCAGTCATACCACTACTCTCAC	20371			
13[B]	4969TNP1	GTGAGAGTAGTGGTATGACTGATGAAG		1470		
	70672	CTGTATTGGTCTGTATGTGGATTTATAAG	18901			

[A]Primers designed to amplify IS*1151* specific tra region sequences

[B]Primers designed to amplify IS*1470-like* specific tra region sequences

Table SVI. Long-range conserved region PCR primers

		F5603		F4969	
Primer name	Sequence	Binding Site	Product Size (bp)	Binding Site	Product Size (bp)
4-21UP3FD1	GTTAATCCAGGATATGAATATTGGTGC	70984		18579	
met-up1	TAACTTGCATACCTATCCTTTTCAC	63167	7817	28964	10385
ORF13-HF	GTTTCAGTCTTTAAAGGTAATCAATCTG	73736		16034	
4-21UP3-4	GTCTCTATTATAATTAGAGTTAGCAGG	70614	3122	18949	2915
damF	GAGATATCTGGAAGGAGTATATTCGATG	2842		9812	
3-3FU4	ATTGAAAGAGAACTTTTGACCTGCTC	73110	5000	16662	6850
cna-F	GTAGGGGAATTGATAGAACAAGACTTC	8366		4671	
DAM-R2	CTTCTGATTGTCTTGAAACTGAATAATG	2392	5974	10262	5591
8-118E	GAAACTATAGGAGTTGCAGTTGC	10400		2671	
C1-6T3D	CTTTTATTTGAGTATCAACCATTTCAGC	7628	2772	5408	2737

Table SVII. PCR results for the conserved region internal PCR assay.

	IS*1151* isolates						IS*1470-like* isolates					
	F5603	NB16	B38	B40	H38094	F4013	F4969	F4396	M26413	T34058	X5722	MR2-4
cpe	+	+	+	+	+	+	+	+	+	+	+	+
IS1469	+	+	+	+	+	+	+	+	+	+	+	+
dcm	+	+	+	+	+	+	+	+	+	+	+	+
ORF15	+	+	+	+	+	+	+	+	+	+	+	-
FLGJ	+	+	+	+	+	+	+	+	+	+	+	+
ORF16	+	+	+	+	+	+	+	+	+	+	+	+
ORF13	+	+	+	+	+	+	+	+	+	+	+	+
FTSK	+	+	+	+	+	+	-	+/-	+	+	-	-
DAM	+	+	+	+	+	+	+	+	+	+	+	+
SRT	+	+	+	+	+	+	+	+	+	+	+	+
CNA	+	+	+	+	+	+	+	+	+	+	+	+
PCP59	+	+	+	+	+	+	+	+	+	+	+	+

Table SVIII. pCPF4969 variable region overlapping PCR survey results.

	IS*1151* isolates						IS*1470-like* isolates						
Rxn #	F5603	NB16	B38	B40	H38094	F4013	F4969	F4396	M26413	T34058	X5722	MR2-4	S13
1	-	-	-	-	-	-	+	+	+	+	+	+	-
2	-	-	-	-	-	-	+	+	+	+	+	+	-
3	-	-	-	-	-	-	+	+	+	+	+	+	-
4	+	+	+/-	+	+	+	+	+	+	+	+	+	-
5	-	-	-	-	-	-	+	+	+	+	+	+	-
6	-	-	-	-	-	-	+	+	+	+	+	+	-
7	-	-	-	-	-	-	+	+	+	+	+	+	-
8	-	-	-	-	-	-	+	+	+	+	+	+	-
9	+	+	-	+	+	+	+	+	+	+	+	+	-
10	-	-	-	-	-	-	+	+	+	+	+	+	-
11	-	-	-	-	-	-	+	+	+	+	+	+	-
12	-	-	-	-	-	-	+	+	+	+	+	+	-
13	-	-	-	-	-	-	+	+	+	+	+	+	-
14	-	-	-	-	-	-	+	+	+	+	+	+	-
15	-	-	-	-	-	-	+	+	+	+	+	+	-
16	-	-	-	-	-	-	+	+	+	+	+	+	-
17	-	-	-	-	-	-	+	+	+	+	+	+	-
18	-	-	-	-	-	-	+	+	+	+	+	+	-
19	-	-	-	-	-	-	+	+	+	+	+	+	-
20	-	-	-	-	-	-	+	-	-	-	-	+	-
21	-	-	-	-	-	-	+	-	+	-	-	+	-
22	-	-	-	-	-	-	+	+	+	+	+	+	-
23	-	-	-	-	-	-	+	-	+	+	+	+	-
24	+	+	+	+	+	+	+	+	+	+	+	+	-

Table SIX. pCPF5603 variable region overlapping PCR survey results.

	IS*1151* isolates						IS*1470-like* isolates						
Rxn #	F5603	NB16	B38	B40	H38094	F4013	F4969	F4396	M26413	T34058	X5722	MR2-4	S13
1	+	+	+	+	+	-	-	-	-	-	-	-	-
2	+	+	+	+	+	-	-	-	-	-	-	-	-
3	+	+	+	+	-	-	-	-	-	-	-	-	-
4	+	+	-	+	+	-	-	-	-	-	-	-	-
5	+	+	+	+	+	-	-	-	-	+	-	+	-
6	+	+	-	+	+	-	-	-	-	-	-	-	-
7	+	+	+	+	+	-	-	-	-	+	-	-	-
8	+	+	+	+	+	-	-	-	-	-	-	-	-
9	+	+	+	+	+	-	-	-	-	-	-	-	-
10	+	+	+	+	+	-	-	-	-	-	-	-	-
11	+	+	+	+	+	-	-	-	-	-	-	-	-
12	+	+	+	+	+	-	-	-	-	-	-	-	-
13	+	+	+	+	+	-	-	-	-	-	-	-	-
14	+	+	+	+	+	-	-	-	-	-	-	-	-
15	+	+	+	+	+	-	-	-	-	-	-	-	-
16	+	+	-	+	+	+	+	-	+	+	+	+	-
17	+	+	-	+	-	+	-	-	-	-	-	-	-
18	+	+	+	+	+	+	-	-	-	-	-	-	-
19	+	+	+	+	+	+	-	+	-	+	-	-	-
20	+	+	+	+	+	-	-	-	-	-	-	-	-
21	+	+	+	+	+	-	+	-	-	-	-	-	-
22	+	+	+	+	+	-	-	-	-	-	-	-	-
23	+	+	+	+	+	-	-	-	-	+	-	-	+
24	+	+	+	+	+	-	-	-	-	-	-	-	-
25	+	+	+	+	+	-	-	-	-	-	-	-	+
26	+	+	+	+	+	-	-	+	+	-	+	-	-
27	+	+	-	+	+	+	+	+	+	+	+	+	-
28	+	+	+	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	-	-	-	-	-	-	-
30	+	+	+	+	+	+	-	-	-	-	-	-	-
31	+	+	+	+	+	+	-	-	-	-	-	-	-
32	+	+	-	+	+	+	-	-	-	-	-	-	-
33	+	+	+	-	+	+	-	-	-	-	-	-	-

Table SX. Transfer region PCR survey results.

	IS*1151* isolates						IS*1470-like* isolates						
Rxn #	F5603	NB16	B38	B40	H38094	F4013	F4969	F4396	M26413	T34058	MR2-4	X5722	S13
1	+	+	+	+	+	+	+	+	+	+	+	+	-
2	+	+	+	+	+	+	+	+	+	+	+	+	-
3	+	+	+	+	+	+	+	+	+	+	+	+	-
4	+	+	+	+	+	+	+	+	+	+	+	+	-
5	+	+	+	+	+	-	+	+	+	+	+	+	-
6	+	+	+	+	+	+	+	+	+	+	+	+	-
7	+	+	+	-	-	+	+	-	-	+	-	+	-
8	+	+	+	+	+	+	+	+	+	+	+	+	-
9	+	+	+	+	+	+	+	+	+	+	+	+	-
10	+	+	+	+	+	+	+	+	+	+	+	+	-
11[A]	+	+	+	+	+	-	-	-	-	-	-	-	-
11[B]	-	-	-	+	-	+	+	+	+	+	+	+	-
12[B]	-	-	-	-	-	+	+	+	+	+	+	+	-
13[B]	-	-	-	-	-	+	+	+	+	+	+	+	-

[A]Primers designed to amplify IS*1151* specific tra region sequences

[B]Primers designed to amplify IS*1470-like* specific tra region sequences

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Epsilon-Toxin Is Required for Most *Clostridium perfringens* Type D Vegetative Culture Supernatants To Cause Lethality in the Mouse Intravenous Injection Model

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***Clostridium perfringens* type D enterotoxemias have significant economic impact by causing rapid death of several domestic animal species. Consequently, domestic animals are commonly vaccinated, at varying efficacy, with inactivated type D vegetative supernatants. Improved type D vaccines might become possible if the lethal toxins produced by type D isolates were characterized and the contributions of those toxins to supernatant-induced lethality were established. Therefore, the current study evaluated the presence of lethal toxins in supernatants prepared from late-log-phase vegetative cultures of a large collection of genotype D isolates. Under this growth condition, most genotype D isolates produced variable levels of at least three different lethal toxins, including epsilon-toxin (ETX). To model the rapid lethality of type D enterotoxemias, studies were conducted involving intravenous (i.v.) injection of genotype D vegetative supernatants into mice, which were then observed for neurotoxic distress. Those experiments demonstrated a correlation between ETX (but not alpha-toxin or perfringolysin O) levels in late-log-phase genotype D supernatants and lethality. Consistent with the known proteolytic activation requirement for ETX toxicity, trypsin pretreatment was required for, or substantially increased, the lethality of nearly all of the tested genotype D vegetative supernatants. Finally, the lethality of these trypsin-pretreated genotype D supernatants could be completely neutralized by an ETX-specific monoclonal antibody but not by an alpha-toxin-specific monoclonal antibody. Collectively, these results indicate that, under the experimental conditions used in the present study, ETX is necessary for the lethal properties of most genotype D vegetative supernatants in the mouse i.v. injection model.**

Clostridium perfringens is an important cause (19) of both histotoxic infections (e.g., human gas gangrene) and enteric diseases (e.g., *C. perfringens* type A human food poisoning and severe enterotoxemias in domestic animals). The virulence of *C. perfringens* is largely attributable to its ability to produce >15 different toxins, several of which have lethal properties (15, 20). However, individual isolates of this bacterium do not express this entire toxin repertoire, providing the basis for a classification scheme (15, 20) that assigns *C. perfringens* isolates to one of five different toxinotypes (type A to E) depending upon their production of four (α, β, ε, and ι) lethal toxins. With the exception of alpha-toxin (CPA), the typing toxins are encoded by genes present on large plasmids (28).

In sheep, goats, and probably other domestic animals, *C. perfringens* type D isolates cause enterotoxemias that initiate with production of toxins in the intestines. Those toxins (including epsilon-toxin [ETX], a CDC/USDA overlap select toxin) can be absorbed through the intestinal mucosa (18) and then spread via the circulation to internal organs, where they cause blood pressure elevation and fluid accumulation in body cavities, as well as edema in several organs, notably brain, heart, lungs, liver, and kidney (24, 29). Type D enterotoxemias can result in peracute, acute, or chronic disease (18). In sheep, these infections primarily produce neurologic signs, which may or may not include classical brain edema-induced focal symmetrical encephalomalacia, often resulting in sudden death (18). Similar peracute and acute neurologic disease, including sudden death, is also observed in type D enterotoxemias of kids and some adult goats, whereas other adult goats develop a chronic gastrointestinal form of type D enterotoxemia that is characterized by a fibrinonecrotic colitis (18).

Understanding the rapid lethality associated with many cases of type D enterotoxemia could lead to improved vaccine design. In the absence of a well-characterized, small animal oral-challenge model, intravenous (i.v.) injection of vegetative culture supernatants into mice is commonly used to study the systemic lethality associated with type D enterotoxemias. However, the potential presence of several lethal toxins in those type D supernatants could complicate interpretation of mouse i.v. injection results. For example, vegetative cultures of type D isolates (by definition) produce at least two potent lethal toxins, i.e., ETX and CPA. Although not yet systematically evaluated with a large isolate collection, some or all type D isolates could produce additional lethal toxins, such as perfringolysin O (PFO), enterotoxin (CPE), or beta2 toxin (CPB2). Variations in lethal toxin levels among type D vegetative culture super-

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natants could impact their lethal activity. For example, some of those supernatants might possess sublethal ETX concentrations but lethal CPA concentrations. However, to date, variations in supernatant lethal toxin levels have not been assessed with a sizeable collection of type D isolates. Finally, although the effects of i.v. injection of some pure *C. perfringens* toxins into animals have been well studied, the relative contribution of different toxins to the lethal properties of type D vegetative culture supernatants has not yet been rigorously determined.

In response, the present study genotypically and phenotypically characterized lethal toxin production by a large collection of type D isolates. Collectively, several results from the present study support the importance of ETX in causing the mouse lethality induced by i.v. injection of late log-phase vegetative supernatants prepared from most type D isolates.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Of the 47 putative type D strains examined in the present study, 30 originated from the Burroughs-Wellcome (BW) collection; those BW isolates were primarily gathered from diseased animals during the 1940s to 1960s and had been stored in a lyophilized form. They were kindly provided by Russell Wilkinson (University of Melbourne). Ten other isolates were recent North American animal disease isolates that were kindly provided by J. Glenn Songer (University of Arizona). The remaining seven isolates examined in the present study came from our laboratory collections and had diverse origins, mostly including veterinary infections.

All *C. perfringens* isolates were initially grown overnight at 37°C under anaerobic conditions on TSC agar media (SFP agar [Difco Laboratories], 0.04% D-cycloserine [Sigma Aldrich]) to ensure culture purity. Unless otherwise specified, FTG (fluid thioglycolate medium; Difco Laboratories) or TGY (3% tryptic soy broth [Becton-Dickinson]; 2% glucose [Sigma Aldrich], 1% yeast extract [Becton-Dickinson], 0.1% L-cysteine [Sigma Aldrich]) were used for growing broth cultures.

Multiplex PCR. Brain heart infusion agar (Becton-Dickinson) plates were inoculated with a putative type D isolate and then grown anaerobically overnight at 37°C. Three or four colonies were picked from each plate and used to prepare template DNA as described previously (37). These DNA preparations were then subjected to a multiplex PCR assay (10) capable of detecting six genes encoding *C. perfringens* lethal toxins or lethal toxin components, i.e., the CPA gene (*plc*), the beta-toxin gene (*cpb*), the CPB2 gene (*cpb2*), the CPE gene (*cpe*), the ETX gene (*etx*), and the *iap* gene encoding the A component of iota toxin. Products from each multiplex PCR were electrophoresed on 2% agarose gels; after electrophoresis, these gels were stained with ethidium bromide for visualization. Isolates carrying both *plc* and *etx* genes are genotypically type D and henceforth are referred to as genotype D isolates (10).

Optimization of vegetative culture conditions for ETX production. A single isolated colony from a TSC plate streaked with a type D isolate was inoculated into 10 ml of FTG medium, which was then incubated overnight at 37°C. A 0.1-ml aliquot of each overnight culture was then inoculated into 10 ml of FTG, TGY, brain heart infusion broth (Difco), or differential reinforced clostridial broth (EM Science). Those cultures were grown at 37°C, with aliquots of each culture removed at specific times. For each removed culture aliquot, optical density at 600 nm values were determined prior to centrifugation. Each resultant supernatant was then mixed with an equal volume of protein sample buffer before boiling for 10 min and loading on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel for ETX Western blotting (see below).

Preparation of vegetative culture supernatants from genotype D isolates. Single isolated colonies of genotype D isolates were inoculated into 10 ml of FTG medium, which was incubated overnight at 37°C. Based upon the pilot ETX production studies described above (see Results), a 0.1-ml aliquot of each overnight culture was inoculated into 10 ml of TGY, and these cultures were grown at 37°C to late log phase. Bacteria were removed from each TGY culture by centrifugation, and the resultant supernatants were filter sterilized with a 0.45-μm-pore-size filter.

Quantification of toxin levels in supernatants from genotype D isolates. (i) ETX. For stronger (>400 ng of ETX/ml) ETX producers, sterile late-log-phase supernatants (prepared as described above) were diluted, as needed, to bring their ETX levels within the standard curve range of purified ETX used for Western blot quantification (see Results). For weaker (<400 ng of ETX/ml) ETX producers, the sterile supernatants were concentrated 10-fold using Amicon Ultra-15 centrifugal ultrafiltration devices (10,000 molecular weight cutoff). All sterile supernatants were mixed with SDS sample buffer, boiled for ~10 min, and electrophoresed on 10% acrylamide gels containing SDS. After electrophoresis, separated supernatant proteins (and pure ETX standards) were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). For ETX Western immunoblotting (31), an ETX-specific monoclonal antibody (5B7; kindly provided by Paul Hauer, Center for Veterinary Biologics, Ames, Iowa) was used as primary antibody, followed by rabbit anti-mouse immunoglobulin G (IgG)-peroxidase conjugate (Sigma) as a secondary antibody. ETX Western blots run with normal (nonconcentrated) or concentrated supernatants were then developed with SuperSignal West Pico or West Femto chemiluminescent substrate (Pierce), respectively. For each genotype D isolate, supernatant ETX levels were quantified for three independent cultures. All Western blot results were analyzed and preserved using a Bio-Rad ChemiDoc imaging system.

(ii) CPB2. To quantify CPB2 toxin levels, an aliquot of sterile late-log-phase supernatant from a culture of *cpb2*-positive genotype D isolate was concentrated 10- to 50-fold using an Amicon Ultra-15 device prior to mixing with protein sample buffer. Each concentrated vegetative supernatant was then boiled and electrophoresed on a 12% acrylamide gel containing SDS. A dilution series of CPB2, purified to homogeneity as described previously (9), was also run on each gel to construct a standard curve. After electrophoresis and sample transfer onto nitrocellulose, Western immunoblotting was performed using a rabbit polyclonal CPB2 antiserum (kindly provided by Michel Popoff, Institut Pasteur, Paris, France) as primary antibody, followed by goat anti-rabbit IgG-peroxidase conjugate (Sigma) as a secondary antibody. Blots were developed with SuperSignal West Pico chemiluminescent substrate. For each *cpb2*-positive genotype D isolate, supernatant CPB2 levels were quantified for three independent cultures.

(iii) CPE. CPE production by sporulating cultures of *cpe*-positive genotype D isolates was assessed by inoculating a 0.1-ml aliquot of an overnight FTG culture into 10 ml of Duncan-Strong (DS) sporulation medium (17, 30). Those cultures were then incubated for ~14 h, at which time sporulation was assessed by phase-contrast microscopy. Sporulating cells in the cultures were sonicated to release their internal CPE. After centrifugation, supernatants from the sonicated sporulating culture lysates were used for CPE Western blotting (17).

For CPE Western blotting, a 100-μl aliquot of supernatant from each sporulating (prepared as described above) or vegetative (prepared as described for ETX Western blotting) culture was mixed with 100 μl of SDS sample buffer, and 30 μl of that mixture was then electrophoresed on 10% acrylamide gels containing SDS (no sample boiling). To quantify CPE levels present in each supernatant, a dilution series of purified CPE (21) was run on the gel to establish a standard curve. After electrophoresis and transfer onto nitrocellulose, CPE Western blotting was performed with a rabbit polyclonal CPE antiserum as primary antibody, followed by a goat anti-rabbit IgG-peroxidase conjugate (Sigma) as a secondary antibody. Blots were developed with SuperSignal West Pico chemiluminescent substrate. For each genotype D isolate, supernatant CPE levels were quantified for three independent sporulating and vegetative cultures.

(iv) CPA. A 10-ml aliquot of sterile late-log-phase supernatant from genotype D vegetative culture supernatant, prepared as described above for the ETX Western blots, was freeze dried. That lyophilized material was then resuspended in 1 ml of phosphate buffered saline (pH 7.4) and subjected to a phospholipase C (PLC) activity assay using nutrient agar supplemented with 4% egg yolk (vol/vol) as described previously (32). *C. perfringens* PLC (Sigma) was used as a standard for quantifying CPA activity. Total protein present in each 10-fold-concentrated vegetative supernatant sample was determined by using the BCA kit (Pierce), and the specific activity was expressed as PLC units/mg of total protein.

(v) PFO. A 1-ml aliquot of sterile late log-phase supernatant from a vegetative genotype D culture (prepared as described above) was subjected to a series of twofold dilutions with 5 mM dithiothreitol (Roche) in DPBS. These diluted samples were then subjected to a quantitative PFO assay using horse red blood cells (Biolab, Melbourne, Australia) as described previously (34). The PFO titer was defined as the reciprocal of the last dilution showing complete hemolysis, as indicated by a significant decrease in absorbance at 570 nm recorded with a Multiskan spectrophotometer (Labsystems).

Mouse lethality assay of genotype D vegetative culture supernatants. For initial toxicity testing, each sterile late log-phase genotype D supernatant (prepared as described above) was divided into two aliquots. Because trypsin activation is necessary for ETX activation (23, 35), one of the paired supernatant aliquots was treated with 0.05% trypsin for 30 min at 37°C, whereas the other aliquot was similarly incubated without trypsin. Two BALB/c mice (male or female, ca. 17 to 20 g; Charles River Laboratories) each received an i.v. injection (tail vein) of 0.5 ml of the trypsinized (i.e., trypsin-treated) supernatant, while

two other mice each received a similar 0.5-ml i.v. injection of the nontrypsinized supernatant. All mice were observed for up to 48 h to monitor the development of significant neurological distress, at which point those mice were immediately euthanized with CO_2. Our IACUC permit did not allow death as a routine expected experimental endpoint, so supernatants that produced significant neurologic distress (defined by the development of one or more of the following signs: incoordination, ataxia, paralysis, blindness, or convulsions) within 48 h were considered to possess lethal activity. However, pilot experiments indicated that death typically follows the onset of neurologic distress in mice receiving i.v. injections of genotype D late-log-phase supernatants.

For vegetative culture supernatants inducing neurologic distress, a 50% lethal dose (LD_{50})/ml was determined with additional pairs of mice, who received i.v. injections containing twofold dilutions (between 1/50 to 1/800) in 1% peptone water of late-log-phase supernatant aliquots that were or were not trypsinized (as described above). Negative and positive control mice were also included in each assay, with negative control mice receiving an i.v. injection of 1% peptone water that did or did not contain trypsin (two mice each). Positive control mice received i.v. injections containing twofold dilutions of a filtered *C. perfringens* type D (CPE-negative and CPB2-negative) late-log-phase supernatant of known toxicity, given at the same dilutions used for the test samples. The toxin titration was calculated as double the reciprocal of the highest dilution inducing lethality, within 48 h, in at least one of the two paired mice; this result was then expressed as the 50% mouse lethal dose/ml.

For these lethality assays, at least two batches of each vegetative culture supernatant were prepared and tested in mice as described above. Lethality results were first averaged for each supernatant batch, followed by averaging the means for the two or more different supernatant preparations tested for each genotype D isolate. If results for different preparations of late-log-phase supernatant for a particular isolate showed >3-fold dilution difference, the samples were retested. All experimental procedures were approved by the Animal Care and Use Committee of the California Animal Health and Food Safety Laboratory, University of California, Davis (permit 34).

Neutralization of genotype D vegetative culture supernatant lethality. To help identify which toxins are responsible for the lethal activity of supernatants prepared from late-log-phase genotype D cultures, monoclonal antibody (MAb) neutralization experiments were performed as follows.

(i) Preincubation of ETX MAb with genotype D vegetative culture supernatants. A 0.6-ml volume of an undiluted genotype D late-log-phase supernatant, prepared as described above, was divided into two aliquots that either were or were not trypsinized as described above. The paired aliquots were then filter sterilized prior to the addition of 0.1 ml of a solution containing 2 mg of ETX-neutralizing MAb 5B7/ml. Those mixtures were then brought to 1.2 ml with 1% peptone water and incubated for 30 min at room temperature. A 0.5-ml aliquot of each mixture was injected i.v. into two mice, while an additional pair of mice received a similar i.v. injection of the same sterile trypsinized or nontrypsinized supernatants that had been identically prepared, except for the omission of ETX MAb.

(ii) Preincubation of CPA MAb with genotype D vegetative culture supernatants. Filter-sterilized supernatants from late-log-phase cultures of six representative *C. perfringens* genotype D strains were incubated with 2 mg of an anti-CPA MAb (kindly provided by P. Hauer)/ml. Mouse inoculations using those mixtures were performed as described above for ETX neutralization studies.

(iii) Preincubation of CPE MAb with genotype D vegetative culture supernatants. Sterile supernatants were prepared from late log-phase cultures of four selected *C. perfringens* genotype D strains as described above. The sterile supernatants were then preincubated at room temperature for 30 min with 0.1 ml of a solution containing 2 mg of the CPE-neutralizing MAb 3C9 (38)/ml before being i.v. injected into mice, as described for ETX supernatant neutralization experiments.

MAb neutralization of semipurified CPA. A 0.5-ml aliquot (containing a 30 LD_{50} dose) of semipurified, ultrafiltered CPA was obtained from a vaccine production batch (CSL, Ltd., Melbourne, Australia) prepared from an ovine isolate of *C. perfringens* type A. That toxin preparation was preincubated at room temperature for 30 min with 0.1 ml of a solution containing 2 mg of either the same CPA- or ETX-neutralizing MAb/ml used in the genotype D supernatant neutralization experiments described above. A 0.5-ml aliquot of each CPA-antibody solution was then injected i.v. into mice.

MAb neutralization of purified ETX. ETX was purified to homogeneity using a modification of the classical method of Habeeb (14). Briefly, late-log-phase supernatant from type D isolate NCTC8346 was precipitated with 45% ammonium sulfate, followed by successive ion-exchange chromatography on Macro-Prep DEAE support (Bio-Rad) and Macro-Prep CM cation-exchange support (Bio-Rad). A 0.5-ml aliquot of pure ETX was then activated with trypsin (as described above) and preincubated at room temperature for 30 min with 0.1 ml of a solution containing 2 mg of either the same CPA- or ETX-neutralizing MAb/ml used in the genotype D supernatant neutralization experiments described above. A 0.5-ml aliquot of each ETX-antibody solution was then injected i.v. into mice.

TABLE 1. Multiplex PCR analysis of genotype D isolates

No. of isolates (%)	Genotype
27 (69)	*plc etx*
5 (13)	*plc etx cpe*
5 (13)	*plc etx cpe cpb2*
2 (5)	*plc etx cpb2*

RESULTS

Lethal toxin gene carriage by putative *C. perfringens* type D isolates. Multiplex PCR analysis was performed to survey the presence of genes encoding six major *C. perfringens* lethal toxins among a collection of 47 putative type D isolates. The 30 surveyed isolates from the BW collection had been previously classified as type D many years ago using the classical toxin neutralization typing method (15, 33). Multiplex PCR analyses confirmed (data not shown) that 22 of the 30 BW isolates are genotype D, since they carry *plc* and *etx* genes but not *cpb* or *iap* genes (10). Of the remaining eight BW isolates formerly classified as type D by classical toxin neutralization approaches, seven were identified as genotype A (i.e., these isolates carry the *plc* gene, but not the *etx* gene required of genotype D isolates), while the other putative type D isolate from the BW collection was found to classify as genotype B (i.e., it carries the *plc*, *etx*, and *cpb* genes). Similar multiplex PCR analysis was also performed on 10 recent animal disease isolates, which confirmed their previous identification by J. G. Songer (unpublished data) as genotype D. Finally, multiplex PCR analysis of seven other putative type D isolates collected from various other sources confirmed their identity as genotype D.

The 39 isolates identified as genotype D by multiplex PCR analysis were heterogeneous with respect to their carriage of genes encoding two other lethal toxins, i.e., CPE and CPB2. Nearly 70% of these genotype D isolates were found to lack both the *cpe* and *cpb2* genes, while the remaining 12 genotype D isolates carried *cpe*, *cpb2*, or both the *cpe* and the *cpb2* genes (Table 1).

Lethal toxin levels in genotype D vegetative culture supernatants. Phenotypic studies were then conducted to evaluate whether the 39 surveyed genotype D isolates express their lethal toxin genes during vegetative growth and to establish whether these isolates produce PFO, a lethal toxin whose gene (*pfoA*) is not detected by the conventional multiplex PCR assay. PLC activity on egg yolk agar plates and hemolytic activity against horse erythrocytes were measured to determine CPA or PFO activity levels, respectively, in vegetative culture supernatants of genotype D isolates. Because specific activity assays are not available for ETX, CPB2, or CPE, the presence of these three lethal toxins in genotype D supernatants was assessed by Western blotting.

Since our genotype D supernatant characterization studies would eventually also include extensive lethality determina-

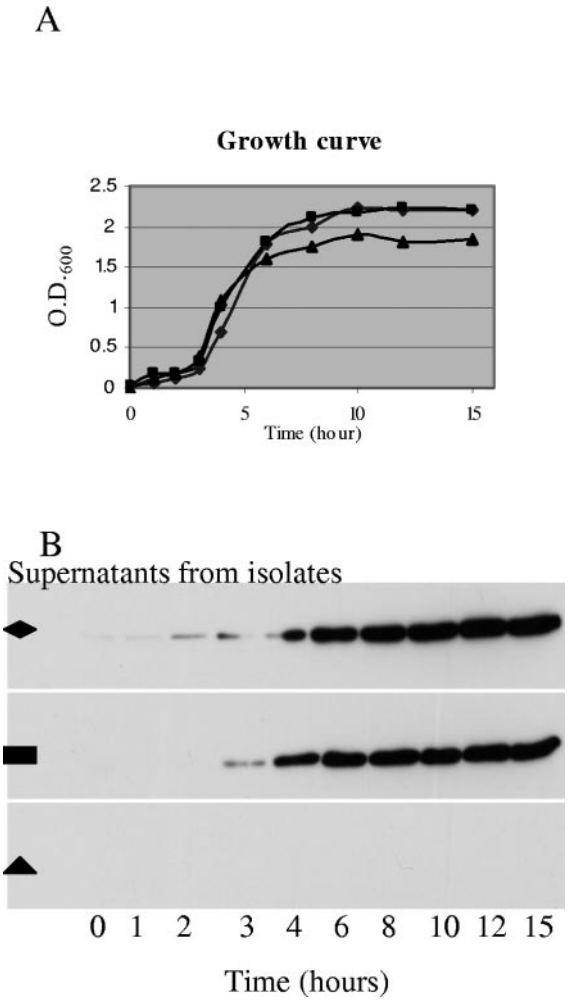


FIG. 1. Pilot studies to optimize ETX expression in genotype D vegetative cultures. Three randomly selected genotype D isolates (■, ▲, and ◆) were inoculated into TGY medium and incubated at 37°C. (A) At the specified times, aliquots were removed from each culture and the optical density at 600 nm of the aliquot was determined with a spectrophotometer. (B) After centrifugation, supernatant ETX levels for each isolate were evaluated by Western blotting, as described in the Materials and Methods. The results shown are representative of two repetitions. Note the absence of any detectable ETX in the supernatant of isolate (▲).

tions, which would use large numbers of mice, we could only choose a single growth condition for preparing vegetative supernatants from genotype D isolates. Before picking that growth condition for vegetative supernatant characterization studies, pilot studies were conducted to optimize conditions for ETX production by type D vegetative cultures. Those studies revealed (data not shown) that, of four tested media (TGY, FTG, brain heart infusion, and differential reinforced clostridial broth), TGY most consistently supported ETX production by several randomly selected D isolates. Additional pilot studies with three genotype D isolates growing in TGY then determined (Fig. 1) that one genotype D isolate failed to produce detectable levels of ETX at any point in the growth curve. However, the other two pilot isolates produced large amounts of ETX by 8 to 10 h (i.e., by late log phase), with their ETX levels then remaining relatively constant for several hours thereafter. Since, (i) late log phase is also optimum for CPB2 and CPA production (5, 9) and (ii) late-log-phase cultures also express PFO (25), supernatants from late-log-phase culture were used for all further studies characterizing the toxin content and lethality of type D vegetative culture supernatants.

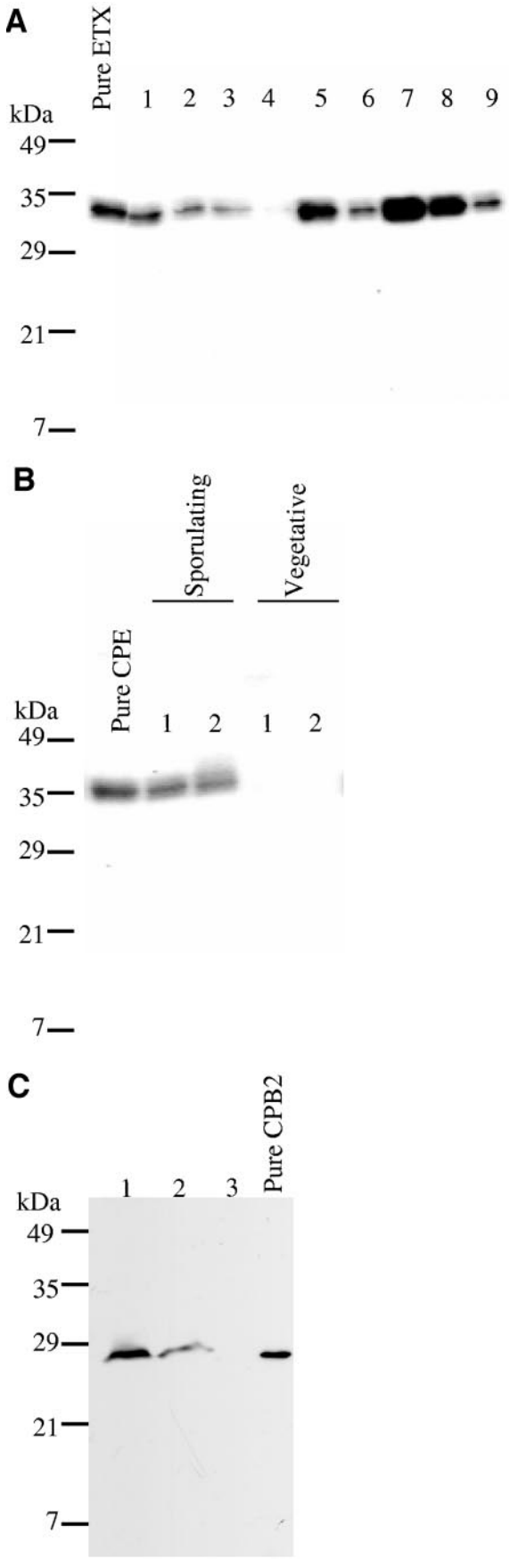
Using those supernatants, Western blot analyses revealed (see Fig. 2A, for representative results) significant variations in supernatant ETX levels among the surveyed genotype D isolates. Late-log-phase culture supernatants from two genotype type D isolates contained no detectable ETX, even using 10-fold-concentrated supernatants and a highly sensitive Western blot detection substrate, while similar supernatants from 13 other genotype D isolates had only low ETX levels (i.e., <1.7 μg of ETX/ml of supernatant). However, medium to high ETX levels (i.e., >1.7 to 53 μg of ETX/ml) were detected in late-log-phase supernatants prepared from the 24 remaining genotype D isolates.

As previously established for *cpe*-positive type A isolates (7), CPE expression by *cpe*-positive genotype D isolates was strongly sporulation associated. None of the 10 surveyed *cpe*-positive isolates produced any CPE during vegetative growth (see Fig. 2B for representative Western blot results). However, the eight *cpe*-positive genotype D isolates that were able to sporulate in DS sporulation medium also could produce CPE in that medium (representative results shown in Fig. 2B). The remaining two *cpe*-positive type D isolates neither sporulated nor produced CPE in DS medium.

Of the seven *cpb2*-positive genotype D isolates surveyed, six were able to express CPB2 during late-log-phase growth (Fig. 2C). However, CPB2 production by those isolates was modest (ranging up to 0.4 μg/ml), becoming detectable only when 50-fold-concentrated vegetative supernatants were used for Western blotting.

Nearly two-thirds of these 39 genotype D isolates were found to produce both CPA and PFO activity during late log phase (Fig. 3). However, similar vegetative supernatants from one third of the surveyed isolates contained no detectable PFO activity (Fig. 3A), while late-log-phase supernatant from one PFO-positive genotype D isolate contained no detectable CPA activity (Fig. 3B). When present, PFO or CPA activity levels in these vegetative supernatants showed considerable isolate-to-isolate variation. PFO activity levels (Fig. 3A) in late-log-phase supernatants ranged from 1 to 5.5 ($\log_2$ titer), while their CPA activity levels (Fig. 3B) ranged from 0.6 to 5.1 PLC units/mg $\times$ 10^{-3}. No consistent correlations (i.e., R^2 values were always <0.4) were observed between the production of ETX, CPA, or PFO by individual genotype D isolates (data not shown).

Lethal properties of genotype D vegetative culture supernatants. Collectively, the results shown in Fig. 2 and 3 indicated that late-log-phase cultures of nearly all genotype D isolates produce multiple lethal toxins, although with considerable variation in amounts. Therefore, 21 of these genotype D isolates were selected for further study to evaluate the contribution of various toxins to the lethal properties of their vegetative supernatants in the mouse i.v. injection model. These 21 isolates included a range of CPA, PFO, and ETX producers (Table 2), including one non-ETX producer that expresses medium amounts of CPA. In addition, those selected genotype D isolates included one expressing CPB2 and 3 others that are



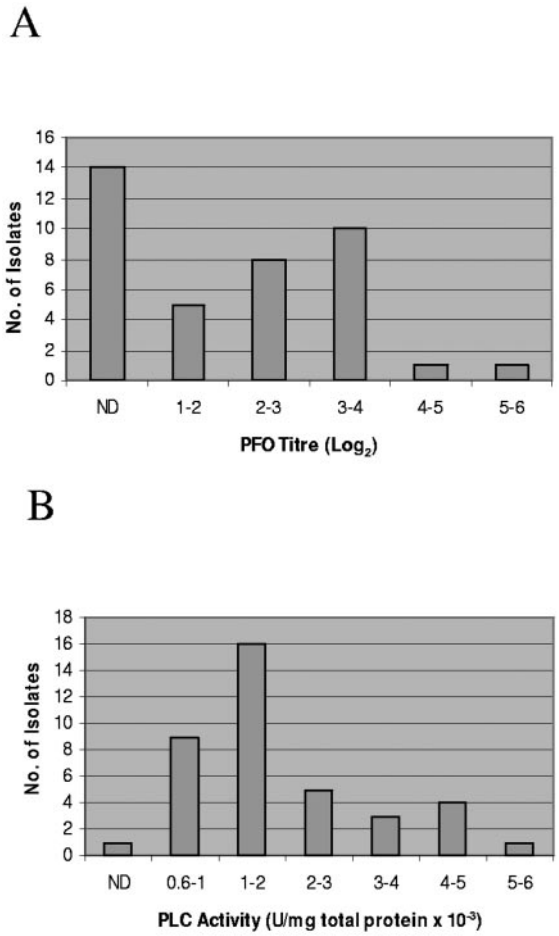


FIG. 3. PFO (A) or alpha-toxin (B) activities in vegetative supernatants of surveyed genotype D isolates. Isolates are grouped based upon the mean of three independent toxin activity determinations. ND, no significant activity detected.

cpe-positive (although they do not express CPE during vegetative growth).

Several of the lethal toxins produced by late-log-phase cultures of genotype D isolates in Fig. 2 and 3 can be trypsin sensitive (11, 13), as confirmed by our pilot study demonstrating that a 30-min pretreatment with 0.05% trypsin completely

FIG. 2. Western blot analysis of ETX, CPE, and CPB2 toxin production by genotype D isolates. (A) Variations in ETX production by representative genotype D isolates probed with a monoclonal antibody against ETX. Pure epsilon-toxin (far left lane) was also electrophoresed as a control. (B) Variations in CPE production by sporulating and vegetative culture of representative *cpe*-positive genotype D isolates probed with CPE polyclonal antiserum. The pure CPE lane shows sporulating culture; sporulating culture lanes 1 and 2 show supernatants of two *cpe*-positive genotype D isolates grown in DS sporulation medium. Vegetative culture lanes 1 and 2 show late-log-phase culture supernatant of these same two isolates grown in TGY medium. (C) Variations in CPB2 toxin production by three representative *cbp2*-positive type D isolates probed with a polyclonal antibody against CPB2 toxin. Pure CPB2 toxin (far right lane) was also electrophoresed as a control.

TABLE 2. Supernatant neutralization with different antibodies

Supernatant	Vegetative culture (phenotype 1)[a]	Epsilon antibody	Alpha antibody
1	$PLC_{(4.2)}$, $PFO_{(5.5)}$, $ETX_{(53)}$	Yes	ND[b]
2	$PLC_{(1.6)}$, $PFO_{(<1)}$, $ETX_{(17)}$	Yes	ND
3	$PLC_{(2.5)}$, $PFO_{(<1)}$, $ETX_{(6)}$	Yes	No
4	$PLC_{(<1)}$, $PFO_{(1.8)}$, $ETX_{(6)}$	Yes	ND
5	$PLC_{(1.9)}$, $PFO_{(<1)}$, $ETX_{(25)}$	Yes	ND
6	$PLC_{(2.1)}$, $PFO_{(<1)}$, $ETX_{(13)}$	Yes	ND
7	$PLC_{(4.6)}$, $PFO_{(3.5)}$, $ETX_{(5)}$	Yes	ND
8	$PLC_{(2.1)}$, $PFO_{(<1)}$, $ETX_{(8)}$	Yes	No
9	$PLC_{(1.5)}$, $PFO_{(<1)}$, $ETX_{(10)}$	Yes	No
10	$PLC_{(1.2)}$, $PFO_{(1.6)}$, $ETX_{(5)}$	Yes	ND
11	$PLC_{(<1)}$, $PFO_{(2.7)}$, $ETX_{(3)}$	ND	ND
12	$PLC_{(5.1)}$, $PFO_{(2.9)}$, $ETX_{(2)}$	Yes	ND
13	$PLC_{(2.1)}$, $PFO_{(<1)}$, $ETX_{(8)}$	ND	ND
14	$PLC_{(3.8)}$, $PFO_{(2.9)}$, $ETX_{(4)}$	Yes	No
15	$PLC_{(1.1)}$, $PFO_{(<1)}$, $ETX_{(4)}$	Yes	No
16	$PLC_{(<1)}$, $PFO_{(2.7)}$, $ETX_{(3)}$	ND	ND
17	$PLC_{(1.4)}$, $PFO_{(<1)}$, $ETX_{(<1)}$	ND	ND
18	$PLC_{(2.0)}$, $PFO_{(3.7)}$, $ETX_{(52)}$, $CPE_{(0)}$	Yes	ND
19	$PLC_{(4.6)}$, $PFO_{(3.5)}$, $ETX_{(2)}$, $CPE_{(0)}$	ND	ND
20	$PLC_{(2.1)}$, $PFO_{(<1)}$, $ETX_{(13)}$	ND	ND
21	$PLC_{(<1)}$, $PFO_{(1.6)}$, $ETX_{(5)}$, $CPB2_{(0.03)}$	ND	ND
Alpha-toxin	PLC	No	Yes

[a] Values shown in subscript parentheses for ETX, CPB2, and CPE are micrograms of protein/ml and for PFO and PLC are activity levels ($\log_2$ titer or U/ml [10^{-3}], respectively).
[b] ND, not determined.

abrogates the lethality of a 30 LD_{50}/ml dose of semipurified CPA. In contrast, the ETX prototoxin must be proteolytically activated to acquire lethal properties (23, 35), which we confirmed by demonstrating that a 30-min preincubation with 0.05% trypsin decreases the LD_{50} of pure ETX prototoxin from 1 to 10 μg/ml to ~12.5 ng/ml (~312 ng/kg). Because of these trypsin sensitivity differences, initial experiments were performed to determine whether similar trypsin pretreatment affects the lethal properties of late-log-phase supernatants prepared from the 21 selected genotype D isolates (Fig. 4). These studies revealed that, without trypsin pretreatment, these supernatants cause little or no lethality when injected i.v. into mice. Nor was lethality observed in mice receiving injections of trypsin-containing buffer alone. However, trypsin pretreatment initiated, or substantially increased, the lethality of late-log-phase supernatants from 19 of the 21 tested genotype D isolates. A non-ETX producer was one of the two genotype D isolates whose vegetative supernatant did not show trypsin enhancement of lethality; even undiluted, late-log-phase supernatant from that ETX-negative isolate was not consistently lethal, with or without trypsin pretreatment.

Since the Fig. 4 results were consistent with ETX playing an important role in genotype D vegetative supernatant-induced lethality, the lethal toxin content of late-log-phase supernatants from these 21 isolates were compared against their trypsin-activated lethality, if any. Those analyses revealed a strong correlation between the ETX levels of these trypsin-activated supernatants and their lethality (Fig. 5, top panel). Similar comparisons indicated that neither CPA (Fig. 5, middle panel) nor PFO (Fig. 5, bottom panel) activity levels significantly correlate with trypsin-activated lethality of late-log-phase supernatants. No attempt was made to correlate CPE or CPB2 expression with lethality because Fig. 2 (middle and bottom panels) results demonstrated that little or none of those two toxins are present in vegetative culture supernatants of genotype D isolates.

MAb neutralization of genotype D vegetative supernatant lethality. To definitively evaluate whether ETX is required for the lethal properties of most trypsin-activated genotype D vegetative culture supernatants, MAb neutralization experiments were performed. Preincubation of neutralizing anti-ETX MAb 5B7 with each of 14 different genotype D late-log-phase supernatants, all containing ETX and exhibiting trypsin-activated lethality in Fig. 4 and 5 experiments, completely neutralized their lethality for mice (Table 2). Furthermore, this anti-ETX MAb also neutralized the lethal activity of late-log-phase supernatant from the one ETX-producing genotype D isolate that did not exhibit enhanced lethality after trypsin pretreatment in Fig. 4 studies. In contrast, preincubation of an anti-CPA MAb with trypsin-activated late-log-phase supernatants from 5 of these 14 genotype D isolates failed to neutralize their lethality, even though these 5 isolates included the strongest CPA and PFO producers analyzed in this survey.

Several controls support the specificity of these neutralization results. First, under preincubation conditions identical to those used above with the genotype D supernatants, the anti-ETX MAb neutralized the lethality of 5 μg of pure trypsin-activated ETX but not a 30 LD_{50} dose of semipurified CPA. However, that CPA dose (but not activated ETX) was neutralized by similar preincubation with an anti-CPA MAb. A final control for antibody specificity included the failure of an anti-CPE MAb to neutralize the lethality of trypsin-pretreated late-log-phase supernatants from five selected genotype D isolates.

DISCUSSION

In domestic ruminants, *Clostridium perfringens* type D isolates are a major cause of fatal enterotoxemias (18, 33). To reduce the economic impact of these enterotoxemias, vaccination is widely practiced using vaccines that typically consist of inactivated supernatant filtrate prepared from a vegetative culture of a type D isolate (18, 33). Whether those type D supernatants commonly contain lethal amounts of ETX and other toxins has been unclear but could be important for improving vaccine efficacy. For example, if vegetative cultures of type D isolates typically produce lethal levels of several toxins, optimally protective vaccines should probably be produced using type D supernatants that contain high levels of all of those lethal toxins and not just ETX.

Classical *C. perfringens* toxin typing studies (15, 20) have demonstrated that antiserum raised against type A vegetative culture supernatants cannot neutralize the lethal properties of type D vegetative culture supernatants in the mouse i.v. injection model, which is consistent with ETX (produced by type D, but not by type A, isolates) playing a role in type D supernatant-induced lethality in mice. However, results from the present study imply that the polyclonal antisera raised against type D supernatants and then used in classical toxin typing studies would typically contain a mix of antibodies against several lethal toxins. Therefore, previous toxin typing results did not preclude the possibility of other lethal toxins (besides ETX) contributing to, or even being sufficient for, the mouse

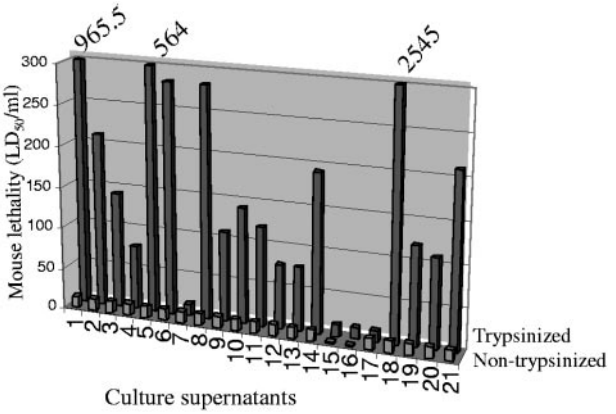


FIG. 4. Mouse lethality assay results with trypsinized or nontrypsinized genotype D supernatants. The front row shows mouse lethality results with a nontrypsinized aliquot of vegetative culture supernatants prepared from 21 different genotype D isolates (see Table 2 for their phenotype). The back row depicts mouse lethality results for the same genotype D vegetative supernatants after trypsinization. Numbers at top of supernatants 1, 5, and 18 had lethality levels too large to depict on this graph. The results shown are the mean of two independent determinations. Note that culture supernatants were arbitrarily assigned numbers 1 to 21 to avoid possible misuse by identifying genotype D isolates producing highly lethal supernatants. Genotype D isolates producing supernatants 1 to 4, 7 to 12, 14, and 18 to 20 are animal disease isolates from the BW collection; isolates producing supernatants 13, 17, and 21 are recent North American animal disease genotype D isolates provided by Glenn Songer; and the remaining supernatants (5, 6, and 16) were from animal disease genotype D isolates obtained from miscellaneous sources.

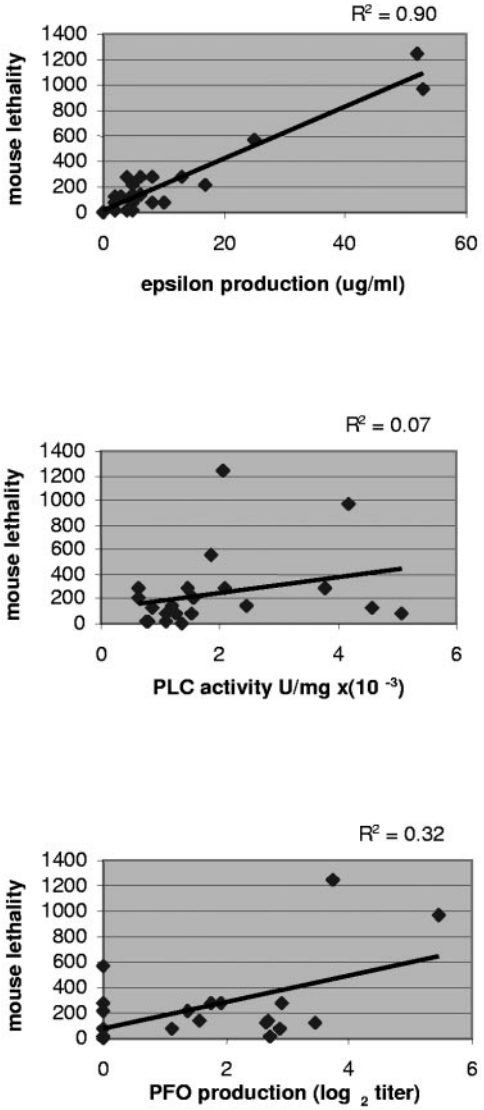


FIG. 5. Correlations between mouse lethality and lethal toxin levels in the vegetative supernatants of genotype D isolates. (Top panel) Regression analysis of late-log-phase supernatant ETX levels (μg/ml) versus mouse lethality. Regression analyses of late-log-phase supernatant alpha-toxin (middle panel) or PFO (bottom panel) levels versus mouse lethality are also shown. For the bottom panel, isolates graphed at 0 on the *x* axis had an undetectable PFO activity level. Mouse lethality results shown represent the mean of two independent determinations and toxin level, or activity determinations represent the mean of three independent determinations. The R^2 shown for each panel is the correlation coefficient.

lethality induced by some or all type D vegetative culture supernatants.

Consequently, the major goals of the present study were to comprehensively characterize lethal toxin production by genotype D isolates and to start identifying which of these exotoxins are important for the lethality of genotype D vegetative supernatants in the mouse i.v. injection model. By determining the carriage and expression of lethal toxin genes among our large collection of genotype D isolates, our analyses now establish that genotype D isolates typically produce widely varying levels of at least three lethal toxins during vegetative growth. The basis for these variations in toxin expression among type D isolates is not clear but could involve such factors as promoter sequence variations or isolate-to-isolate variations in the activity of two-component regulatory systems (e.g., VirR/VirS), which are known to positively regulate CPB2, CPA, and PFO expression levels in type A isolates (2, 6, 26). Currently, little or no information is available regarding the regulation of ETX production.

With respect to identifying which specific toxins are important for the lethality of genotype D vegetative supernatants, three results from the present study indicate that, under the experimental conditions used, ETX is required for the lethal properties of most late-log-phase type D supernatants in the mouse i.v. injection model. First, our studies identified a strong correlation between genotype D vegetative supernatant ETX levels and mouse lethality, whereas CPA or PFO activity levels in those supernatants showed no correlation with mouse lethality. The current results also revealed that ~5% of the surveyed genotype D isolates produce no detectable ETX, at least under the current experimental conditions. Further study of these non-ETX-producing isolates is under way, but the apparent discovery of silent *etx* genes in some genotype D isolates has potential diagnostic significance, i.e., genotype D isolates with silent *etx* genes would classify differently using classical toxin typing neutralization techniques compared to multiplex PCR genotyping approaches. To our knowledge, silent *etx* genes have not been identified previously, but a precedent exists for silent *C. perfringens* lethal toxin genes, e.g.,

type E isolates usually carry silent *cpe* genes (3) and some type A isolates (and possibly some type D isolates, see Fig. 2) apparently carry silent *cpb2* genes (4, 9, 16, 36). Similarly, several isolates previously classified as type D by toxin neutralization typing approaches were shown in our study to belong to genotype B; this classification discrepancy might be attributable to isolates carrying silent *cpb* genes.

A second line of evidence in support of ETX as the major toxin contributing to the lethality of most genotype D vegetative supernatants is provided by our trypsin pretreatment results. These studies indicate that trypsin pretreatment was either required for, or substantially increased, the mouse lethality of vegetative supernatants prepared from 19 of the 21 tested genotype D isolates. That determination is consistent with ETX playing the major role in type D supernatant lethality given (i) the known requirement for proteolytic activation of ETX toxicity (23) and (ii) observations that CPB2 (11) and CPA (13) can be trypsin sensitive. ETX activation explaining the trypsin pretreatment-induced increase in mouse lethality observed for most surveyed genotype D vegetative supernatants is also consistent with our observation that an ETX-negative genotype D isolate produced a late-log-phase supernatant that was nonlethal, with or without trypsin pretreatment.

Our antibody neutralization results provide the final piece of evidence that ETX plays a major role in genotype D vegetative supernatant-induced mouse lethality. An anti-ETX MAb was shown to specifically block the lethality of trypsin-activated late-log-phase supernatants from most genotype D isolates, which indicates ETX is necessary for the lethal properties of these supernatants in the mouse i.v. injection model. Notably, this anti-ETX MAb (but not an anti-CPA MAb) even neutralized the one ETX-containing supernatant that exhibited the same lethality in the presence or absence of trypsin-pretreatment. Besides confirming ETX is responsible for the lethal properties of that supernatant, this result also suggests that genotype D isolate produces extracellular proteases to self-activate ETX, a phenomenon noted previously for some type D isolates (22). For other type D isolates, ETX is activated by intestinal proteases prior to its absorption (14).

The present study's conclusion that, under the experimental conditions used, ETX is necessary for the lethality of most genotype D vegetative culture supernatants in the mouse i.v. injection model is consistent with the potency of this toxin, which has the lowest LD_{50} (0.1 to 0.3 μg/kg) of all *C. perfringens* toxins when administered via the i.v. route (12). By comparison, the other lethal toxins consistently present in type D supernatants, i.e., CPA and PFO, have LD_{50} values of 3 or 13 μg/kg, respectively, by the i.v. route (12). Establishing that ETX is required for the lethality of most genotype D vegetative supernatants is also consistent with results from a previous study by el Idrissi and Ward (8), who noted a correlation between mouse i.v. lethality and ETX levels (measured by enzyme-linked immunosorbent assay) in late-log-phase supernatants prepared from six type D isolates. These authors noted that their putative correlation between ETX levels and lethality was preliminary and required confirmation by analysis of additional *C. perfringens* type D isolates; in that regard, only a single genotype D isolate was common to both studies. Although el Idrissi and Ward did not attempt to neutralize type D supernatant lethality with an ETX-specific antibody, our results demonstrating that ETX is required for genotype D vegetative supernatant lethality are consistent with, and offer support for continuing, the common practice of evaluating type D veterinary vaccination efficacy by measuring serum anti-ETX titers in immunized animals (18). These new findings are also consistent with a previous study showing that immunization with an anti-idiotypic antibody raising antibodies against an ETX neutralizing epitope can protect mice against challenge with a virulent type D isolate (27).

There are also several obvious limitations to our study. First, although this survey included a large number of genotype D isolates with diverse geographic origins and dates of isolation, rare genotype D isolates could exist that cause ETX-independent mouse lethality by producing exceptionally large amounts of another (non-ETX) lethal toxin. Supporting that possibility, a few type A isolates have been identified that express exceptionally large amounts of CPA activity, e.g., late-log-phase supernatants of type A strain ATCC 13124 produce CPA activity levels of 18.8 U/mg of total protein $\times 10^{-3}$ (determined in the present study). Second, variations in culture conditions could affect the relative contributions to mouse lethality by different lethal toxins in genotype D vegetative supernatants. That possibility should be evaluated by future studies, but the culture conditions used in our study for preparing vegetative supernatants were chosen for maximal toxin content (see Results) and closely resemble the culture conditions used by el Idrissi and Ward, thus allowing direct comparisons between these two studies. Third, our studies do not necessarily rule out the possibility that additive or synergistic interactions between ETX, CPA, and PFO could affect lethality. For example, such toxin interactions might hasten time to death of mice, a factor not investigated in the current study. There is precedent for interactions between *C. perfringens* toxins, since CPA and PFO have been shown to act synergistically in a mouse model of *C. perfringens* type A myonecrosis (1). Fourth, species differences could affect the relative sensitivity of animals to type D lethal toxins (see Introduction). Finally, although injecting supernatants i.v. into mice is useful for modeling the rapid lethality of type D enterotoxemias, this approach obviously does not completely mimic the natural infection. Conceivably, other lethal toxins could play a role in, or even be required, for ETX absorption across the intestinal mucosa. This important question cannot currently be addressed for large numbers of genotype D isolates in the absence of a well-characterized small animal oral-challenge model amenable to antibody neutralization approaches. Such oral challenge models would also be useful for dissecting the lethal properties of type D isolates using toxin knockout mutants. Therefore, efforts are currently under way to develop improved animal models for studies aimed at elucidating the virulence of type D isolates.

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Dissecting the Contributions of *Clostridium perfringens* Type C Toxins to Lethality in the Mouse Intravenous Injection Model

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The gram-positive anaerobe *Clostridium perfringens* produces a large arsenal of toxins that are responsible for histotoxic and enteric infections, including enterotoxemias, in humans and domestic animals. *C. perfringens* type C isolates, which cause rapidly fatal diseases in domestic animals and enteritis necroticans in humans, contain the genes for alpha toxin (*plc*), perfringolysin O (*pfoA*), beta toxin (*cpb*), and sometimes beta2 toxin (*cpb2*) and/or enterotoxin (*cpe*). Due to the economic impact of type C-induced diseases, domestic animals are commonly vaccinated with crude type C toxoid (prepared from inactivated culture supernatants) or bacterin/toxoid vaccines, and it is not clear which toxin(s) present in these vaccines actually elicits the protective immune response. To improve type C vaccines, it would be helpful to assess the contribution of each toxin present in type C supernatants to lethality. To address this issue, we surveyed a large collection of type C isolates to determine their toxin-producing abilities. When late-log-phase vegetative culture supernatants were analyzed by quantitative Western blotting or activity assays, most type C isolates produced at least three lethal toxins, alpha toxin, beta toxin, and perfringolysin O, and several isolates also produced beta2 toxin. In the mouse intravenous injection model, beta toxin was identified as the main lethal factor present in type C late-log-phase culture supernatants. This conclusion was based on monoclonal antibody neutralization studies and regression analyses in which the levels of alpha toxin, beta toxin, perfringolysin O, and beta2 toxin production were compared with lethality. Collectively, our results highlight the importance of beta toxin for type C-induced toxemia.

Clostridium perfringens is a gram-positive, anaerobic bacterium that causes histotoxic and enteric infections (including enterotoxemias) in humans and domestic animals by virtue of production of a large number of toxins (25, 33). The differential production of four *C. perfringens* toxins, alpha toxin (PLC, also referred to as CPA), beta toxin (CPB), epsilon toxin, and iota toxin, is the basis for classifying *C. perfringens* isolates into five types, types A to E. By definition, type C isolates must produce PLC and CPB, but they could also produce several other biomedically relevant lethal toxins not used in the current toxinotyping classification scheme, including perfringolysin O (PFO), beta2 toxin (CPB2), and/or enterotoxin (CPE).

Type C isolates cause several enteric diseases in domestic animals and have a significant impact on the agricultural industry, in terms of both livestock loss and vaccination costs (34). The type C infections include acute enterotoxemia in adult sheep (also known as "struck"), hemorrhagic or necrotic enterotoxemias in piglets, lambs, calves, kids, and foals, and necrotic enteritis in poultry. Newborn animals, especially piglets, are particularly susceptible to type C infections, which can result in herd morbidity rates of 30 to 50%. While the pathology and case fatality rates associated with type C infections are different in different species, most animals exhibit obvious hemorrhage and necrosis of the intestines, followed by death that is generally attributed to beta toxemia and/or the intestinal disorders mentioned above. In adult sheep, evidence of toxemia, including accumulation of protein-rich peritoneal, pleural, and pericardial fluid, is often apparent.

C. perfringens type C isolates are also the only non-type A isolates known to cause human disease (15, 17). Type C isolates are responsible for human enteritis necroticans (also known as pigbel or Darmbrand). The risk factors for enteritis necroticans include low intestinal trypsin levels and/or low intestinal motility due to a protein-poor diet or preexisting health problems (such as pancreatic disease) that affect the gastrointestinal tract. In pigbel, the type C isolates are introduced by consumption of undercooked meat products (often pork) (15). In severe cases of this disease, patients exhibit signs of toxemia, and segmental necrotizing enteritis is visible in the proximal intestine (15). Prior to control with a type C toxoid vaccine (16), enteritis necroticans was the most common cause of death in children who were more than 1 year old in the highlands of Papua New Guinea (22).

It has been commonly assumed, but never directly proven, that CPB (the second most potent *C. perfringens* toxin, which has a mouse 50% lethal dose [LD_{50}] of ~310 ng/kg [27]) is responsible for the deaths resulting from type C infections (34). However, administration of CPB alone does not result in the intestinal pathology or lethality of a type C infection (which requires culture supernatants or bacteria), suggesting that CPB

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alone does not cause classic type C disease (34). Furthermore, the current type C vaccines administered to domestic animals generally contain inactivated, relatively crude culture supernatants prepared from type C isolates (35, 38, 40). Consequently, it has not been definitively established which toxins present in type C supernatants (or the type C bacteria themselves) are needed to elicit protective immunity.

The mouse intravenous (i.v.) injection model is used to assess protection by type C vaccines prepared for domestic animals. This model is believed to mimic the systemic phase of type C infections, in which type C toxins circulate systemically after absorption from the intestine (34). However, since type C supernatants contain a number of lethal toxins, it is not clear which toxin(s) in type C supernatants is responsible for mouse lethality. Furthermore, while the effects of i.v. injection of individual purified toxins into mice have been reported, the contributions of different toxins to type C supernatant-associated lethality have not been rigorously assessed previously.

Therefore, the aim of this study was to use the mouse i.v. injection model to assess the role in lethality of individual toxins present in type C vegetative culture supernatants. Initially, the toxin repertoires (at the genotypic and phenotypic levels) of a large collection of type C isolates were determined. Select isolates were then tested further using the mouse i.v. injection model to assess the relative contributions to lethality of toxins produced during type C vegetative growth. Our results suggest that the CPB toxin is the primary mediator of lethality when type C vegetative culture supernatants are injected i.v. into mice.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 55 putative *C. perfringens* type C strains surveyed in this study either were obtained from our laboratory collection (4 human or domestic animal disease isolates) or were kindly provided by J. Glenn Songer (University of Arizona) (7 domestic animal disease isolates) or Russell Wilkinson (University of Melbourne) (44 isolates originating from the Burroughs-Wellcome collection [BW] of *C. perfringens* human or domestic animal disease isolates). The 11 type A isolates surveyed were obtained from our laboratory collection of environmental and disease isolates. Stock cultures of *C. perfringens* isolates were prepared in cooked meat medium (Difco Laboratories) and were stored at −20°C. Prior to use in experiments, bacteria were initially cultured overnight at 37°C in fluid thioglycolate medium (FTG) (Difco Laboratories). To ensure culture purity, samples of overnight FTG cultures were streaked on TSC agar plates (SFP agar [Difco Laboratories] containing 0.1% D-cycloserine [Sigma-Aldrich]), which were incubated overnight at 37°C under anaerobic conditions. Unless indicated otherwise, bacteria were grown in TGY broth (3% tryptic soy broth [Becton-Dickinson], 2% glucose [Sigma Aldrich], 1% yeast extract [Becton-Dickinson], 0.1% L-cysteine [Sigma Aldrich]) to assess vegetative toxin production and to prepare vegetative culture supernatants for mouse i.v. challenge studies.

Multiplex PCR toxin genotyping of *C. perfringens* isolates. *C. perfringens* isolates were inoculated onto brain heart infusion agar (Difco Laboratories) plates and incubated overnight at 37°C under anaerobic conditions. The resultant colonies were then used to prepare template DNA, as previously described (41). To detect the presence of toxin genes in our putative type C isolate collection, PCR primers designed to amplify internal regions of six *C. perfringens* lethal toxins or lethal toxin components (*plc*, *cpb*, *etx*, *ia*/*b*, *cpe*, and *cpb2*) were mixed with TaqComplete master mixture (GeneChoice), and PCR amplification was performed as previously described (9). The PCR products were separated on 2% agarose gels and were visualized with ethidium bromide.

PCR detection and sequencing of the *pfoA* gene. Two separate PCR assays designed to amplify sequences in the *pfoA* open reading frame (ORF) were used to determine if selected genotype C isolates carry the *pfoA* gene. All primer annealing positions indicated below are the positions relative to the first nucleotide of the initiation codon. The annealing start site for forward and reverse primers was the 5′ end of the primer. The primers used were primers pfoAF1 (5′-ATCCAACCTATGGAAAAGTTTCTGG) and pfoAR1 (5′-CCTCCTAAA ACTACTGCTGTGAAGG), which amplified the ORF region from position 443 to position 975, and primers pfoAF2 (5′-GTATTGATTCTGGAATATCAAGT TTAAG) and pfoAR2 (5′-CTTCAAACTGTGCAACATAGGCTC), which amplified the ORF region from position 110 to position 1223. The expected product sizes were 532 bp and 1,113 bp for the pfoAF1-pfoAR1 and pfoAF2-pfoAR2 reactions, respectively.

To evaluate the basis for the PFO-negative phenotype of the type C isolates that were PCR positive for *pfoA*, the *pfoA* gene was completely sequenced (including the promoter region and ORF). For this *pfoA* sequencing, the following three separate PCR were performed: RXN1 (756 bp), in which pfoAproF (5′-GATACGTGGAAATGATTTAGAGGTAGA-3′) and pfoAF2(R) (5′-CTT AAACTTGATA TTCCAGAATCAATAC-3′) were used to amplify the ORF region from position −619 to position 138; RXN2 (898 bp), in which pfoAproF2 (5′-CTGAACTGAATTTTAAGTTTAGAGAGAGT-3′) and pfoAmidR (5′-G TCTA CTCCAAGTGAGTTTTCAAGG-3′) were used to amplify the ORF region from position −268 to position 630; and RXN3 (1.29 kbp), in which pfoAF1 and pfoAendR (5′-CTGCTCTTAAAATCAATGCCTCAGC-3′) were used to amplify the ORF region from position 443 to position +232. The PCR products were then sequenced with the primers used for each PCR (the RXN3 product was also sequenced with pfoAR2). Sequencing was performed at the University of Pittsburgh core sequencing facility (http://www.genetics.pitt.edu /services.html), and the resulting sequences were analyzed and aligned using Bioedit (11).

All primers (except pfoAF1 and pfoAR1, which were obtained from Garry Myers, The Institute for Genome Research) were designed by using the strain 13 genome *pfoA* sequence (30). Primers were used at a concentration of 1 μM with TaqComplete master mixture. *C. perfringens* template DNA was prepared as described above for multiplex PCR analysis. Each PCR was performed using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min and a final extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

Optimization of vegetative growth conditions for CPB production. An isolated genotype C colony on a TSC plate was inoculated into FTG, which was then incubated overnight at 37°C. A 0.1-ml aliquot of the overnight FTG culture was transferred into 10 ml of either FTG, TGY medium, brain heart infusion broth (Difco Laboratories), or differential reinforced clostridial broth (EM Science). The cultures were incubated at 37°C until the late log phase, when samples were removed for Western blot analysis of CPB (see below). Growth of the cultures was monitored by determining the optical density at 600 nm (OD_{600}) at hourly intervals. Samples of cultures grown in TGY medium (determined to be the most consistent medium for CPB production [see Results]) were taken at 1-h intervals; after centrifugation of these samples, the supernatants were mixed 1:1 with 2× sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) loading buffer, boiled for 5 min, and then loaded on 10% SDS–PAGE gels for Western blotting of CPB (see below).

Growth conditions used for preparation of genotype C vegetative culture supernatants. Based on the results of the CPB production optimization studies described above, FTG (10 ml) was inoculated with a single colony of a type C isolate grown on a TSC plate. After overnight incubation at 37°C, a 0.1-ml aliquot was transferred into 10 ml of TGY medium, which was then incubated until the culture reached the late log phase based on measurement of the OD_{600} (the levels of lethal toxins expressed during vegetative growth were maximal during the late log phase, based on pilot experiments and previously described data [see Results]). Samples were removed from the cultures and centrifuged, and the resultant culture supernatants were filtered with 0.45-μm-pore-size filters (Millipore). The amounts of PLC, CPB, PFO, and CPB2 (for *cpb2*-positive isolates) present in the sterile vegetative supernatant filtrates were then determined using either Western blotting or activity assays as described below.

Quantification of toxin levels in genotype C culture supernatants. (i) CPB. Late-log-phase supernatants from three independent cultures of each genotype C isolate were prepared, mixed 1:1 with 2× SDS-PAGE loading buffer, and boiled for 5 min prior to loading on 10% SDS–PAGE gels for Western blot analysis of CPB. Different amounts of purified CPB were also loaded on each gel to create a standard curve for quantifying the CPB toxin present in each vegetative culture supernatant. After electrophoresis, protein was transferred onto nitrocellulose membranes (Bio-Rad Laboratories) and immunoblotted with neutralizing anti-CPB monoclonal antibody (MAb) CPCN10A2 (a gift from Paul Hauer, Center for Veterinary Biologics, Ames, Iowa), followed by a rabbit anti-mouse immunoglobulin G (IgG) peroxidase-conjugated antibody (Sigma). The blots were developed using the Supersignal West Pico (Pierce) substrate. The results of all

Western blot experiments were analyzed using a Bio-Rad ChemiDoc imaging system with the Quantity One quantitation software (Bio-Rad).

(ii) CPB2. CPB2 toxin levels in late-log-phase culture supernatants prepared from *cpb2*-positive genotype C isolates were determined as previously described (8). Briefly, cultures were either concentrated 10- to 50-fold using Amicon Ultra-15 centrifugal devices (10,000-molecular-weight cutoff; Millipore) or used without concentration, mixed 1:1 with 2× SDS-PAGE loading buffer, boiled for 5 min, and then electrophoresed on 12% SDS–PAGE gels. Purified CPB2 was electrophoresed on each SDS-PAGE gel to create a standard curve for quantifying the CPB2 in each vegetative supernatant. After transfer to nitrocellulose, the blots were probed with rabbit polyclonal anti-CPB2 antibodies, followed by goat anti-rabbit IgG peroxidase-conjugated antibody (Sigma). Immunoreactivity was detected using the Supersignal West Pico substrate.

(iii) CPE. The amounts of CPE in sporulating and vegetative culture supernatants from *cpe*-positive genotype C isolates were determined using methods described previously, with minor modifications (28). To assess CPE production by sporulating cultures, 0.2-ml aliquots of overnight FTG cultures were used to inoculate 10 ml of either Duncan Strong (DS) sporulation medium or modified Duncan Strong (MDS) sporulation medium (13). After growth for 15 h, sporulation was assessed using phase-contrast microscopy. The medium resulting in the highest percentage of sporulating bacteria (DS or MDS medium) for each isolate was used for CPE analysis. DS or MDS medium sporulating cultures were sonicated to disrupt any sporulating cells that remained intact in order to release CPE for immunodetection.

All other methods used to detect CPE in supernatants of sporulating or late-log-phase, vegetative cultures were identical to methods described previously (28). Briefly, supernatants were electrophoresed on 10% SDS–PAGE gels, and protein was subsequently transferred to nitrocellulose for Western blot analysis using a polyclonal rabbit anti-CPE antibody, followed by a goat anti-rabbit IgG peroxidase-conjugated antibody. Purified CPE was electrophoresed on each gel to create a standard curve for quantifying the CPE present in sporulating supernatants.

(iv) PLC. PLC production by genotype A or C isolates was assessed as previously described (28). Sterile (filtered) late-log-phase culture supernatants were concentrated 10-fold by lyophilization. Lyophilized material was then resuspended in phosphate-buffered saline and used in a phospholipase C assay in which nutrient agar supplemented with 4% (vol/vol) egg yolk agar was used, as described previously (32). A standard curve used to quantify PLC toxin was created using *C. perfringens* PLC (Sigma). Specific activity was expressed in PLC units/mg of total protein. The total protein in each lyophilized sample was determined using a BCA kit from Pierce.

(v) PFO. The levels of PFO activity present in genotype A or C culture supernatants were determined using a limiting dilution hemolysis assay, as previously described (36). Briefly, late-log-phase culture supernatants and Dulbecco's phosphate-buffered saline containing 5 mM dithiothreitol (Roche) were used to create a series of twofold dilutions. PFO-induced hemolytic activity was determined using horse erythrocytes (which were not lysed by other *C. perfringens* toxins under the assay conditions used [36]). PFO activity was then expressed as the reciprocal of the last dilution showing complete hemolysis (defined as the point at which a significant decrease in A_{570} was observed).

Assessing the lethality of genotype C vegetative supernatants using a mouse i.v. injection model. In initial screening experiments, sterile late-log-phase culture supernatants were prepared in duplicate as described above for toxin quantification and stored at −80°C until they were tested. To reduce animal use during lethality testing, the up and down method for calculating mouse LD_{50} was used (3). Briefly, this involved each BALB/c mouse (male or female; ca. 17 to 20 g; two mice per supernatant sample; Charles River Laboratories) receiving an i.v. (tail vein) injection of a 0.5-ml supernatant sample. Death as a routine experimental endpoint was not allowed by our Animal Care and Use Committee protocol. However, in pilot experiments we found that the monitored signs of distress (including nonresponsiveness [mice did not move when they were touched] and neurological distress [rolling over or rapid movement of the rear legs]) were typically followed soon by death when mice were inoculated with genotype C culture supernatants. For routine experiments, mice were observed for 48 h after i.v. injection to monitor development of significant distress as a marker for lethality. In pilot experiments, which were performed for up to 1 week, mice always showed signs of distress within 48 h after i.v. injection.

Vegetative culture supernatants that elicited significant distress signs (lethality) in mice following i.v. injection were titrated to calculate a mouse LD_{50}/ml, as described previously (39). Briefly, supernatants were diluted twofold (between 1:50 and 1:800) in 1% peptone water and injected i.v. into pairs of mice. Positive control mice received twofold dilutions of the same supernatant from a sterile genotype C isolate (*cpb2* negative and *cpe* negative) whose toxicity was known. As a negative control, some mice were injected i.v. with 1% peptone water, which never induced signs of distress. The supernatant lethality titer was then calculated as twice the reciprocal of the highest dilution that induced lethality within 48 h in at least one of the two paired mice; the result was then expressed as the LD_{50}/ml. For these LD_{50}/ml experiments, two independently prepared batches of each vegetative culture supernatant were injected into pairs of mice (two mice per batch and two batches per isolate resulted in four mice tested per isolate at each dilution). The results were expressed as the average of the LD_{50}/ml values determined for each independent batch.

Both batches of vegetative culture supernatant were retested if there was a >3-fold dilution difference in the calculated supernatant LD_{50}/ml values for supernatant batches from the same isolate. LD_{50}/ml values for a limited number of isolates were determined again using freshly prepared supernatants approximately 2 months after the initial experiments to confirm the reproducibility of the results. Experimental procedures involving animals were approved by the Animal Care and Use Committee of the California Animal Health and Food Safety Laboratory, University of California, Davis (permit 04-11593).

Treatment of genotype C vegetative culture supernatants with neutralizing monoclonal antibodies. To help determine which toxin(s) was responsible for the lethal activity of genotype C vegetative culture supernatants, MAb neutralization experiments were performed. To neutralize CPB activity, two 0.8-ml aliquots of a late-log-phase sterile vegetative culture supernatant were mixed with 0.2 ml of the CPB-neutralizing MAb CPCN10A2 (used at a concentration of 2 mg/ml). Pilot experiments with semipurified CPB (see below) confirmed the neutralizing properties of MAb CPCN10A2. The supernatant-MAb mixtures were incubated at room temperature for 30 min. Mice were then injected i.v. with 0.5 ml of the supernatant-MAb mixture (two mice per neutralization trial) or with a control supernatant prepared similarly except for omission of the MAb. The same process was used to neutralize PLC or CPE activity, except that either 2 mg of an anti-PLC MAb (kindly provided by P. Hauer)/ml or 2 mg of anti-CPE MAb 3C9/ml (42) was substituted for the anti-CPB MAb. The semipurified, ultrafiltered PLC used for neutralization studies was obtained from an ovine *C. perfringens* type A isolate (CSL, Ltd., Melbourne, Australia).

Partial purification of CPB. Genotype C strain CN684 was grown to the late log phase in TGY medium at 37°C, chilled to 4°C, and then centrifuged at 10,000 × *g* for 20 min. The supernatant was retained, and the protein was precipitated with 40% ammonium sulfate (Fisher Scientific). The precipitated protein was then pelleted by centrifugation at 10,000 × *g* for 30 min. The pellet was resuspended in 30 mM Tris-HCl buffer (Bio-Rad) (pH 7.5) and dialyzed overnight against the resuspension buffer. The solution was filtered with a 0.45-μm filter and loaded unto a DEAE-CL6B Sepharose column preequilibrated with 30 mM Tris-HCl buffer (pH 7.5). CPB was then eluted from the column using 0.1 M NaCl in 30 mM Tris-HCl buffer (pH 7.5). Fractions were assessed for the presence of CPB by Western blot analysis (as described above), and purity was analyzed using Coomassie brilliant blue staining. Fractions containing CPB were pooled and analyzed with the Lowry assay to determine the protein concentration. A 10-μg aliquot of the pooled CPB sample was then loaded unto a 10% SDS–PAGE gel and stained with Coomassie brilliant blue to assess the purity of the final product. Analysis of the stained gel using a Bio-Rad ChemiDoc imaging system indicated that CPB comprised ~95% of the final purified material.

Statistical analysis. The correlation between toxin production and LD_{50}/ml values was determined using linear regression analysis (performed using Microsoft Excel) and was expressed as an R^2 value. Average toxin production levels were compared using the Student *t* test to determine if differences between the averages were statistically significant ($P < 0.05$). The Instat 2.03 software from Graph Pad was used to perform statistical calculations.

Nucleotide sequence accession number. Nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers DQ673097, DQ673098, DQ673099, and DQ673100.

RESULTS

Identification of toxin genes in putative *C. perfringens* type C isolates. To determine which toxin genes were present in our collection of 55 putative type C isolates, we used a multiplex PCR assay that can detect six major *C. perfringens* toxins. This analysis confirmed that seven isolates obtained from J. Glenn Songer and four isolates obtained from our collection were genotype C isolates (i.e., they possessed the *plc* and *cpb* genes but not the *etx* or *iap* gene). Multiplex PCR analysis of 44

TABLE 1. Type C isolate genotype summary

Genotype	No. of isolates (%)
plc$^+$ *cpb*$^+$	20 (44)
plc$^+$ *cpb*$^+$ *cpb2*$^+$	19 (42)
plc$^+$ *cpb*$^+$ *cpe*$^+$	5 (11)
plc$^+$ *cpb*$^+$ *cpb2*$^+$ *cpe*$^+$	1 (2)

putative type C isolates from the BW collection, which had been typed many decades previously using the classical toxin neutralization method, confirmed that 34 of these isolates were genotype C isolates (data not shown). The 10 remaining BW collection isolates that had been initially classified as type C isolates by the toxin neutralization method were identified as genotype A isolates by multiplex PCR because they carried the *plc* gene but not the *cpb*, *etx*, or *iab* gene; these isolates were not studied further.

Nearly one-half (44%) of the 45 confirmed genotype C isolates were shown by multiplex PCR to have a simple *cpb plc* toxin genotype, while 42% had a *cpb cpb2 plc* toxin genotype (Table 1). Interestingly, all seven of the North American veterinary genotype C disease isolates surveyed were positive for *cpb2*. The remaining genotype C isolates surveyed were either *cpb*, *plc*, and *cpe* positive (11%) or *cpb*, *plc*, *cpe*, and *cpb2* positive (2%). It is also noteworthy that both the *cpe* and *cbp2* genes were detected in several BW collection strains isolated in the 1930s and 1940s, well before the CPE and CPB2 toxins were identified.

Presence of lethal toxins in genotype C vegetative culture supernatants. Following multiplex PCR analysis, the verified genotype C isolates were assayed to determine their toxin production phenotypes. The production of PLC and the production of PFO were assessed using either an egg yolk agar hydrolysis assay (which measured the phospholipase C activity of PLC) or a horse erythrocyte hemolytic assay (which measured PFO activity). Currently, there are no functional activity assays for specific measurement of CPB, CPB2, or CPE levels. In lieu of such assays, quantitative Western blotting was used to measure CPB, CPB2, and CPE levels in culture supernatants.

The optimal culture conditions for CPB production have not been well established. Therefore, a few randomly chosen genotype C isolates were initially grown in four vegetative growth media (FTG, TGY medium, brain heart infusion broth, and differential reinforced clostridial broth) to identify optimal vegetative growth conditions for CPB production. Western blot analysis of CPB (using an anti-CPB MAb) showed that TGY medium was the most consistent medium for strong CPB production (data not shown). It was also determined (Fig. 1) that CPB production peaks during the late log phase for most genotype C isolates and then decreases during the late stationary phase. One genotype C isolate, CN3715, did not produce detectable levels of CPB (unless it was concentrated 50-fold) during any growth stage in TGY medium.

Since late-log-phase TGY medium cultures produced maximal levels of CPB and also contained maximal or significant levels of CPB2, PFO, and PLC (as determined in previous studies [28]), these growth conditions were used to determine toxin levels in our study. The vegetative culture supernatants of selected genotype C isolates were eventually tested to determine their levels of lethality using a mouse lethality model, so toxin levels were compared only using these growth conditions in order to minimize the number of animals that were required later for lethality studies.

Using these optimized conditions, CPB production by our collection of genotype C isolates was quantified by densitometric analysis of Western blot CPB bands compared with a CPB standard curve (Fig. 2A). These analyses indicated that for genotype C isolates there were wide variations in the levels of CPB produced, which ranged from <1 to ~50 μg/ml (Fig. 2B). Interestingly, *cpb2*-positive genotype C isolates produced low levels of CPB (average, 3.9 μg/ml) compared to the levels produced by *cpb2*-negative genotype C isolates (average, 13.3 μg/ml), and only one *cpb2*-positive genotype C isolate was among the 33% of the genotype C isolates surveyed that produced >10 μg/ml of CPB (Fig. 2B). The difference between the averages was statistically significant ($P < 0.01$). There was no association between CPB toxin production levels and the date of isolation or origin of the genotype C isolates.

CPB2 toxin production in vegetative culture supernatants prepared from *cpb2*-positive genotype C isolates was also measured using Western blotting and densitometry (Fig. 3A). The

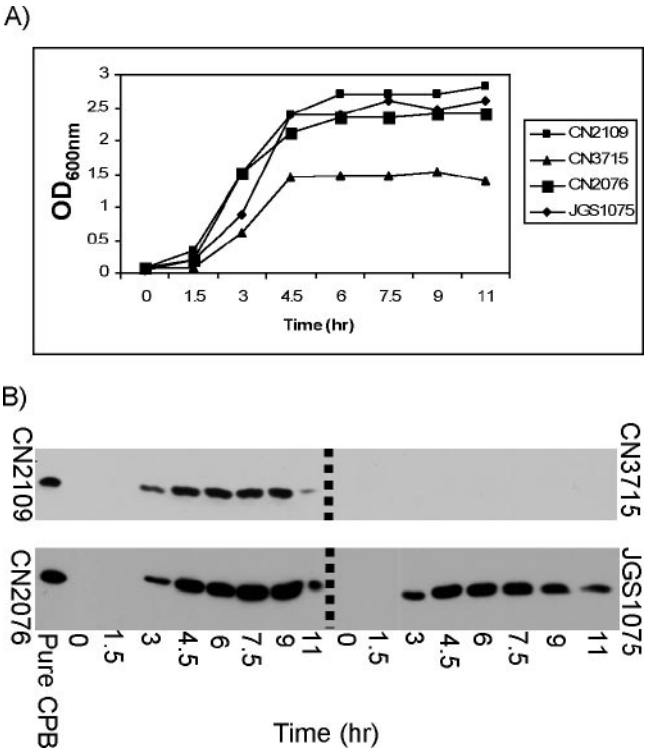

FIG. 1. Optimization of growth conditions for production of CPB by genotype C vegetative cultures. Four representative genotype C isolates were grown in TGY medium at 37°C. Every ~1.5 h the OD_{600} was determined in order to assess growth (A), and samples were removed to determine the amount of CPB present (B). (B) For CPB detection, vegetative culture supernatant samples were processed for Western blotting using anti-CPB MAb. Purified CPB was loaded in the left lane of each blot as a positive control, and sample times are indicated below the blots.

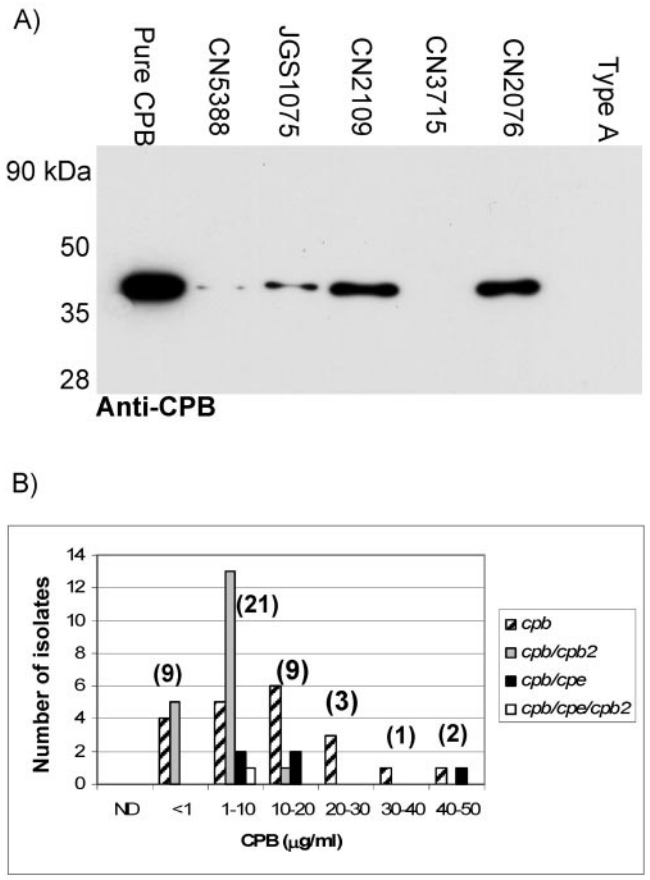

FIG. 2. Western blot quantification of CPB in genotype C vegetative culture supernatants. Genotype C isolates were grown to the late log phase in TGY medium at 37°C and then processed for Western blot analysis of CPB. (A) Results for representative genotype C isolates (indicated at the top). The genotypes are as follows: CN5388, *plc cpb cpe cpb2*; CN2076, *plc cpb cpe*; JGS1075 and CN3715, *plc cpb cpb2*; and CN2109, *plc cpb*. The positions of molecular weight markers are indicated on the left (in kDa), and a *cpb*-negative type A isolate, as a negative control, was electrophoresed in the right lane. Pure CPB was electrophoresed in the left lane as a positive control. CPB levels in vegetative culture supernatants were determined by densitometric comparisons with a CPB standard curve generated using purified CPB. (B) CPB levels for isolates, expressed in μg/ml. Results are shown for each toxin subgenotype (all isolates are also *plc*$^+$), and the total numbers of isolates for the different ranges are indicated in parentheses above the bars. ND, toxin could not be detected in culture supernatants.

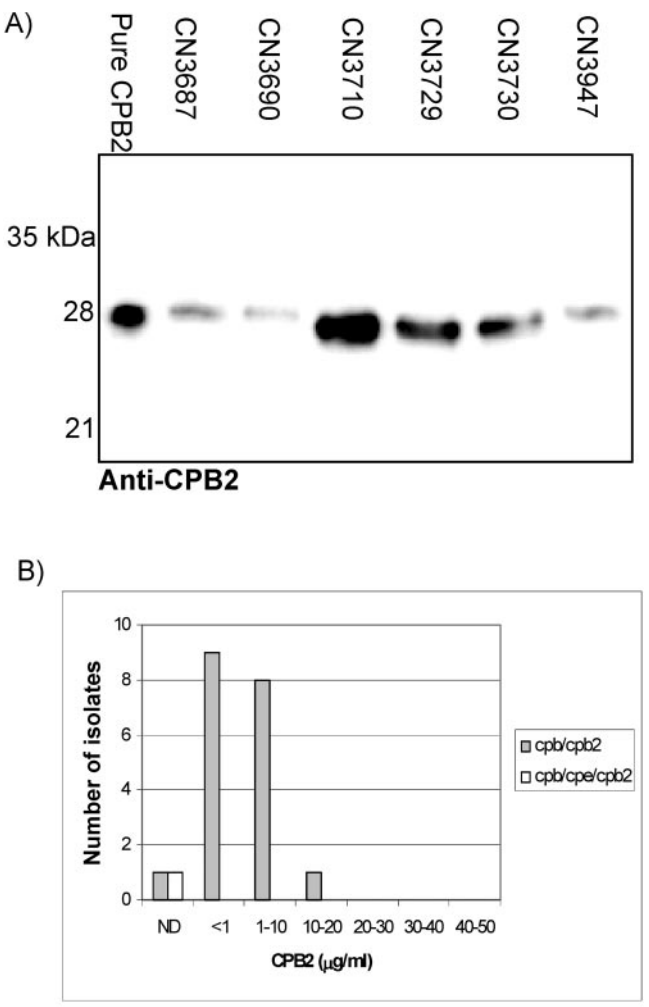

FIG. 3. Western blot analysis of CPB2 levels in *cpb2*-positive genotype C vegetative culture supernatants. Genotype C isolates carrying the *cpb2* gene were grown in TGY medium at 37°C to the late log phase. Culture supernatant samples from these cultures were then processed with or without concentration (10- to 50-fold) and used for Western blot analysis of CPB2. (A) Representative Western blot for CPB2. The positions of molecular weight markers are indicated on the left, and pure CPB2 was electrophoresed in the left lane as a positive control. All isolates shown have toxin genotype *plc cpb cpb2*. The amount of CPB2 in each sample was determined by comparing the CPB2 signal density to a CPB2 standard curve generated using purified CPB2. (B) Amount of CPB2 in each sample. ND, toxin could not be detected in culture supernatants.

CPB2 toxin levels were found to vary from nondetectable to 13 μg/ml (Fig. 3B). No association between CPB2 production levels and isolate disease origin was apparent.

Genotype C isolates carrying the *cpe* gene were assessed to determine their abilities to produce CPE under both sporulating and vegetative culture conditions. Consistent with previous studies which showed that *cpe*-positive type A and D isolates produce CPE only during sporulation (7, 28), Western blot analysis of CPE (Fig. 4A) demonstrated that all of the *cpe*-positive genotype C isolates surveyed produce CPE during sporulation but not during vegetative growth (Fig. 4B). The higher-molecular-weight species apparent in the purified CPE lane and supernatant samples tested (Fig. 4B) are CPE aggregates. The CPE aggregation phenomenon, particularly when higher concentrations of CPE are subjected to SDS-PAGE, has been well documented (20).

An analysis of the PFO expression of our genotype C isolate collection, performed using a PFO-specific horse red blood cell hemolysis activity assay, showed that most (83%) genotype C isolates produced PFO (Fig. 5A), although the levels ranged from nondetectable to a $\log_2$ titer of 4 to 5. It is noteworthy that five of the six *cpe*-positive genotype C isolates surveyed (the *plc*-, *cpe*-, and *cpb2*-positive genotype C isolate was the lone exception) did not produce PFO, while PFO was produced by nearly all genotype C isolates carrying only the *plc*

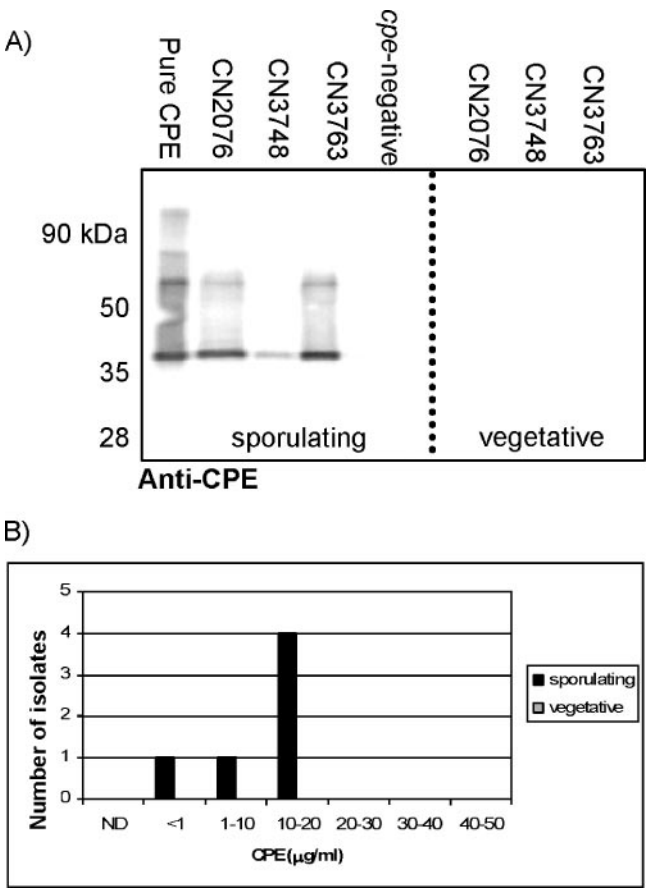

FIG. 4. Detection and quantification of CPE in *cpe*-positive genotype C culture supernatants using Western blotting. Isolates were grown in either MDS or DS medium (for sporulating conditions) or TGY medium (for vegetative conditions) for 15 h at 37°C. Culture supernatants were sonicated, and lysates were subjected to Western blot analysis of CPE. (A) Purified CPE was electrophoresed in the left lane as a positive control, and a *cpe*-negative type A isolate was electrophoresed as a negative control. The positions of molecular weight markers are indicated on the left. Whether samples were obtained from sporulating or vegetative culture supernatants is indicated at the bottom. The amount of CPE in each isolate supernatant was determined by comparing the CPE signal intensity to a standard curve created using pure CPE. (B) CPE levels. ND, toxin could not be detected in culture supernatants.

and *cpb* genes or only the *plc*, *cpb*, and *cpb2* genes. For comparison, PFO production during vegetative growth was also assessed (Fig. 5B) for 11 representative genotype A isolates (genotype A isolates are the predominant *C. perfringens* isolates [34]). These comparisons indicated that the PFO titers of vegetative culture supernatants derived from genotype A isolates and genotype C isolates are similar under the experimental conditions used in our study; i.e., the difference between the averages was not statistically significant.

To determine whether PFO-negative genotype C isolates lack the *pfoA* gene as a possible explanation for why these isolates did not produce detectable levels of PFO, two *pfoA* PCR assays in which different regions in the *pfoA* ORF were amplified were performed. These PCR assays did not result in amplification of either of the expected internal *pfoA* PCR products from the five PFO-negative, *cpe*-positive genotype C isolates (data not shown). In contrast, *pfoA* PCR products of the expected size were amplified from the three PFO-negative, *cpe*-negative genotype C isolates and from two PFO-positive genotype C positive control isolates (data not shown).

Sequencing of the *pfoA* gene was performed for the three type C isolates that were PCR positive for *pfoA* but did not produce detectable PFO activity. This sequencing, which included the promoter region containing the VirR binding box (5) and the ORF, revealed that there were no nucleotide differences in the promoter region of these isolates compared to the *pfoA* sequence of type A strain 13. Compared to the strain 13 PFO amino acid sequence, an A71T amino acid substitution was present in all genotype C isolates sequenced (including three PFO-negative isolates and one PFO-positive genotype C isolate), while an A215V mutation was also present in one of the PFO-negative isolates (data not shown).

Alpha toxin production by genotype C isolates was determined using an egg yolk agar hydrolysis assay that measures phospholipase C activity. The PLC activity (expressed as U/mg total protein $\times 10^{-3}$) in late-log-phase supernatants of genotype C isolates was found to range from nondetectable levels (for 15% of the genotype C isolates surveyed) to ~10 U/mg total protein $\times 10^{-3}$ (Fig. 6A). Most of the *cpb2*-positive genotype C isolates produced lower levels of PLC (PLC activity, <2 U/mg total protein $\times 10^{-3}$) than *cpb2*-negative genotype C isolates produced (average PLC levels, 0.5 and 3.2 U/mg total protein $\times 10^{-3}$), and *cpb2*-positive genotype C isolates accounted for most (18/31) of the isolates that produced low levels of PLC. The difference between the averages was statistically significant ($P < 0.01$). For comparison, the PLC activities in late-log-phase supernatants from 11 representative genotype A isolates (from environmental [five isolates] and disease [6 isolates] sources) ranged from 0.6 to >18 U/mg total protein $\times 10^{-3}$ (Fig. 6B), and only 1 of the 11 type A isolates produced >4 U/mg total protein $\times 10^{-3}$. The difference between the average PLC values for type C and type A isolates was not significant.

Assessing the contributions of individual toxins to the lethality of genotype C vegetative culture supernatants using a mouse i.v. injection model. The results shown in Fig. 2 to 6 demonstrated that late-log-phase culture supernatants from genotype C isolates typically contained multiple toxins with lethal activity. To assess the contributions of individual toxins to lethality, late-log-phase culture supernatants from select genotype C isolates were prepared for lethality testing with the mouse i.v. injection model. In the absence of a well-characterized small-animal oral challenge model for type C infection, the mouse i.v. injection model is commonly used to assess the lethality of genotype C vegetative culture supernatants (12, 35). For our mouse lethality experiments, sterile-filtered dilutions of vegetative culture supernatants were injected i.v. into mice to determine an LD_{50}/ml for each isolate. The results were then compared with the amount of each lethal toxin (CPB, CPB2, PFO, or PLC) previously shown to be present in the samples in order to evaluate whether there was a correlation between the level of each toxin and lethality (Fig. 7).

For the 22 genotype C isolates tested, there was a strong positive correlation between mouse lethality and the level of CPB toxin (Fig. 7A) in the late-log-phase supernatants. By contrast, lethality showed only a weak positive correlation with

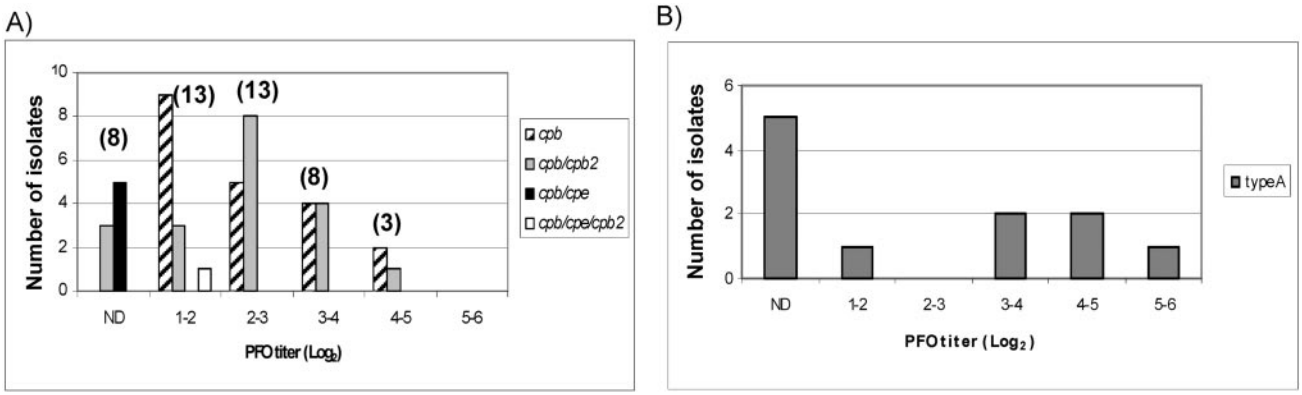

FIG. 5. PFO titers of genotype C isolates and selected genotype A isolates. Serial twofold dilutions of late-log-phase vegetative culture supernatants were incubated with horse erythrocytes to measure hemolysis. The last dilution that produced complete hemolysis (defined as a significant change in A_{570}) was defined as the $\log_2$ titer. (A) PFO titers for genotype C isolates for each toxin subgenotype (all isolates are also *plc*$^+$). The total numbers of isolates in the titer groups are indicated in parentheses. (B) PFO titers for representative type A isolates. ND, toxin could not be detected in culture supernatants.

PLC and CPB2 levels and no correlation with PFO (Fig. 7B to D). Additionally, the lethal properties of the supernatants were rapidly destroyed (data not shown) by treating the vegetative culture supernatants with trypsin (0.5% for 30 min at 37°C). Since CPB is very trypsin sensitive (14), this observation is consistent with the hypothesis that CPB is an important factor in the lethality observed when mice receive injections of genotype C vegetative culture supernatants.

Neutralization of genotype C vegetative culture supernatant lethality with MAb. To definitively discern the role of CPB in mouse lethality, monoclonal antibody neutralization experiments were performed. Fourteen genotype C vegetative culture supernatants were pretreated with neutralizing monoclonal antibodies specific for either CPB or PLC. When these pretreated supernatants were tested for lethality in the mouse i.v. injection model, only the late-log-phase supernatants pretreated with a CPB-neutralizing MAb had lost the ability to induce mouse lethality (Table 2). ATCC 13124, a strain that produces a high level of PLC, was used in this study to demonstrate that we could neutralize lethal amounts of toxins other than CPB in our mouse model, if they were present. It is worth noting that while mice treated with anti-PLC MAb still died, the time until death was longer when mice were injected with supernatants treated with PLC-neutralized supernatants than when they were injected with nonneutralized supernatants; six of seven mice showed a >1.5-fold increase (range, 1.1- to 11.8-fold) in the time until death.

The specificity of anti-CPB monoclonal antibody neutralization was confirmed by demonstrating that preincubation of semipurified CPB with this MAb protected mice from lethal i.v. challenge (Table 2). However, lethality in mice challenged i.v. with the semipurified CPB was not prevented if the toxin was preincubated with either anti-PLC MAb or anti-CPE MAb (Table 2). To further confirm the specificity of the anti-CPB MAb in these neutralization experiments, we showed that semipurified PLC remained lethal when it was preincubated with the anti-CPB MAb prior to i.v. injection into mice, but that anti-PLC MAb was able to completely neutralize the lethality of this PLC preparation (Table 2).

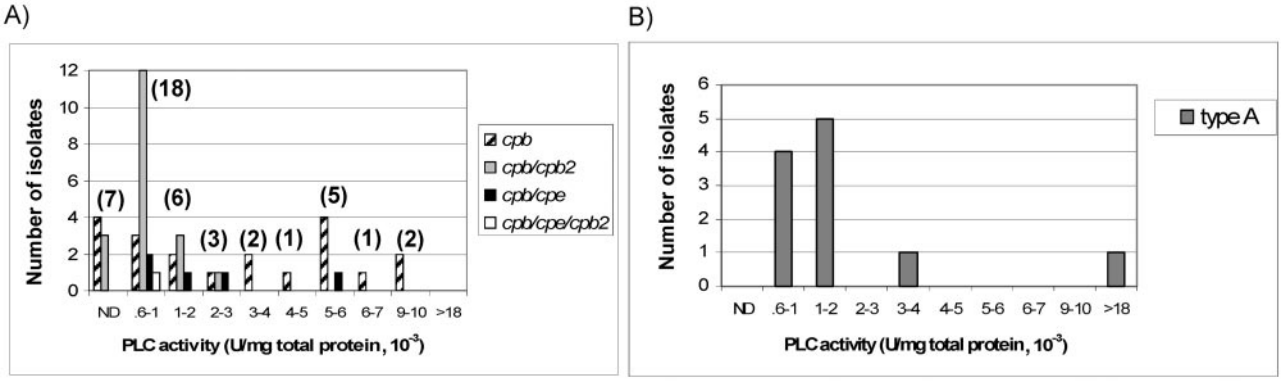

FIG. 6. PLC levels in genotype C and selected genotype A vegetative culture supernatants. Late-log-phase vegetative culture supernatants were assessed to determine their PLC activities using an egg yolk hydrolysis assay. A standard curve was generated using semipurified PLC. PLC activity is shown for genotype C isolates (A) and selected type A isolates (B). PLC results for genotype C isolates are shown for the different toxin subgenotypes (all isolates carry the *plc* gene). The total numbers of isolates in the PLC activity ranges are indicated in parentheses. ND, toxin could not be detected in culture supernatants.

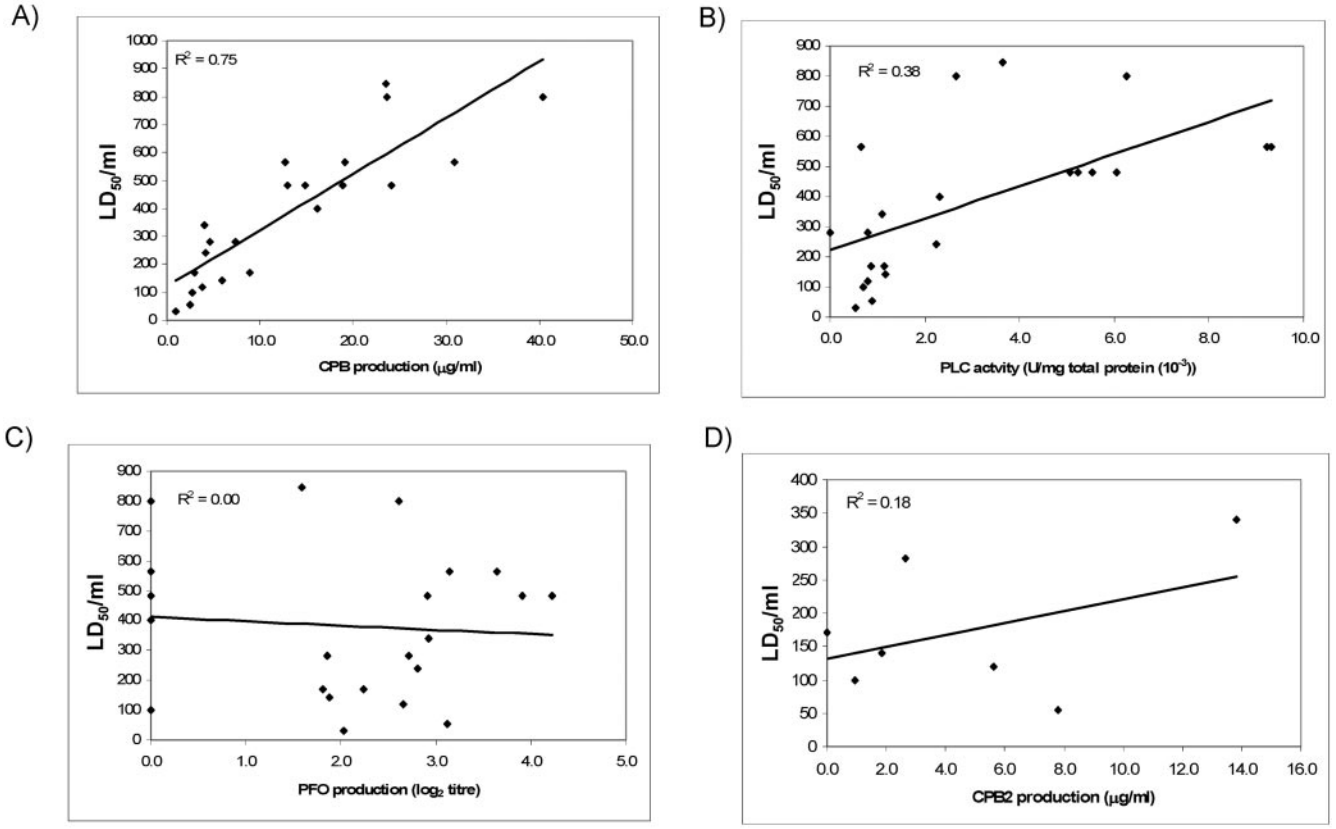

FIG. 7. Correlation of specific toxin levels of genotype C isolates and mouse lethality. Vegetative culture supernatants from select genotype C isolates were assessed to determine their levels of lethality using a mouse i.v. injection model. Pairs of mice were injected i.v. in the tail vein with dilutions of vegetative culture supernatants. The reciprocal of the last dilution that caused lethality in at least one of the two mice was defined as the LD_{50}/ml (see Materials and Methods). The LD_{50}/ml values were then correlated with the amounts of CPB, PLC, PFO, or CPB2 present in the vegetative culture supernatants. A linear equation was used to draw a best-fit line based on the data points, and the R^2 for this line is indicated on each graph.

DISCUSSION

C. perfringens type C isolates remain an important cause of disease in domestic animals, as indicated by the widespread type C vaccination of domestic animals (24). Type C isolates also are the only non-type A isolates documented to cause human disease (15, 17). Type C-associated human disease is most closely associated with Papua New Guinea, where it was once the second leading cause of death in children who were more than 1 year old, which led to a childhood vaccine program that helped to reduce this problem (22). In addition, type C human necrotic enteritis is now seen in developed countries, typically in patients with pancreatic disease (10, 18, 23, 29, 37).

Despite the obvious importance of type C isolates for both human and animal disease, the virulence of these isolates is poorly understood. While these bacteria must produce at least CPB and PLC in order to be classified as type C isolates, our results indicate that most type C isolates also produce a third lethal toxin (PFO) and that ~40% of these isolates produce a fourth lethal toxin (CPB2) during late-log-phase growth. This complex lethal toxin repertoire raises the possibility that type C-induced disease results from the combined activities of several toxins. Consistent with this possibility, animal model studies have shown that CPB alone cannot elicit the symptoms of a type C infection (34). Furthermore, the current type C veterinary vaccines are based on crude toxoids or bacterin/toxoid (killed bacteria plus inactivated supernatants) (35, 38, 40). In addition, the human vaccine previously used in New Guinea was not prepared with a pure CPB toxoid but instead was prepared with ammonium sulfate-precipitated supernatants from type C vegetative cultures that were formalin inactivated (40; Gregor Lawrence, personal communication). Therefore, the protective immune response elicited by these type C toxoid vaccines against type C infections could involve protective antibodies not only against CPB (which is the only component for which type C vaccines are validated [19]) but also against any other lethal toxins produced by the type C isolates used for producing the toxoid vaccine. To our knowledge, type C strains used for vaccine preparation do not have a well-characterized toxin repertoire.

As a first step in determining which toxins play a role in type C-induced disease, we determined the roles of individual type C toxins in the mouse i.v. supernatant injection lethality model. In this research we initially determined the toxin genotypes and phenotypes of a large number of putative type C isolates, which confirmed that all isolates previously genotyped by multiplex PCR were genotype C isolates. However, a number of

TABLE 2. Neutralization of type C toxins with MAb

Isolate	Type	Phenotype[a]	Lethality neutralized by:		
			Anti-CPB MAb	Anti-PLC MAb	Anti-CPE MAb
CN685	C	$CPB_{8.9}$ $PLC_{1.0}$ $PFO_{2.2}$	Yes		
CN5383	C	$CPB_{23.5}$ $PLC_{3.6}$ $PFO_{1.6}$	Yes		
CN885	C	$CPB_{23.6}$ $PLC_{6.3}$ $PFO_{2.6}$	Yes	No	No
CN886	C	$CPB_{24.1}$ $PLC_{5.1}$ $PFO_{4.2}$	Yes	No	
CN1797	C	$CPB_{18.9}$ $PLC_{5.5}$ $PFO_{3.9}$	Yes	No	
CN2109	C	$CPB_{12.8}$ $PLC_{9.2}$ $PFO_{3.1}$	Yes	No	
CN3685	C	$CPB_{13.0}$ $PLC_{6.0}$ $PFO_{2.9}$	Yes	No	
CN3717	C	$CPB_{<1}$ PLC_{ND} PFO_{2}	Yes		
CN3955	C	$CPB_{30.9}$ $PLC_{9.3}$ $PFO_{3.6}$	Yes	No	No
NCTC 10719	C	$CPB_{2.7}$ $CPB2_{0.9}$ $PLC_{<0.7}$ PFO_{ND}	Yes		
JGS1071	C	$CPB_{2.5}$ $CPB2_{7.8}$ $PLC_{0.9}$ $PFO_{3.1}$	Yes	No	
CN3686	C	$CPB_{6.0}$ $CPB2_{1.9}$ $PLC_{1.2}$ $PFO_{1.9}$	Yes		
CN5388	C	$CPB_{3.0}$ $CPE_{<0.05}$ $CPB2_{ND}$ $PLC_{0.9}$ $PFO_{1.8}$	Yes		
CN3758	C	$CPB_{14.8}$ $CPE_{11.2}$ $PLC_{5.2}$ PFO_{ND}	Yes	No	No
ATCC 13124	A	$PLC_{18.8}$ $PFO_{5.1}$	No	Yes	No
	A	Semipurified PLC (from CSL)	No	Yes	
	C	Semipurified CPB	Yes	No	

[a] The subscript numbers indicate the supernatant concentrations (μg/ml for CPB, CPB2, and CPE; U/mg total protein $\times 10^{-3}$ for PLC; and $\log_2$ titer for PFO) of the toxins. ND, not detectable.

isolates assigned to type C many years ago using the classical guinea pig skin test were instead classified as type A isolates by multiplex PCR. The explanations for the difference in classification include the loss of the *cpb*-containing plasmid after toxin neutralization typing and, perhaps, the relatively crude nature of historical guinea pig skin neutralization testing.

Interestingly, our genotypic analyses showed that ~40% of the genotype C isolates surveyed carry the *cpb2* gene, which is a considerably higher percentage than the 18% *cpb2*-positive isolates identified in a recent survey of type D isolates (28). The percentage of *cpb2*-positive genotype C isolates in our collection is lower than the percentage determined by Bueschel et al., who reported that 64% of their genotype C isolates ($n = 178$) were *cpb2* positive (4). The *cpb2* carriage rate for type C isolates is also less than the 80% *cpb2* carriage rate determined for type A isolates carrying a plasmid-borne *cpe* gene (8). The very strong association between *cpb2* and type A isolates carrying a *cpe* plasmid is partially attributable to the fact that many, although not all, of the type A isolates carry *cpb2* and *cpe* on the same plasmid. In this regard, it is interesting that we identified only a single *cpe*-positive, *cpb2*-positive type C isolate in the current survey. Studies are now under way to determine whether *cpb* and *cpb2* reside on the same plasmid in our genotype C isolates.

Phenotypic analyses showed that under our assay conditions, genotype C isolates carrying the *cpb2* gene typically produce lower levels of both CPB and PLC than the *cpb2*-negative genotype C isolates produce. Since the regulation of PLC or CPB in type C isolates has not been studied yet, we have no explanation for these observations. However, the explanation might involve differences in the VirR/VirS two-component system, which positively regulates both *cpb2* and *plc* expression in type A isolates via its ability to regulate expression of VR-RNA regulatory RNA (2, 21, 31). Another possibility is that genes present on the putative *cpb2* plasmid might specifically downregulate *cpb* and/or *plc* expression.

Phenotypic analyses also revealed that five of the six *cpe*-positive genotype C isolates surveyed produced no detectable PFO activity. Two PCR analyses indicated that the *pfoA* gene was not present in these isolates. In contrast, similar PCR analyses of the *pfoA* gene in *cpe*-positive *C. perfringens* type A isolates demonstrated that the *pfoA* gene is present in most *cpe* plasmid isolates but is not present in most chromosomal *cpe*-positive type A isolates (unpublished observations). Studies should be performed to address the *cpe* gene location (chromosomal versus plasmid) in *cpe*-positive genotype C isolates, which would reveal whether the genotypes of these isolates resemble the *pfoA*-negative genotype of most chromosomal *cpe* type A isolates.

Sequencing of the *pfoA* gene from the three *cpe*-negative genotype C isolates that failed to produce PFO yet were PCR positive for *pfoA* revealed no nucleotide substitutions in the promoter region that could explain the lack of PFO activity for these isolates (5). Nor could the lack of PFO activity for these isolates be attributed to consistent nucleotide changes in the *pfoA* ORF. However, compared to the *pfoA* gene of type A strain 13, there were consistent nucleotide changes in the *pfoA* ORF of the type C isolates sequenced that resulted in two different amino acid substitutions. The A71T substitution was identified in all four type C isolates sequenced, including a PFO-positive isolate, while an A215V substitution was present in a single PFO-negative isolate. Based on these sequencing and PCR results, it appears that the lack of detectable PFO activity for some type C isolates results either from an inability to make PFO (for most or all *cpe*-negative, type C isolates that were PCR positive for *pfoA*), implying that there is a regulatory

gene mutation, or from the actual absence of the *pfoA* gene (for the *cpe*-positive type C isolates negative in both *pfoA* PCR assays). However, our results do not rule out the possibility that some *pfoA*-positive, PFO-negative isolates could produce PFO under culture conditions different than those tested or that the A215V substitution encoded by the *pfoA* gene of one *pfoA*-positive PFO-negative isolate eliminates its PFO activity.

Finally, the results of our toxin phenotype analyses of genotype C isolates allowed us to compare the toxin production abilities of these isolates with those of genotype D and A isolates. No statistically significant differences were found between the average amounts of PLC or PFO produced when we compared genotype C isolates to genotype A or D isolates (28; this study). However, it should be noted that not all of the genotype A isolates used to measure PFO and PLC levels in this study were isolated from gas gangrene cases, which are believed to be associated with genotype A isolates that produce high levels of PFO and PLC. The average levels of CPE (which is produced only during sporulation) in culture supernatants from sporulating genotype C isolates, genotype A isolates (6), and genotype D isolates (28; this study) were also not significantly different. It is notable that while some genotype D isolates did not produce detectable levels of their typing toxin (ETX) (28) even when their supernatants were concentrated, all genotype C isolates tested produced detectable amounts of CPB toxin, although detection sometimes required concentration of the culture supernatants. It is important to note that these findings are specific for the media and test conditions used in this study; i.e., isolates could make more or less PLC (or other toxins) in brain heart infusion media in the late log phase.

The timing of CPB toxin production has not been well documented. Therefore, in this study we tried to determine when this toxin is produced during in vitro growth, because quantifying CPB toxin levels in vegetative culture supernatants is important both for our mouse i.v. lethality studies and for preparing type C vaccines. The results of these analyses indicated that CPB toxin production reaches peak levels during late-log-phase growth in TGY medium. The timing of CPB production agrees with the results of a previous study in which the workers measured levels of CPB activity in culture supernatants from a single type C isolate using a guinea pig skin test animal model (26). Interestingly, Sakurai and Duncan also found that fermentable sugars can increase CPB production under non-pH-controlled conditions (26). In the current study, the levels of CPB production were consistently higher in TGY medium (which contained 2% glucose/liter) than in FTG (0.55% glucose/liter), brain heart infusion broth (0.2% glucose/liter), or differential reinforced clostridial broth (0.1% glucose/liter). These analyses also revealed that CPB production varies substantially (>40-fold) among type C isolates, suggesting that there are isolate-dependent variations in *cpb* gene regulation. Furthermore, CPB toxin levels were typically found to decrease during the stationary phase, possibly due to decreased CPB production or protease activity present in stationary-phase cultures. Together, the variability in CPB toxin production observed among type C isolates and the reductions in CPB toxin levels detected during longer growth periods highlight the importance of selecting the proper strain and growth conditions for preparing type C toxoid vaccines. To our knowledge, the identities of genotype C strains and the growth conditions used to prepare type C vaccines have not been clearly reported in refereed publications.

To gain further understanding regarding which toxin(s) present in type C supernatants is responsible for virulence (and therefore must be neutralized for a vaccine to elicit protective immunity), sterile late-log-phase supernatants from approximately one-half of our type C isolates were injected i.v. into mice. When the LD_{50}/ml values obtained for these vegetative culture supernatants were correlated with the levels of lethal toxins produced by the isolates, no positive correlation was observed between LD_{50}/ml and PFO levels. In contrast, the levels of three other lethal toxins (CPB, CPB2, and PLC) showed at least some positive correlation with supernatant LD_{50}/ml values, and the CPB levels exhibited a much higher LD_{50}/ml correlation (R^2 >0.7) than either the PLC or CPB2 levels (R^2, 0.38 and 0.18, respectively). These correlation results suggest that CPB plays a major role in the lethality of type C supernatants in the mouse i.v. injection model.

The importance of CPB for type C supernatant lethality in the mouse i.v injection model was then conclusively demonstrated by toxin neutralization experiments using neutralizing monoclonal antibodies specific for CPB, PLC, or CPE. In these neutralization studies, the lethal properties of late-log-phase type C supernatants were consistently neutralized by anti-CPB monoclonal antibodies, but neutralization was not observed using either anti-PLC or anti-CPE monoclonal antibodies. Interestingly, the time until death for mice treated with supernatants incubated with anti-PLC antibodies was longer than the time until death for mice treated with untreated supernatants. This observation along with the CPB neutralization data may indicate that when our experimental conditions are used, PLC is not the primary mediator of lethality (since the mice still died), but it does make a minor contribution to i.v. lethality, possibly explaining the small positive correlation between supernatant LD_{50}/mls and PLC concentration (R^2 = 0.38) (Fig. 7D).

Previous oral challenge and intestinal loop studies suggested that CPB cannot cause the full pathology of a natural type C infection (34). This finding along with the current i.v. challenge results suggests that CPB is required for the systemic lethality of type C supernatants, but a secondary factor(s) may facilitate the intestinal absorption of CPB into the circulation during natural disease. This possibility cannot be conclusively addressed by the mouse i.v. challenge model or monoclonal antibody neutralization approaches, which do not address factors such as the breaching of the intestinal permeability barrier or possible synergistic toxin interactions. There is a precedent for such synergistic interactions since previous studies have shown that PLC and PFO act synergistically in the pathogenesis of histotoxic type A infections in mice (1). While the current results provide important insights into type C pathogenesis, further research is clearly needed to determine which toxin(s) (or the bacteria themselves) contributes to the early stages of type C-induced enteric disease. To address this issue, efforts are being made to develop a small-animal oral challenge model that better mimics the natural type C disease. When available, this oral challenge model should be useful for virulence testing of type C toxin knockout mutants in order to dissect the specific roles of various toxins at each stage of type C disease.

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