A STUDY OF THE BETA-2 TOXIN GENE AND THE BETA-2 TOXIN IN *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM HUMAN SOURCES

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To the memory of Dr. Stanley L. Erlandsen.

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ABSTRACT

Heidi M. Roskens Dalzell

A STUDY OF THE BETA-2 TOXIN GENE AND THE BETA-2 TOXIN IN *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM HUMAN SOURCES

Clostridium perfringens is an important human pathogen known to cause a range of diseases including diarrhea, necrotizing bowel disease and gas gangrene. Though potentially pathogenic, this microorganism is commonly identified in the fecal microbiota of healthy individuals. The major clinical findings associated with *C. perfringens* diseases are linked to production of potent toxins. In 1997, Gibert et al. identified a new toxin, the beta2 toxin, from a *C. perfringens* strain from a piglet with necrotic enteritis. Subsequently, this new beta2 toxin gene (*cpb2*) has been identified in *C. perfringens* from dogs, horses, and other animals. The principal objective of this investigation was to study *cpb2* and the beta2 toxin in *C. perfringens* isolates from human sources. The *C. perfringens* isolates were grouped into three different populations: 1) fecal samples from patients suspected of having *C. perfringens* gastrointestinal illnesses (e.g. antibiotic-associated diarrhea or colitis), 2) extraintestinal specimen sources (e.g. wounds, abscesses, blood cultures), 3) a control group of isolates from healthy volunteers. Results of studies using different PCR methods and nucleotide sequencing revealed that *cpb2* was present in the genome of isolates from all populations, and that the genetic variation between *cpb2* from the different *C. perfringens* isolates was greater then expected. Using western immunoblotting

techniques, it was found that the beta2 protein was not expressed by all *cpb2* positive *C. perfringens* isolates. Finally, different variants of *cpb2* were cloned into *E. coli*, and the recombinant beta2 protein used in cell cytotoxicity assays. Results from these assays demonstrated that recombinant beta2 proteins caused a range of cellular damage at different levels of protein concentration and different lengths of time. Our results from these experiments provided new information regarding *cpb2* in *C. perfringens* isolates from human sources; as well as on the range of variation of *cpb2* genes, differences in beta2 toxin expression, and differences in the effects of recombinant beta2 toxin on enterocytes. This information could help to explain differences in virulence between *C. perfringens* isolates, differences in diseases and disease severity.

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INTRODUCTION

Clostridium perfringens: Classification, Morphology, and Biochemical Features

Clostridium perfringens (referred to as *C. welchii* especially in Great Britain) is an anaerobic, gram-positive rod capable of forming environmentally resistant spores. It is a ubiquitous microorganism found in a variety of habitats, including soil, water, lower mammals, and humans. The first recognizable description of this bacterium was by Welch and Nuttall in their 1892 paper describing an organism they called *Bacillus aerogenes capsulatum* (Lucey and Hutchins, 2004; Welch and Nuttall, 1892; and reviewed by Hatheway, 1990). Veillon and Zuber also provided a recognizable description of *C. perfringens* in brief form in 1897, and again in 1898 in a more descriptive paper which described numerous anaerobes isolated from patients with pulmonary gangrene or appendicitis (referred to by Finegold, 1994; Veillon and Zuber, 1897; Veillon and Zuber, 1898). In the "classical" classification scheme, *C. perfringens* belonged to the kingdom *Monera*, phylum Bacteria, class *Schizomycetes*, order *Eubacteriales*, family *Clostridiaceae*, genus *Clostridium*, and species *perfringens* (Euzeby, 1997; Sneath et al., 1986). According to *Bergey's Manual of Systematic Bacteriology*, classification of bacteria in the higher taxonomic groupings is described as irregular and unreliable (Sneath et al., 1986). By the 1990s the genus *Clostridium* had become one of the largest and most diverse of

all bacterial genera and was in need of taxonomic revision (Collins et al., 1994). Therefore, recent phylogenetic studies have focused on using ribosomal RNA analysis for the formation of new molecular data-based taxonomy for classification of microorganisms (Schmidt and Relman, 1994). Interest in the use of ribosomal RNA analysis in the phylogeny and taxonomy of microorganisms in recent years has lead to the development of the Ribosomal Database Project (RDP-II), an online resource for ribosomal RNA sequence analysis (Cole et al., 2005). Studies by Collins and associates using 16S ribosomal RNA (rRNA) gene sequencing have revealed extreme heterogeneity in the genus *Clostridium*, and suggest that extensive reclassification of the clostridia is necessary (Collins et al., 1994). Based on rRNA analysis, the new phylogeny of the clostridia would place *C. perfringens* into Cluster I (equivalent to the rRNA group I proposed by Johnson and Francis (1975)). This division contains almost half of the known *Clostridium* species but is phylogenetically distinct from the other 18 clusters described (Collins et al., 1994). Beyond the species level, *C. perfringens* is further divided into five subtypes: A, B, C, D, or E, based on the production of the four commonly named major lethal toxins.

In clinical microbiology laboratories today, the identification of *C. perfringens* is based on demonstrating a number of phenotypic characteristics. In Gram's stained preparations, the organism typically is a large, boxcar-shaped, gram-positive rod (figure 1a); sub-terminal spores are produced but are rarely observed in the laboratory. When grown on Centers for Disease Control and Prevention (CDC) formulated anaerobe blood agar plates under anaerobic

conditions at 35º C, creamy yellow to gray colonies are formed within 24 hours. The colony margins can be smooth, giving the appearance of a round, slightly raised colony, or rough, in which case the colonies are relatively flat. A characteristic feature of *C. perfringens* is a double-zone of hemolysis (figure 1b). The inner zone is an area of complete lysis of the red blood cells caused by production of the theta toxin (figure 1b, arrow). The outer, wider zone of incomplete hemolysis is due to the alpha toxin (figure 1b, arrowhead). A positive test for lecithinase activity also aids in identifying *C. perfringens* and is based on the presence of the alpha toxin. Lecithinase activity is visible as a cloudy precipitate in the agar surrounding colonies grown on egg yolk agar (figure 1c).

A number of biochemical tests are performed to confirm a bacterial isolate as *C. perfringens* in the laboratory. These tests include the determination of the carbohydrate fermentation profile, hydrolysis of esculin, production of indole, reduction of nitrate, hydrolysis of gelatin, and “stormy” fermentation reaction in the milk proteolysis test (figure 1d). Expected results for the traditional biochemical tests for *C. perfringens* and other clostridia that give results similar to *C. perfringens* are shown in table 1. Short-chain fatty acid analysis using gas-liquid chromatography also aids in identification. Typically, *C. perfringens* displays peaks that indicate production of acetic acid, propionic acid, and butyric acid on the volatile fatty-acid chromatograph (Koneman et al., 1997), and may show peaks that indicate production of succinic acid and lactic acid (table 1) (Reig et al., 1981). Chromatographic profiles of volatile and non-volatile short-chain fatty acids characteristically produced by *C. perfringens* are in figure 2.

Clinically Important Diseases Associated with *C. perfringens*

Clostridial Myonecrosis

As previously mentioned, *C. perfringens* is commonly present in the fecal microbiota of healthy humans and animals, but it is also a clinically significant pathogen that causes a range of human and animal diseases. The symptoms and signs of *C. perfringens* diseases are due to the production of numerous toxins that exhibit cytotoxic, hemolytic, hemorrhagic, necrotic, or other damaging effects on cells (Petit et al., 1999). The best-known major human disease caused by *C. perfringens* is clostridial myonecrosis or gas gangrene. The toxins of *C. perfringens* that are attributed with causing the clinical features of clostridial myonecrosis include the alpha toxin and the theta toxin (Bryant et al., 2000); the genes for both of these toxins are present in all subtypes of *C. perfringens*. This disease typically occurs when *C. perfringens* (exogenous or endogenous) is introduced into a soft tissue wound caused by trauma or surgery, although cases in healthy humans have been reported without antecedent trauma (Zelic et al., 2004). Clostridial myonecrosis is a life-threatening disease characterized by a rapid progression with severe muscle necrosis, extensive gas production, shock, and renal failure. Diagnosis is usually based on the clinical presentation because prompt treatment is associated with better patient outcomes and because the presence of bacteria that have the typical Gram reaction and cellular morphology of *C. perfringens* may simply indicate normal microbiota in the patient specimen

(Halpin and Molinari, 2002). Patients with clostridial myonecrosis have a poor prognosis even with aggressive therapies such as surgical débridement, high-dose antibiotics, or amputation (Bangsberg et al., 2002).

Gastrointestinal Diseases

Although clostridial myonecrosis is the disease caused by *C. perfringens* that may be most familiar, the gastrointestinal diseases produced by this microorganism are more common. *Clostridium perfringens* type A is the third most common cause of food-borne illness in the United States (Sparks et al., 2001) and the second most common cause in the United Kingdom (Adak et al., 2002). Like other food-borne zoonoses, such as those caused by *Campylobacter spp*., *Salmonella spp*. and *Escherichia coli* (O'Brien, 2005), *C. perfringens* food-borne disease is also attributed to animal food sources (Labbe, 1989; Lin and Labbe, 2003).

Many other types of bacteria also cause food-borne illness by various pathogenic mechanisms. For example, *Staphylococcus aureus* and *C. botulinum* grow and produce toxins within improperly handled foods. The clinical manifestations observed with disease due to these organisms are due to ingestion of the pre-formed toxin that is in the food (Rasooly et al., 1997; Sharma and Whiting, 2005). In contrast, viable *C. perfringens* is ingested, grows, and

produces toxins directly within the upper small bowel. *Clostridium perfringens* toxins having multiple effects on the intestinal lumen (Popoff, 1998). The clinical symptoms of *C. perfringens* type A food-borne disease are typically mild and follow an 8 to 24 hour incubation period. There is often abdominal pain, nausea, and diarrhea lasting 12 to 18 hours. Fatal complications in the young and elderly are rare and usually due to dehydration (Brynestad and Granum, 2002).

Another gastrointestinal disease that may be associated with *C. perfringens* type A is antibiotic-associated diarrhea (AAD), and it is second in incidence only to *Clostridium difficile* AAD (Modi and Wilcox, 2001; Sparks et al., 2001). It has not been determined whether AAD associated with *C. perfringens* is due to proliferation of resident *C. perfringens* or acquisition of *C. perfringens* after exposure to antibiotics (Vaishnavi et al., 2005). The evidence supports *C. perfringens* as a potential cause of AAD rather than simply a harmless member of the patient's microbiota isolated by chance (Borriello et al., 1984). The incidence of AAD associated with *C. perfringens* has varied from less then one percent to greater then six percent (Heimesaat et al., 2005). The higher rate of greater then six percent approaches the incidence of *C. difficile* associated AAD (Abrahao et al., 2001). In a study by Asha and others (2006), risk factors found in patients with *C. perfringens* AAD were female gender and the use of antacids. No specific antibiotic classes that increased the risk of AAD due to *C. perfringens* were identified. Metronidazole has been associated with successful treatment of AAD due to *C. perfringens* and is also an effective treatment for AAD due to *C. difficile* (Borriello and Williams, 1985). Thus, treatment of patients with AAD with

metronidazole could succeed without regard to determining if the agent is *C. difficile* or *C. perfringens*.

In addition to food-borne disease and AAD, *C. perfringens* is associated with other gastrointestinal illnesses in humans, including sporadic diarrhea and enteritis necroticans. Sporadic diarrhea caused by *C. perfringens* type A is usually a non life-threatening illness with spontaneous resolution occurring after 2 to 7 (Brett et al., 1992). Unlike sporadic diarrhea, enteritis necroticans is a rare, often fatal severe intestinal disease of humans caused by *C. perfringens* type C, a type not found in the normal fecal microbiota of humans; however it can be found in the intestinal microbiota of sheep and can cause an enteritis necroticans-like disease in pigs (Lawrence and Cooke, 1980). Also known as Darmbrand, enteritis necroticans due to *C. perfringens* type C was prevalent in post-World War I Europe (Gui et al., 2002). In Papua New Guinea, “pigbel” is synonymous with Darmbrand and enteritis necroticans in terms of etiology and clinical findings. Pigbel is associated with ceremonial village feasts of large quantities of sweet potatoes and pork contaminated with *C. perfringens* type C spores (Davis, 1984). A few cases of enteritis necroticans due to *C. perfringens* type C have recently been reported in the United States, primarily in diabetic patients who ingested contaminated pork products (Gui et al., 2002; Petrillo et al., 2000; Severin et al., 1984). Although most cases of enteritis necroticans have been associated with *C. perfringens* type C, a recent report identified *C. perfringens* type A as the cause of enteritis necroticans in an outbreak consisting of seven patients in which two died (Brynestad and Granum, 2002). Patients

with enteritis necroticans typically present with symptoms of abdominal pain and severe, bloody diarrhea. Examination of the bowel following removal of the necrotic portion or at autopsy often reveals intestinal distention with increased fluid, mucosal necrosis, pseudomembranes (Gui et al., 2002), and evidence of gas-formation in the mucosa and sub-mucosa (Severin et al., 1984). Current treatment of enteritis necroticans includes administration of fluids or resection of the affected bowel when symptoms are severe (Severin et al., 1984), along with antimicrobial agents (e.g., penicillin and metronidazole) administered intravenously (Watson et al., 1991). Immunization with a vaccine composed of a beta toxoid (a toxin modified to be non-harmful) developed from the beta toxin effectively prevented disease in the endemic area of Papua, New Guinea (Lawrence et al., 1990). Unfortunately, the vaccine is difficult to obtain since few companies presently produce the beta toxoid. In addition, neither anti-gas-gangrene antitoxin nor specific beta antitoxin are effective as treatment options for necrotic enteritis (Watson et al., 1991).

Economically important animal species are also susceptible to *C. perfringens* diseases. *Clostridium perfringens* gastrointestinal diseases in animals include diarrhea in piglets and foals, necrotic enteritis in calves, piglets, lambs and foals, and enterotoxaemia in sheep and horses (Rood, 1998). These diseases can be mild and self-limiting or highly virulent and lethal. The severity and range of the diseases caused by *C. perfringens* in humans and animals make *C. perfringens* a clinically, socially, and economically important microorganism to study.

Genomics of *C. perfringens*

The genome of *C. perfringens* is a circular chromosome of approximately 3.6 mega-base pair in size (due to strain variation the range is 3.07 to 3.65 mega-base pair), with a low guanine + cytosine (G + C) content of about 25% (Canard et al., 1992; Casjens, 1998). The genome of *C. perfringens* also includes extrachromosomal genetic elements (e.g. plasmids and phage encoded mobile genes) that can vary in size and composition (Bruggemann, 2005; Rooney et al., 2006). A role for phages in the transfer of genes between strains of *C. perfringens* has not been identified (Zimmer et al., 2002). Many of the virulence genes that are important in the pathogenesis of illnesses due to *C. perfringens* are located on plasmids, including the beta, epsilon, and iota toxin genes (Katayama et al., 1996). The enterotoxin gene associated with *C. perfringens* gastrointestinal diseases can be located either on the chromosome or a plasmid (Brynestad et al., 1997; Cornillot et al., 1995). Both the epsilon toxin and the beta toxin genes are located on plasmids. These plasmids can be lost during maintenance of *C. perfringens* stock cultures in the laboratory (Katayama et al., 1996). This has implications with respect to investigations of genes encoded on a plasmid. However, loss of a plasmid encoded gene has not been described for other genes in *C. perfringens*, and stability of some plasmids in stock cultures has been demonstrated (Mahony et al., 1987). Conjugative transfer of plasmids between *C. perfringens* strains has been demonstrated (Brefort et al., 1977), including transfer of plasmids encoding resistance genes

for tetracycline and chloramphenicol (Abraham and Rood, 1985; Abraham et al., 1985). However, few plasmids have been shown to change the defined phenotype of a strain of *C. perfringens* (Rood and Cole, 1991). The potential for transfer of other plasmids that contain toxin or other virulence genes has obvious potential for affecting the pathogenecity of *C. perfringens* strains; therefore further studies are needed in this area.

The Toxins of *C. perfringens*

The pathogenesis of *C. perfringens* diseases is directly linked to the many toxins and enzymes this organism produces. The major lethal toxins are termed alpha, beta, epsilon, and iota. These four toxins are used to assign *C. perfringens* to one of five toxin types (table 2). Determination of the toxin type of an isolated strain of *C. perfringens* was classically accomplished using cultured organisms and type-specific anti-sera to neutralize the effects of the strain in experimental animals, such as mice and guinea pigs (Sterne and Warrack, 1964; Yamagishi et al., 1997).

Since the development of modern molecular biology and the identification of the *C. perfringens* toxin genes, techniques such as Polymerase Chain Reaction (PCR) are now more commonly used to determine the toxin type of an isolated strain of *C. perfringens* (Yoo et al., 1997). Each *C. perfringens* toxin

type is generally associated with specific diseases and is found in certain types of animals. In addition to the four major lethal toxins, there are at least 15 other *C. perfringens*-produced toxins (Rood and Lyristis, 1995; Rood, 1998) that may participate in the pathogenesis of disease (Hatheway, 1990). Two of these toxins, the beta2 toxin and the *C. perfringens* enterotoxin (CPE), are of particular interest for their possible association with disease in humans and animals.

Control and Regulation of Toxin Production

There is increasing evidence that many *C. perfringens* virulence genes are regulated by elements of a two-component regulatory system similar to that described in other bacteria (Lyristis et al., 1994; Shimizu et al., 1994). These two-component systems include a histidine kinase sensor and a response regulator component (Gross, 1993; Stock et al., 1989). In *C. perfringens* the components involved in transcriptional control of some virulence genes are termed VirS and VirR (figure 3). Aspects of the histidine kinase family of sensor proteins have been shown in VirS, in addition to a membrane spanning region and a region that can interact with the organism's environment to receive external stimuli (Cheung et al., 2004; Lyristis et al., 1994). The external stimuli have not yet been identified for *C. perfringens*, nor have the stimuli been shown to be environmental factors versus related to growth phase factors. Further

evidence for VirS as a sensor protein for this system is the presence of a highly conserved histidine residue that is capable of undergoing autophosphorylation at histidine residue-255 (Cheung et al., 2004; Lyristis et al., 1994).

VirR is the response regulator, the second element of this two-component system. VirR is activated by phosphorylation at an aspartic acid residue (Asp-57) by the activated VirS; the phosphorylated VirR then functions as a DNA-binding protein to regulate transcription from specific promoters (Lyristis et al., 1994; Shimizu et al., 1994). Direct regulation of transcription by phosphorylated VirR can be positive or negative, depending on the target promoter (Banu et al., 2000). Studies of the *C. perfringens* VirR protein have shown that a pocket of basic amino acids in the C-terminal region of the VirR protein, referred to as the SKHR motif, is essential for proper function of the protein (McGowan et al., 2003). Studies have also revealed that the VirR protein binds independently to two imperfect direct repeats (CCCAGTTNTNCAC) located upstream of the gene promoter (Cheung and Rood, 2000), and that the spatial organization of these repeats is important for transcription of the gene (Cheung et al., 2004).

VirR can also play an indirect role in the regulation of many genes by affecting regulatory genes, including the VirR-RNA regulatory element (VR-RNA) (Ohtani et al., 2002; Shimizu et al., 2002b). The VR-RNA has been shown to positively and negatively regulate gene transcription in *C. perfringens*. Although the 3'-region is essential for function, VR-RNA does not regulate gene transcription by binding to complementary DNA or RNA of a promoter region. The actual mechanism of gene regulation has not been described (Shimizu et al.,

2002b). Fifteen proteins have been identified as regulated by the VirS/VirR system in studies that compare a wild type *C. perfringens* proteome profile to a VirR mutant (Shimizu et al., 2002a). The affected genes can be located on the chromosome or on plasmids (Banu et al., 2000; Ohtani et al., 2003). *Clostridium perfringens* genes regulated by the VirS/VirR system include positive regulation of the theta toxin (perfringolysin O), collagenase (kappa toxin) (Shimizu et al., 1994), and phospholipase C (alpha toxin) genes (Ba-Thein et al., 1996) and negative regulation of the cystathionine gamma-synthase and cysteine synthase genes (Banu et al., 2000). The beta2 toxin gene, *cpb2*, is of particular interest in this study. It is indirectly regulated in a positive manner by the VR-RNA component of the VirS/VirR regulatory system (Ohtani et al., 2003). In addition to the VirS/VirR system, phylogenetic analysis has identified other potential two-component signal transduction systems; 23 sensor kinases and 17 response regulator genes are conserved in three strains of *C. perfringens* (Myers et al., 2006).

Quorum sensing via cell-cell communication allows for regulation of gene expression in many different pathogenic bacteria as a response to changes in population density (Waters and Bassler, 2005). Ohtani and others (2002) identified a homologue of the *luxS* gene in *C. perfringens*, which appears to be regulated by the VirS/VirR system (Ohtani et al., 2000). The *luxS* gene has been identified in a number of pathogenic bacteria, first in *Vibrio harveyi* and then in *Escherichia coli* and *Salmonella typhimurium* (Surette et al., 1999). In *C. perfringens* the *luxS* gene is involved in stimulation of production of the theta,

alpha, and kappa toxins through a cell-cell signaling mechanism, at either the transcriptional or post-transcriptional level (Ohtani et al., 2002). Additional data by Ohtani and others (2002) provides evidence that the VirS/VirR system is an essential component for *luxS* regulation of these toxins.

The *luxS* gene is responsible for production of a molecule initially identified as autoinducer-2. Autoinducer-2 appears to be involved in quorum sensing and cell-cell signaling in type III secretion systems for *E. coli* (Sperandio et al., 1999; Sperandio et al., 2003). A role for autoinducer-2 activation of the VirS/VirR system has not been shown. Furthermore, Walters and Sperandio (2006) found that the autoinducer signaling molecule dependent on the *luxS* gene was not autoinducer-2, but was a different signaling compound that was then named autoinducer-3. Studies are needed to determine if autoinducer-3 is produced by *C. perfringens* and whether it plays a role in the *luxS* regulation of the alpha, kappa, theta toxins, or other toxin genes.

The Alpha Toxin

All strains of *C. perfringens* (types A to E) carry the alpha toxin gene (*cpa,* sometimes referred to as *plc*). Of the four major toxins, type A *C. perfringens* produces only the alpha toxin (table 2). Type A is the most common *C. perfringens* type in the environment and the most common type identified in

humans, either as part of the normal microbiota or in disease (Petit et al., 1999). Outbreaks of *C. perfringens* food-borne illness in the United States are due to primarily to type A strains (Koneman et al., 1997), as are most cases of *C. perfringens* myonecrosis in humans (Stevens and Bryant, 1999).

Unlike many of the virulence genes of *C. perfringens* that are located on plasmids, the alpha toxin gene is located on the chromosome (Rood, 1998). It is 1197 base pair in length and encodes a protein that contains 398 amino acids, with a 28 amino acid signal sequence that is cleaved before the mature protein is secreted (Titball et al., 1989). The molecular weight of the alpha toxin (determined using SDS-PAGE) is approximately 43,000 kDa, and the isoelectric point is pH 5.4 (Jolivet-Reynaud et al., 1988). Although the alpha toxin gene is found in all types of *C. perfringens*, differences in the level of alpha toxin production between types have been observed (Mollby and Holme, 1976). This may be due to differences in the transcription rate (Katayama et al., 1993). How differences in transcription rates are controlled has not been described, although there is an A+T-rich region upstream of the -35 promoter that may be involved in negative regulation of expression of the alpha toxin gene (Toyonaga et al., 1992). There is also evidence that the alpha toxin gene is positively regulated by the VirS/VirR system through *luxS* mediated signaling (Ohtani et al., 2002).

The alpha toxin is a zinc-metallophospholipase C sphingomyelinase that hydrolyzes phospholipids and sphingomyelin and causes disorganization of plasma membranes (Sakurai et al., 2004). Other pathogenic bacteria, including *Bacillus cereus* (Gilmore et al., 1989) and *Listeria monocytogenes* (Camilli et al.,

1991), as well as other clostridia such as *C. bifermentans* (Tso and Siebel, 1989) and *C. novyi* (Taguchi and Ikezawa, 1978) produce phospholipase C enzymes/toxins that are homologous to the alpha toxin. There are two distinct domains of the alpha toxin. The enzymatic phospholipase C activity is restricted to the N-terminal domain (Naylor et al., 1998). The C-terminal domain is responsible for the binding and insertion of the alpha toxin into the lipid bilayer of a cell (Nagahama et al., 2002) and may contribute to the membrane damaging effects observed with the alpha toxin. The non-enzymatic C-terminal domain is also essential for the alpha toxin's hemolytic activity (Titball et al., 1991).

The alpha toxin is hemolytic, dermonecrotic, and lethal to mice (Rood and Lyristis, 1995). The alpha toxin is also capable of directly affecting myocardial contractility, which accounts for the decrease in cardiac index and mean arterial pressure when alpha toxin is administered to mice (Stevens et al., 1988). Recombinant alpha toxin produced in *E. coli* induces hemolysis of murine erythrocytes (Titball et al., 1989). Unlike other toxins of *C. perfringens*, alpha toxin activity is only slightly decreased by treatment with chymotrypsin and is not affected by treatment with trypsin (Ginter et al., 1996). Additional features of the alpha toxin include its ability to cause tissue necrosis, inhibit polymorphonuclear cell recruitment, and produce thrombosis (Awad et al., 2001). All of these effects are observed in clostridial myonecrosis (Bryant et al., 2000; Zelic et al., 2004). The extensive array of cellular damage caused by the alpha toxin explains why investigators have concluded that it is the primary mediator of the tissue damage is associated with gas gangrene (Stevens and Bryant, 1997).

The Beta Toxin

The beta toxin is produced by *C. perfringens* types B and C. Type C strains are associated with enteritis necroticans (also known as “Darmbrand” and “pigbel”) in humans. Newborn animals (in particular piglets) are especially susceptible to infection with *C. perfringens* type C (Tweten, 2001). Enterotoxemias associated with types B and C are of concern in the veterinary sciences, impacting neonatal and young horses, sheep, goats, cows and pigs (Sterne and Warrack, 1964). Type B and C strains of *C. perfringens* have also been isolated from the intestines of a variety of healthy animals (Songer, 1996), apparently as part of the intestinal microbiota.

The beta toxin gene, *cpb*, has been located on different large plasmids that are carried by *C. perfringens* types B and C (Duncan et al., 1978) that have received little study to date. The gene for the beta toxin is 1007 base pair long; it is translated into a protein of 336 amino acids. Twenty-seven of these amino acids comprise a signal sequence that is cleaved prior to secretion (Hunter et al., 1993). The secreted toxin has similarities (based on 17% to 28% identity) to other toxins that are known to form pores in the plasma membranes of eukaryotic cells. These include the *S. aureus* alpha toxin, components of the gamma toxin, components of leukocidin, and the cytotoxin K of *Bacillus cereus* (Smedley et al., 2004). Mechanisms for regulation of transcription or secretion of the beta toxin of *C. perfringens* have not been described to date.

The beta toxin is heat labile and is readily inactivated by denaturation, dilution, oxidizing agents (Sakurai and Fujii, 1987), or trypsin (Lawrence, 1997). When injected intravenously or intraperitoneally, the beta toxin is lethal to adult mice, whereas oral administration is lethal to neonatal mice (Sakurai and Fujii, 1987). The beta toxin has been demonstrated to cause toxemia in mice, cardiac failure in rats (Steinthorsdottir et al., 1998), and fluid accumulation with mucosal hemorrhage in rabbit ileal loops (Yamagishi et al., 1987). Recent reports suggest disease manifestations result from the ability of the beta toxin to cause cation dependent pore formation in susceptible cell membranes (Nagahama et al., 2003; Shatursky et al., 2000; Steinthorsdottir et al., 2000). However, the question of what makes some cell membranes susceptible remains unanswered.

The importance of the beta toxin in human and animal diseases has been demonstrated by immunization studies with beta toxoid (a non-toxic version of the beta toxin protein). The beta toxoid vaccine has resulted in decreases in the incidence of pigbel caused by *C. perfringens* type C in humans (Lawrence et al., 1990) and similar diseases in animals (Kennedy et al., 1977).

The Epsilon Toxin

Epsilon toxin is produced by *C. perfringens* types B and D, which are causes of rapidly fatal enterotoxemias in animals. Recently, *C. perfringens*

epsilon toxin was classified as a category B potential bioweapon. This category includes agents of the second highest priority that are relatively easy to spread, have moderate to low mortality rates, and require diagnostic capabilities not available in the routine microbiology laboratory (http://www3.niaid.nih.gov/topics/ BiodefenseRelated/Biodefense/ research/CatA.htm, accessed March 12, 2008). However, no human cases of disease associated with epsilon toxin producing strains of *C. perfringens* or purified toxin have been reported (Marks, 2004). The epsilon toxin gene is 986 base pair and codes for a 328 amino acid prototoxin (Hunter et al., 1992). The inactive prototoxin is secreted extracellularly, and proteolytic activation (e.g., by trypsin or chymotrypsin) is necessary for it to be toxic (Miyata et al., 2001). The epsilon toxin gene is located on a large plasmid (Katayama et al., 1996). It has not been the focus of many studies, so little information is available.

Regulation of production of the epsilon toxin has not been elucidated. The activated epsilon toxin binds to vascular endothelial cells of lamb, guinea pig, mouse, rabbit and sheep, and causes vascular damage and edema in the brain, heart, lungs, kidneys, and other organs (Buxton, 1978; Gardner, 1973). Neurological illnesses due to the ability of the toxin to cross the blood-brain barrier include opisthotonos and convulsions (Miyamoto et al., 1998). The epsilon toxin is particularly active against hippocampal cells and kidney cells (Tamai et al., 2003).

The Iota Toxin

The iota toxin is produced by type E *C. perfringens*, and has been implicated in cases of sporadic diarrhea in lambs and calves (Barth et al., 2004). Unique among *C. perfringens* extracellular toxins, the iota toxin is a binary toxin that consists of two separate proteins, designated iota-a and iota-b. The iota toxin genes are located on a plasmid, and both are located within the same stretch of DNA in the same reading frame (Perelle et al., 1993). The iota-a gene is 1,163 base pair in length. It codes for a peptide that is 387 amino acids in length that is cleaved to form the mature 346 amino acids peptide. The iota-b gene is 2,627 base pair in length, coding for a peptide of 875 amino acids in length (Perelle et al., 1993). Regulation of the iota toxin at the transcriptional or translational level has not been described. However, there is evidence the iota toxin may be activated extracellularly by a VirR/VirS regulated trypsin-like enzyme secreted by type E *C. perfringens* strains (Gibert et al., 2000). Both proteins, iota-a and iota-b, are necessary for the toxic activities associated with the iota toxin (Nagahama et al., 2004). The binding component iota-b is activated by trypsin removal of a 20kDa N-terminal peptide. After activation the iota-b component can bind to a cell surface receptor (Stiles et al., 2000). The bound iota-b facilitates internalization of the enzymatic iota-a component via receptor-mediated endocytosis (Blocker et al., 2001). The internalized iota-a acts directly on actin monomers by ADP-ribosylation, impairing cell division and migration (Aktories, 1994).

Other Toxins and Enzymes

Clostridium perfringens strains area also capable of producing up to 15 minor toxins and enzymes, in addition to the four major lethal toxins. The actions of these additional toxins and enzymes can affect the severity and course of an infection caused by *C. perfringens*.

The theta toxin (also known as perfringolysin O) many be present in all strains of *C. perfringens*. Like the alpha toxin, it is chromosomally encoded and positively regulated via the *luxS* system by the VirR/VirS two component regulatory system (Ohtani et al., 2002). By binding cholesterol and causing lysis of red blood cells, the theta toxin can cause edema and ischemia that leads to decreased oxygen delivery to affected tissue (Iwamoto et al., 1993; Stevens et al., 1997). Through a cardiac pressor effect, the theta toxin injected intravenously is lethal for mice (Stevens et al., 1988). These actions of the theta toxin contribute to the necrotic effects seen in *C. perfringens* diseases, including clostridial myonecrosis.

Some minor toxins have been found only in specific types of *C. perfringens*. The delta toxin is a hemolysin with affinity for sheep, goat, and pig erythrocytes but is less active against erythrocytes from other mammals. It is associated with only types B and C *C. perfringens* (Alouf and Jolivet-Reynaud, 1981). The lambda toxin is a proteinase that digests gelatin, hemoglobin, and casein. The gene for the lambda toxin is located on a large plasmid and is associated with *C. perfringens* types B, D, and E (Jin et al., 1996). Other

extracellular toxins associated with all types of *C. perfringens* include the following: the kappa toxin, a collagenase; the mu toxin, a hyaluronidase; as well as a neuraminidase (Rood, 1998); a hemolysin; a nuclease; and an extracellular endonuclease (Rood and Cole, 1991). Because many of the genes for these minor toxins are located on the variably sized plasmids of *C. perfringens*, it has been suggested that it is possible to gain or lose these virulence genes (Katayama et al., 1996; Petit et al., 1999).

The Enterotoxin

The *C. perfringens* enterotoxin (CPE) has been extensively studied. CPE is known to play a key role in outbreaks of *C. perfringens* food-borne illness (McClane, 1992), some cases of AAD (Modi and Wilcox, 2001; Sarker et al., 1999; Sparks et al., 2001), and cases of sporadic diarrhea (Collie et al., 1998). The *C. perfringens* enterotoxin gene (*cpe*) has been identified in approximately 5% of all *C. perfringens* isolates (Sarker et al., 2000; Sparks et al., 2001) and can be located on either the chromosome or a plasmid (Collie et al., 1998; Collie and McClane, 1998; Cornillot et al., 1995). There is a positive correlation between strains with a chromosomally located *cpe* and food poisoning (Sarker et al., 1999; Sarker et al., 2000; Sparks et al., 2001), while *C. perfringens* strains associated with non-food-borne gastrointestinal diseases carry *cpe* on a plasmid

(Collie and McClane, 1998; Sarker et al., 2000; Sparks et al., 2001). The enterotoxin gene is 957 base pair long, encoding a polypeptide that is 319 amino acids (Czeczulin et al., 1993). It is highly conserved regardless of whether the gene is located on the chromosome or a plasmid (Cornillot et al., 1995). The enterotoxin is primarily associated with *C. perfringens* type A, although it has also been identified in some type C and type D strains (McClane, 1998; Skjelkvale and Duncan, 1975). There is evidence that most type E strains of *C. perfringens* carry *cpe* on a plasmid; however, CPE is not produced by these strains. Although *cpe* is still highly conserved in these type E strains, there are a number of minor mutations that result in nonsense or frameshift mutations, as well as an absence of promoters upstream of the gene (Billington et al., 1998).

Unlike other toxins produced by *C. perfringens*, CPE is not secreted from the cell; it is produced while the organism undergoes sporulation and is released when *C. perfringens* is lysed to release the spore (Labbe, 1989; Smedley et al., 2004). Regulation of CPE production occurs at the transcriptional level and appears to rely on a sporulation-specific sigma factor or transcriptional activator (McClane, 1998; Melville et al., 1994). In the intestinal lumen, CPE binds to protein receptors on enterocytes. Enterocyte receptors include claudins, proteins that serve a role in the formation of tight junctions between cells (Katahira et al., 1997). CPE forms protein complexes after binding cells that express the appropriate receptor in the host cell plasma membrane, including a small complex that corresponds in size to receptor-bound CPE (Wieckowski et al., 1994). The small complex cannot induce cytotoxicity, but the formation of a large

complex with an unknown composition precedes plasma membrane permeability changes and initiation of cytotoxicity (Kokai-Kun et al., 1999; Wnek and McClane, 1989). CPE induces pore formation in intestinal cell membranes, which results in fluid and electrolyte loss leading to cell death (McClane, 2000; Popoff, 1998).

The main effects of CPE are on intestinal epithelial cells, especially those at the tips of the villi. The highest activity is in the ileum, with lower activity in the jejunum (McDonel, 1979; Meer et al., 1997). High concentrations of CPE can result in inflammation in the intestine, which likely contributes to some of the clinical manifestations associated with *C. perfringens* gastrointestinal diseases (Chakrabarti et al., 2003). Although the mode of action of CPE has been well described and its association with food-borne disease in the United States has been documented, laboratory testing for CPE is seldom performed except in public health or research laboratory settings. This is due in part to a lack of reliable commercially available rapid tests for identification of CPE in human clinical specimens. Rapid tests are needed because CPE degrades very quickly in stool. Also, the techniques and materials involved in the induction of sporulation and subsequent CPE production in *C. perfringens* strains isolated from humans with disease are complex and difficult for routine clinical laboratories to perform.

The Beta2 Toxin

The beta2 toxin was first differentiated from the beta toxin by Gibert et al. in a 1997 publication. However, the beta2 toxin was actually first described in a 1986 publication by Jolivet-Reynaud et al. (of the same laboratory as Gibert), although it was thought to be the beta toxin. These investigators were attempting to purify and characterize the beta toxin from CWC245, a type C strain of *C. perfringens* isolated from a piglet with necrotizing enterocolitis. The purified toxin was lethal to mice, induced a dermonecrotic effect in guinea pigs, and was inactivated when trypsin was present (Jolivet-Reynaud et al., 1986). All of these findings correlated with activities previously reported for the beta toxin (Lawrence and Cooke, 1980). Additionally, the unknown toxin isolated by Jolivet-Reynaud et al. (1986) was cytotoxic to Chinese Hamster Ovary (CHO) cells and caused hemorrhagic necrosis of intestinal mucosa when a guinea pig ligated intestinal loop assay was performed. However, the molecular weight of this isolated toxin was 27,670 Da (Jolivet-Reynaud et al., 1986), which differed from the molecular weight of 34,861 Da reported for the beta toxin (Hunter et al., 1993). This difference in molecular weight prompted further study and characterization of the unknown toxin. The new toxin was termed the beta2 toxin because of the similarity in biological activity to the beta toxin (Gibert et al., 1997). Confirmation of the beta2 toxin as a novel toxin was shown by a lack of nucleotide sequence or amino acid sequence similarity with the beta toxin (Hunter et al., 1993) or any other known protein or toxin (Gibert et al., 1997). It was suggested that the beta2

toxin was associated with some veterinary diseases because *C. perfringens* strains isolated from piglets with necrotic enteritis and from horses with enterocolitis were positive for the beta2 toxin gene (Gibert et al., 1997).

Since the first publication identified the beta2 toxin in 1997 (Gibert et al., 1997), other investigators have identified the presence of the beta2 toxin gene (*cpb2*) in all types (A-E) of *C. perfringens* isolated from a variety of animals (Baums et al., 2004; Bueschel et al., 2003; Garmory et al., 2000). Manteca et al. (2002) identified strains of *C. perfringens* positive for *cpb2* using a *cpb2* gene probe followed by intestinal ligated loop assays in a study of bovine enterotoxaemia. They postulated a synergism between the beta2 toxin and the alpha toxin in these bovine diseases. Other investigators have identified *cpb2* using PCR or gene probes in *C. perfringens* strains associated with veterinary diseases in studies involving the following: diarrheic dogs (Thiede et al., 2001), an African elephant with ulcerative enteritis (Bacciarini et al., 2001), lambs with dysentery (Gkiourtzidis et al., 2001), a goat with enterotoxemia (Dray, 2004), reindeer (Aschfalk et al., 2002), cod fish (Aschfalk and Muller, 2002), hooded seals (Aschfalk and Muller, 2001), and chickens (Engstrom et al., 2003). Because the beta2 toxin was first identified in a strain of *C. perfringens* isolated from a piglet with necrotic enteritis, some studies of the beta2 toxin focused on porcine strains of *C. perfringens*. Several studies found *cpb2* in *C. perfringens* strains from pigs with gastrointestinal diseases, often associated with *cpb* (the beta toxin gene, in types B and C) (Bueschel et al., 2003; Garmory et al., 2000; Klaasen et al., 1999). These studies also confirmed production of the beta2 toxin

in *C. perfringens* strains positive for *cpb2* (Waters et al., 2003). Other studies have focused on the possible relationship between the beta2 toxin and *C. perfringens* related diseases in horses. In horses *cpb2* was identified in *C. perfringens* type A; this was in contrast to porcine studies in which *cpb2* was primarily associated with types B and C (Herholz et al., 1999; Tillotson et al., 2002). Immunohistochemistry was used to localize beta2 toxin in the gastrointestinal tract of horses with *C. perfringens* related disease (Bacciarini et al., 2003). Others reported that they did not identify the beta2 toxin in their *cpb2* positive strains from horses and suggested that the beta2 toxin was below the detection level of their Western blotting assay (Waters et al., 2005). Variation in production of the beta2 toxin by *cpb2* positive strains of *C. perfringens* of all types (A-E) has been reported (Bueschel et al., 2003; Jost et al., 2005; Vilei et al., 2005).

Nucleotide sequencing of *cpb2* revealed that there was variation between *cpb2* from different isolates. The "consensus" sequences described by Jost et al. (2005) were similar to the sequence of the first *cpb2* described (Gibert et al., 1997), and isolates with these sequences were usually found to express the beta2 toxin. However, strains of *C. perfringens* with "atypical" *cpb2* sequences often did not produce the beta2 toxin (Jost et al., 2005). The extent of the variability of *cpb2* and the role of the beta2 toxin in virulence in *C. perfringens* diseases remains unclear.

At the commencement of this investigation, there was a lack of information regarding beta2 in *C. perfringens* isolates from human sources. We were

intrigued by questions concerning the possibility of a role for beta2 in *C. perfringens* diseases. For instance, was *cpb2* present in *C. perfringens* isolates from humans? If so, how frequently or infrequently was it present? Another question was whether the beta2 toxin was expressed in *cpb2* positive isolates. Also of interest was whether the beta2 toxin could be implicated as a virulence factor in *C. perfringens* related diseases in humans. Veterinary studies provided support for the hypothesis that beta2 toxin produced by *C. perfringens* from humans could be involved in human gastrointestinal diseases.

Additionally, there were *cpb2* sequences in GenBank that were not described in any publication we could find. These were identified by the submitters as pseudogenes of *cpb2* (accession numbers AJ537533-AJ537551). This seemed to suggest that these investigators had identified *cpb2* nucleotide sequences that were not expressed. If these genetic variants contained information necessary for transcription, then it was possible they might contain mutations that would prevent translation, or would change the structure of the protein such that function was affected. As such, they would be unlikely to play a role in any disease process; therefore, it would be relevant to determine the functionality of *cpb2* and the product of this gene, the beta2 toxin. Studies focusing on these questions would provide much needed information concerning the beta2 toxin and its possible link to disease in humans, and could also aid in the general understanding of how *C. perfringens* causes disease.

As this investigation progressed, new reports were published which provided further information about *cpb2* and the beta2 toxin. Specifically, reports

were published that focused on *cpb2* and the beta2 toxin in *C. perfringens* isolates from humans with gastrointestinal diseases. The *C. perfringens* type A strains from humans with antibiotic-associated diarrhea (AAD) or sporadic diarrhea (SD) carried the *cpe* gene on a plasmid, while most isolates from humans with food-borne disease carried the *cpe* gene on the chromosome (Fisher et al., 2005). The association of *cpe* with *cpb2* varied remarkably depending on the location of *cpe*; greater than 75% of *C. perfringens* isolates were *cpb2* positive if *cpe* was located on a plasmid, while less then 15% were positive for *cpb2* if *cpe* was on the chromosome (Fisher et al., 2005). Other studies have provided additional information regarding *cpb2* positive *C. perfringens* strains from humans with AAD or SD, including evidence that *cpb2* is regulated at the transcriptional level (Harrison et al., 2005). The plasmids that carry *cpb2* have been described (Miyamoto et al., 2006). These reports help to support the results and conclusions from this investigation, namely that the beta2 toxin may be a virulence factor in *C. perfringens* diseases in humans.

Research Aims

The principal objective of this study was to determine the prevalence of *cpb2*, the beta2 toxin gene, in *C. perfringens* isolates from a variety of human sources, including isolates from a population of healthy volunteers. Reports in

the literature have described the identification of *cpb2* and a possible role for the beta2 toxin in gastrointestinal diseases of animals. At the commencement of this study, information was lacking regarding the question of the possibility of a role for *cpb2* and the beta2 toxin in *C. perfringens* related diseases in strains isolated from humans.

After determining whether *cpb2* was present in the *C. perfringens* isolates from the different human populations, we formed two interconnected hypotheses concerning *cpb2* and the beta2 toxin of *C. perfringens* and their possible role in diseases of humans. First, we hypothesized that *cpb2* was present in isolates from humans with *C. perfringens* related diseases at a higher frequency than isolates from healthy volunteers. The second hypothesis was that *cpb2* positive *C. perfringens* isolates from healthy volunteers either contained a gene that was expressed at low levels, expressed a non-functional variant of the toxin, or encoded a gene that was not expressed.

To determine the validity of our hypotheses, we defined three additional research aims. First, we sought to identify any genetic variations of *cpb2*, which could predict the sequences of the protein toxin or variants of the protein toxin. This knowledge could aid in our understanding of differences in disease presentation, disease severity, and virulence of *C. perfringens* isolates in humans. In addition, we also investigated the expression of beta2 toxin in *cpb2* positive isolates. Finally, the last aim of this study was to determine the activity of recombinant beta2 toxin from *C. perfringens* strains from human sources produced by transformed *E. coli* in caco-2 cultured cells.

MATERIALS AND METHODS

Clostridial Strains

Growth and Storage of Organisms

All strains of *C. perfringens* used in this study, with the exception of the reference strains, were selected from the stock culture collection of the Anaerobe Laboratory, Division of Clinical Microbiology, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine. These strains had been isolated from clinical specimens from volunteers for previous research studies. The types of clinical specimens included: feces, wound or abscess material, blood, peritoneal fluid, and other normally sterile body fluids. Organisms were identified as *C. perfringens* based on Gram reaction and microscopic features, aerotolerance testing, colony characteristics, short-chain fatty acid profile, and reactions in biochemical tests. Stock cultures were maintained in cooked meat (CM) storage medium (Remel Inc., Lenexa, KS). The following scheme was used to grow and verify the correct identities of the bacteria included in this study (figure 4): each clostridial isolate was first cultured from the cooked meat (CM) storage medium onto a CDC formulation anaerobe blood agar plate (CDC-AnBAP) (Remel). If there was no initial growth on the

CDC-AnBAP, the organism was inoculated into pre-reduced anaerobically sterilized (PRAS) cooked meat carbohydrate broth (PRAS-CMC) (Remel), and incubated under anaerobic conditions (in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2) at 35º C overnight in an anaerobic chamber (Anaerobic Chamber model B, Coy Laboratory Products Inc., Grass Lake, MI). Organisms grown on the CDC-AnBAP were subcultured a minimum of three times on CDC-AnBAP to ensure purity and viability of the organism. After subculturing, traditional phenotypic methods were used to confirm the identity of each isolate, including colony morphology, appearance of the growth on the CDC-AnBAP, and Gram's stain features. Colonies from the CDC-AnBAP were also used to inoculate two PRAS-CMC broth tubes and incubated under anaerobic conditions at 35º C overnight. One PRAS-CMC tube was used for short-chain fatty acid analysis (both volatile and non-volatile fatty acids) using gas-liquid chromatography (GLC), and the other tube used to inoculate the appropriate PRAS media to determine the biochemical characteristics of the organism (table 1 and figure 2). If the organism was confirmed as *C. perfringens* based on colony characteristics, Gram's stain features, short-chain fatty acid profiles, and biochemical reactions, then genomic DNA was collected from the isolate. Typical colonies of *C. perfringens* on a CDC-AnBAP and the microscopic appearance on Gram's stain of a pure culture of *C. perfringens* are shown in figure 1. Organisms that were not verified as *C. perfringens* were not maintained or used in this study.

Short-Chain Fatty Acid Analysis

Short-chain fatty acid analysis of volatile and non-volatile fatty acids using GLC was performed as part of the identification process for each clostridial strain (figure 2) (Jousimies-Somer et al., 2002). Isolated colonies were used to inoculate a PRAS-CMC broth culture tube. Following overnight incubation under anaerobic conditions at 35º C, the broth in the PRAS-CMC culture tube was acidified with 400 μl of a 50% (w/v) H_2SO_4 solution (Fisher Scientific International Inc., Hampton, NH) and divided equally into two glass tubes (2.0 ml each). For analysis of volatile fatty acids, one tube containing 2.0 ml of acidified broth culture was extracted once using an equal volume of ethyl ether (Fisher). The organic layer was then removed and the volatile fatty acid profile determined using a Varian 3700 Gas Chromatograph (Varian Instrument Group, Walnut Creek, CA) fitted with an SP 1000 column (Supelco Inc., Bellefonte, PA) and flame ionization detector. For analysis of the non-volatile fatty acids, the other tube containing 2.0 ml acidified culture was used. Accordingly, 1.0 ml of methanol (Fisher) was added along with 100 μl of a 50% (w/v) solution of H_2SO_4. Then the tube was vortexed vigorously and heated to 60º C for 30 minutes. After cooling to room temperature, 500 μl of chloroform (Fisher) was added and the tube was centrifuged in a bench top clinical centrifuge for 5 minutes to allow the chloroform and water layers to separate. The water layer was removed by gentle vacuum suction, and the bottom chloroform layer collected for determination of the non-volatile fatty acid profile using GLC analysis with a Varian 3700 Gas

Chromatograph (Varian Instrument Group, Walnut Creek, CA) fitted with an SP 1000 column (Supelco Inc., Bellefonte, PA) and flame ionization detector. Typical profiles for both volatile fatty acids and non-volatile fatty acids are shown in figure 2.

Biochemical Characterization

The biochemical characteristics of each strain of *C. perfringens* were determined using the identification scheme of the Anaerobe Laboratory, which was originally based on data from Holdeman et al. (1977), but updated recently (Winn et al., 2006; Jousimies-Somer et al., 2002). Broth from an overnight culture of PRAS-CMC (figure 4) was used to inoculate the PRAS biochemical media (Remel; by injection of three to five drops through the rubber stopper). The PRAS biochemical media was incubated at 35º C in ambient air. The following day, the carbohydrate fermentations were determined by direct measurement of the pH of the cultures using a Zeromatic SS-3 pH meter (Beckman, Palo Alto, CA). Other biochemical tests were either interpreted after addition of appropriate indicators (e.g., for nitrate reduction, indole production, and bile esculin) or directly interpreted (e.g., for milk digestion, and hydrolysis of gelatin). Typical findings for *C. perfringens* carbohydrate fermentations are

shown in table 1, and an image of a typical result for digestion of milk is shown in figure 1d.

Reference Strains

Reference strains of *C. perfringens* were obtained from two sources: the American Type Culture Collection (ATCC) (Manassas, VA) and the National Collection of Type Cultures (NCTC) (London, United Kingdom). Strains ATCC 13124 and ATCC 8009 were both type A; ATCC 12917 was type A and *cpe* positive; ATCC 27324 was type E and *cpe* positive. NCTC 4964 was type B; NCTC 3227 and NCTC 10719 were both type C; NCTC 8503 was type D. The lyophilized materials were reconstituted per the instructions provided by the supplier with the strains. These bacteria were grown on CDC-AnBAP under anaerobic conditions at 35º C and checked for purity and rapidity of growth. The reference strains were verified as *C. perfringens* by using the same procedure described above for the isolates obtained from the Anaerobe Laboratory culture collection at Indiana University. Gram reaction, cellular features, aerotolerance testing, colony characteristics, short-chain fatty acid profile, and biochemical characteristics were all used to confirm the identity of the reference strains.

Genomic DNA Isolation from *C. perfringens* Isolates

After confirming the identity of an isolate as *C. perfringens*, a single colony from the CDC-AnBAP was used to inoculate 10 ml of brain heart infusion broth (BHIB) and grown under anaerobic conditions at 35º C overnight. Genomic DNA was then isolated from each overnight culture according to the following procedure. First, the culture was centrifuged at 2500 x *g* at 4º C for 15 minutes in a Sorvall RC-3B using the H-4000 rotor to pellet the bacterial cells, and the cell supernatant was removed. The cells were then washed in 10 ml of 10X TE (100mM Tris-HCl (pH 8.0) and 10mM EDTA), and centrifuged again at 2500 x *g* at 4º C for 15 minutes in a Sorvall RC-3B using the H-4000 rotor to pellet the bacterial cells, and the 10X TE buffer was aspirated. The cells were resuspended in 2.0 ml of 10X TE buffer, to which 10 μl of lysozyme (100 mg/ml in 0.25 M Tris-HCl, pH 8.0) was added, and incubated for 1 hour at 35º C with agitation. Next, 200 μl of 10% (w/v) sodium dodecyl sulfate (SDS) was added, and the mixture incubated at 60º C for 10 minutes. This was followed by addition of 20 μl of 0.5 M EDTA, and 6.0 μl of proteinase K (15.1 mg/ml) (Sigma), with incubation at 35º C for 2 hours. Then 440 μl of 5 M $NaClO_4$ (Sigma) was added, and the mixture was incubated at 65º C for 30 minutes. Following this incubation, the cell lysate was extracted by addition of an equal volume of phenol:chloroform:isoamyl alcohol in a 25:24:1 ratio (PCI), and mixed well for 5 minutes at room temperature. The phases were then separated by centrifugation at 2,500 x *g* at 4º C for 5 minutes in a Sorvall RC-3B (Thermo Electron

Corporation, Ashville, NC) using the H-4000 rotor, and the upper (aqueous) phase was removed to a fresh tube. The cell lysate was extracted again with an equal volume of chloroform:isoamyl alcohol in a 24:1 ratio for 30 minutes with agitation, and separated by centrifugation at 2,500 x *g* at 4º C for 15 minutes in a Sorvall RC-3B (Thermo Electron) using the H-4000 rotor. Next, the upper (aqueous) phase was removed to a fresh tube, and the DNA was precipitated with an equal volume of 2-propanol at -20º C overnight. The DNA was pelleted by centrifugation at 14,000 x *g* in a microcentrifuge for 10 minutes, and the 2-propanol was removed. The pellet of DNA was washed with 70% ethanol and centrifuged for 5 minutes at 14,000 x *g* in a microcentrifuge. Finally, the 70% ethanol was removed, the pellet of DNA allowed to dry, and the DNA resuspended in 200 μl of H_2O. After collection of the total DNA, aliquots were made of each sample. Some aliquots were stored at 4º C and the others stored at -20º C, for later use in multiple experiments.

Quantitation and Qualification of DNA

To determine the concentration of DNA isolated from each *C. perfringens* strain, ultraviolet absorbance spectrophotometry was performed using a Beckman DU 640 UV/Visible spectrophotometer (Fullerton, CA). Samples were diluted 1:100 in distilled deionized water for total volume of 100 μl. The

absorbance of the sample at 260 nm and 280 nm was read by the spectrophotometer. At an absorbance of 260 nm, 1.0 correlates with 50 μg/ml of double stranded DNA. The concentration of DNA (μg/ml) was calculated by multiplying the absorbance of the sample at 260 nm by 100 (the dilution factor) and then multiplying by 50 because the sample is double stranded DNA. To estimate the purity of the genomic double stranded DNA isolated from each *C. perfringens* strain, the ratio of the absorbance of the sample at 260 nm to the absorbance of the sample at 280 nm was determined. A ratio of 1.8 indicated a more pure, or better quality, sample of double stranded DNA. A ratio above or below 1.8 signified a sample contaminated with either phenol or protein.

Polymerase Chain Reaction (PCR)

Oligonucleotide primers used for PCR were purchased from Integrated DNA Technologies, Inc. (IDT) (Coralville, IA). The oligonucleotides were ordered at approximately 25 nmol concentration and purified using standard desalting techniques to remove contaminating organic salts. Upon receipt in the laboratory, oligonucleotides were resuspended in DNAse-free H_2O (HyClone, Logan, UT) to make a stock concentration of 100 pmol/μl.

All PCR reactions were performed using TaKaRa *Taq* Polymerase, along with the supplied 10X PCR buffer, dNTPs, and 25 mM $MgCl_2$ (TAKARA BIO, Inc., Otsu, Shiga, Japan).

Temperature cycling, which varied depending on the PCR protocol used, utilized the Applied Biosystems GeneAmp PCR 2700 (Foster City, CA) thermocycler.

Multiplex PCR

The genotype of each isolate was determined by using a multiplex polymerase chain reaction described by Garmory et al. (2000), with minor changes (http://microvet.arizona.edu/research/ClostridiumWeb/methods.html, accessed September 8, 2003). This multiplex PCR allowed us to determine the genotype of the strains of *C. perfringens*. It enabled the identification of the four major toxin genes; *cpa* (alpha toxin), *cpb* (beta toxin), *etx* (epsilon toxin), and *iA* (iota toxin), if they were present in the genome of that strain. This PCR method also detected the presence of *cpe* and/ or *cpb2* if either was present in the genomic DNA of the organism. Each of these six genes had a region that was amplified by a set of primers specific for that toxin gene (table 3). The final genotype for each strain was based on results from three separate multiplex PCR reactions. To assay for the six possible gene fragments, the following were

mixed together in one reaction tube: 2.6 μl of cpa-F-1438/cpa-R-1743 (10 pmol/μl), 1.8 μl of cpb-F-871/cpb-R-1046 (10 pmol/μl), 2.6 μl of iA-F-275/iA-R-701 (10 pmol/μl), 2.2 μl etx-F-227/etx-R-862 (10 pmol/μl), 1.8 μl of cpb2-F-492/cpb2-R-1058 (10 pmol/μl), and 1.7 μl of cpe-F-439/cpe-R-650 (10 pmol/μl), 5.0 μl 10X PCR buffer, 1.0 μl of 25 mM $MgCl_2$ (for a final concentration of 2 mM), 4.0 μl of dNTPs, 12.1 μl H_20, 0.5 μl (2 units) of *Taq*, and 2.0 μl of *C. perfringens* genomic DNA (approximately 50 ng). The gene fragments were amplified using the PCR with denaturing for 2 minutes at 95º C, followed by 35 cycles consisting of: 1 minute at 94º C, 1 minute at 55^{o}C, 1 minute at 72º C, and an additional period of extension for 10 minutes at 72º C. Eighteen microliters of each PCR product was mixed with 3.0 μl of loading dye (Promega, Madison, WI) and loaded onto a 1.5% agarose gel and electrophoresed for approximately 1 hour with 90 volts. The gel was stained using ethidium bromide and band patterns visualized with ultraviolet light. A negative control using H_2O in place of the sample DNA was processed along with the sample DNA for each PCR performed and run along with the sample DNA to ensure that reagents were free of any contaminating DNA. Genomic DNA from the *C. perfringens* reference strains was also included with each PCR batch to ensure that the six genes being assayed for were indeed amplified with that mix of reagents, serving as positive controls.

Simplex PCR

Simplex PCR reactions, using three separate sets of primers, were used to confirm the presence and further characterize *cpb2* from isolates positive for *cpb2* by the multiplex PCR. Different primer sets, for different regions of *cpb2*, were used for each simplex PCR (table 4). Three separate simplex PCR reactions were performed for each isolate because of reports that some strains were *cpb2* positive using a set of PCR primers designed by Garmory et al. (2000), but that these same strains were not positive for *cpb2* using primers designed by Herholz et al. (1999) (Marks and Kather, 2003). We chose to perform a PCR reaction using the primer set for *cpb2* described by Garmory et al. (2000) to verify the results from the multiplex PCR. The simplex PCR using the primer set of Herholz et al. (1999) was used to determine if our strains remained *cpb2* positive using this primer set. The third set of PCR primers were designed in this laboratory in an attempt to amplify the entire *cpb2* sequence according to the *cpb2* nucleotide sequence described by Gibert et al. (1997). The protocol for each simplex PCR was essentially the same, differing only in the primer set used and the annealing temperature. For each simplex PCR: 5.0 μl of each forward and reverse primer (10 pmol/μl) were mixed with 5.0 μl 10X PCR buffer, 1.0 μl of 25 mM $MgCl_2$ (for a final concentration of 2 mM), 4.0 μl dNTPs, 21.25 μl H_20, 0.5 μl (2 units) of Taq, and 2.0 μl of *C. perfringens* genomic DNA (approximately 50 ng). The gene fragment was amplified by denaturing for 2 minutes at 95º C, followed by 35 cycles consisting of: 30 seconds at 94º C, 30

seconds at the annealing temperature required by the primers (table 4), 45 seconds at 72º C, and finally an additional period of extension for 10 minutes at 72º C. A total of 18 μl of each PCR product was then mixed with 3.0 μl of 6X loading dye and was loaded onto a 1.5% agarose gel and run for approximately 1 hour at 90 volts. The gel was stained using ethidium bromide and visualized with ultraviolet light. A negative control using H_2O in place of the sample DNA was processed along with the sample DNA for each PCR performed and included with the sample DNA to ensure that reagents were free of any contaminating DNA. Additionally, a reference strain of *C. perfringens* that was negative for *cpb2* (ATCC 13124) was also run with each PCR batch of samples processed as a negative control. A positive control using the *cpb2* positive reference strain (NCTC 10719) was included with each PCR batch to ensure amplification of *cpb2*, if the gene was present in the genomic DNA being assayed.

Triplex PCR

Jost et al. (2005) described what they called a triplex PCR that was used in this study to supplement the DNA sequencing results (described in a subsequent section). It was also used to determine whether the *cpb2* gene was a “consensus” type (group-1 *cpb2*, the designation used by our laboratory) or an “atypical” type (group-2 *cpb2*, the designation used by our laboratory). For the

triplex PCR, three individual primers were used, including two forward primers, one specific for the group-1 *cpb2* and the other specific for the group-2 *cpb2*; and a reverse primer common to both sequences (table 5), which is the same reverse primer for *cpb2* used in the multiplex PCR. For each triplex PCR reaction, 2.5 μl of each primer (10 pmol/μl) were mixed with 5.0 μl of 10X PCR buffer, 1.0 μl of 25 mM $MgCl_2$ (for a final concentration of 2 mM), 4.0 μl dNTPs, 35.25 μl of H_20, 0.5 μl (2 units) of Taq, and 2.0 μl of *C. perfringens* genomic DNA (approximately 50 ng). The gene segments were amplified using the protocol described by Jost et al. (2005), with minor modifications. Briefly, denaturation was performed for 2 minutes at 95º C, followed by 35 cycles consisting of: 30 seconds at 94º C, 30 seconds at 50º C, 30 seconds at 72º C, with an additional period of extension for 10 minutes at 72º C. A total of 18 μl of each PCR product was mixed with 3.0 μl of 6X loading dye, loaded onto a 1.5% agarose gel, and run for approximately 1 hour at 90 volts. The gel was stained using ethidium bromide and visualized with ultraviolet light. Expected amplification products were a 304 base pair product for group-1 *cpb2*, and a 741 base pair product for group-2 *cpb2*. A negative control using H_2O in place of the sample DNA was processed along with the sample DNA for each PCR performed and run along with the sample DNA to ensure that reagents were free of any contaminating DNA. As a positive control, DNA collected from *C. perfringens* ATCC 27324, which contains both the group-1 *cpb2* gene and the group-2 *cpb2* gene, was processed with our unknown sample to ensure that each type of *cpb2* gene would amplify if present in the genomic DNA sample.

Nucleotide Sequencing and Amino Acid Sequence Prediction

Nucleotide sequences of the *cpb2* gene from the genomic DNA of *C. perfringens* strains were produced using a D-rhodamine matrix on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) automated sequencer.

At the commencement of this portion of this investigation, two *cpb2* sequences had been published by others (Gibert et al., 1997; Shimizu et al., 2001). The first published sequence, termed CWC245-*cpb2* (our laboratory's designation), came from a type C strain of *C. perfringens*, from a piglet with necrotic enteritis (Gibert et al., 1997). The second sequence was CP13-*cpb2* (our laboratory's designation), from a type A strain of *C. perfringens*, identified during a whole genome sequencing project (chromosomal and plasmid) of strain 13 (Shimizu et al., 2001; Shimizu et al., 2002a).

The primers we designed for sequencing *cpb2* were primarily based on CP13-*cpb2* because the strains we tested were all identified as type A *C. perfringens* originally isolated from humans. We designed three sets of primers to amplify *cpb2*, or regions of *cpb2*. The first set of primers, 13G-cpb2-492/1058, corresponded to the same primers used in the multiplex PCR (table 3), with a few base changes made when the CP13-*cpb2* sequence was used for the primer design. The PCR primers 13G-cpb2-492/1058 amplified a 567 base pair region of *cpb2* (figure 5). The second set of primers, 13H-cpb2-377/950, were the same primers as those used in cpb2PCR-2 (Herholz et al., 1999), with minor base

changes according to the CP13-*cpb2* sequence. This second set of PCR primers, 13H-cpb2-377/950 amplified a 574 base pair region of *cpb2*, which overlaps with the region amplified by the first set of primers by 459 base pair (figure 5). The third primer set, 13M-cpb2(-)22/(+)33, was designed to amplify the entire open reading frame of *cpb2*, and was also based on the CP13-*cpb2* sequence (figure 5). Sequences were obtained for all *cpb2* positive strains, using a minimum of one of the primer sets described above. Multiple primer sets were used as needed for a *cpb2* positive *C. perfringens* strain in an effort to obtain the entire *cpb2* nucleotide sequence for each strain.

PCR was performed as described previously (see previous section on polymerase chain reaction, simplex PCR). Four microliters of the PCR reaction was combined with 2.0 μl of H_20 and 1.0 μl of 6X loading dye, and electrophoresed through a 1.5% agarose gel at 100 volts for 1 hour. If only one band at the expected size was seen, the remaining PCR product was purified by spin dialysis through a Sepharose CL-6B (Sigma) column. If multiple bands were observed in the PCR product, the remaining 46 μl of PCR product was combined with 7.0 μl of 6X loading dye and run in two adjacent wells of a 1.5% agarose gel at 100 V for 1 hour. The band at the expected size was cut out of the gel and purified by electroelution using cellulose dialysis tubing with a MW cut-off of 12,000-14,000 (Sigma). The quantitation and qualification of DNA was performed as previously described under the section heading “Genomic DNA isolation from *C. perfringens* isolates” prepared for nucleotide sequencing

according to the protocol provided with the ABI Prism 310 Genetic Analyzer (Applied Biosystems) automated sequencer.

The Basic Local Alignment Search Tool (BLAST) was used to search for similar or related sequences that were published in the NCBI database (http://www.ncbi. nlm.nih.gov/BLAST/) (Altschul et al., 1990).

Alignment studies of DNA sequences and amino acid sequences were performed using ClustalW, through the European Bioinformatics Institute (EBI) website: http://www.ebi.ac.uk/clustalw/ (Higgins et al., 1994). The ClustalW program calculates the best matches for submitted sequences to sequences within its database producing an alignment of sequences that aides investigators in the determination of identity, similarity, and possible homology between sequences. The pairwise alignment scores produced by the ClustalW program correspond to the percent identity between sequences that are reported in the results section. Similarity between sequences means that the sequences share a statistically significant number of bases or amino acids. Homology indicates shared ancestry between two sequences, and can be inferred for sequences that have a high percentage of identity or similarity. However, a high percentage of identity or similarity does not automatically imply homology. Consensus sequences were determined using the nucleotide sequence alignment generated by the ClustalW program for the *cpb2* sequences (and partial sequences) for our *C. perfringens* strains. For differences in nucleotide bases between sequences, the base that was found in the majority of sequences was selected and used in the consensus sequence.

Nucleic acid sequences were then translated using an online translation tool to convert the nucleotide sequence (DNA or RNA) into a deduced protein sequence (http://au.expasy.org/tools/dna.html) available from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (Gasteiger et al., 2003).

Isolation of Culture Supernatant Proteins

Proteins were isolated from the culture supernatant essentially as described by Waters et al. (2003). Briefly, an isolated colony of *C. perfringens* from a CDC-AnBAP was used to inoculate two 10 ml tubes of Brain Heart Infusion Broth (BHIB) (Gibco) and grown overnight under anaerobic conditions at 35º C. The overnight culture was centrifuged to pellet the bacterial cells, and the culture supernatant was then transferred to a clean tube. Proteins were precipitated overnight at 4º C, after the addition of 4.76 grams of ammonium sulfate ($(NH_4)_2SO_4$) (Fisher Scientific) per 10 ml of culture supernatant. Precipitated proteins were collected by centrifugation at 27,000 x *g* in a Sorvall RC-5B plus centrifuge (Thermo Electron) using the SS-34 rotor at 4º C for 20 minutes, followed by a wash with 2.0 ml of cold acetone, and another centrifugation at 27,000 x *g* in a Sorvall RC-5B plus centrifuge (Thermo Electron) using the SS-34 rotor at 4º C for 20 minutes. The remaining acetone was

allowed to evaporate, and the proteins were then resuspended in 1.5 ml of H_2O. The concentrated cell culture supernatant proteins were stored at -20º C.

Western Blotting

Western Blotting with Polyclonal Anti-Beta2 Antibody

Western blotting was performed according to the basic protocol provided with the Invitrogen NuPAGE® Novex Bis-Tris gels using the XCell II™ Blot Module, with minor modifications. Thirty microliters of the concentrated cell culture supernatant proteins were mixed with 30 μl of lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, Carlsbad, CA), and this mixture was heated in boiling H_2O for 5 minutes. The samples were then immediately placed on ice, and 15 μl of each sample was loaded into a single well of a NuPAGE® Novex 10% Bis-Tris 15 well (1.5mm) gel or 10 well (1.5 mm) gel (Invitrogen). Proteins were separated by electrophoresis at 200 volts for 40 minutes, using MES SDS running buffer (Invitrogen). The separated proteins were transferred to an Immobilon™-P Polyvinylidene Difluoride (PVDF) membrane (Millipore Co., Billerica, MA), using the XCell II™ Blot Module set at 35 volts for 2 hours. The PVDF membrane was stained using a Ponceau S solution [0.1% (w/v) (Sigma) in 5% (v/v) acetic acid (Sigma)] to verify separation of the proteins and then washed

with H_2O to remove the Ponceau S solution. The membrane was blocked overnight at 4º C with Tris buffered saline plus 0.1% (v/v) Tween-20 (Sigma) (TBST) and 5% Carnation® non-fat dried milk (Nestlé® USA, Glendale, CA). Next, the membrane was probed with a 1:5,000 dilution (in TBST + 5% Carnation® non-fat dried milk) of polyclonal rabbit anti-beta2 antibody and incubated at room temperature for 3 hours. This antibody was kindly provided by Dr. Popoff (Gibert et al., 1997), and produced in rabbits using the CWC245 type C strain of *C. perfringens*. This was followed by four, 15 minutes washes of TBST. The membrane was then incubated with a 1:500,000 dilution (in TBST + 5% Carnation® non-fat dried milk) of a donkey anti-rabbit horseradish peroxidase (HRP) labeled antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature, and followed again by four, 15 minutes washes in TBST. Finally, the membrane was incubated for 5 minutes with ECL Advance™ (Amersham Biosciences) which is a chemiluminescent substrate, and apposed to autoradiography film for 30 seconds to 15 minutes. The film was developed using a Kodak Medical X-Ray processor (Rochester, NY).

Western Blotting with Monoclonal Anti-Beta2 Antibody

A second Western blotting procedure was performed using a monoclonal goat anti-beta2 antibody kindly provided by Dr. Songer (Bueschel et al., 2003),

according to the same procedure as described above with a few changes. The monoclonal antibody was generated using the *cpb2* sequence described by Gibert et al. (1997), minus the coding region for the signal sequence; characterization of the specific epitope was not described. The blocking buffer used was TBST plus 3% bovine serum albumin (BSA) (Sigma), plus 0.1% sodium azide (NaN_3) (Sigma); this solution was also used as the diluent for the primary and secondary antibodies. The membrane was blocked for 3 hours at room temperature, followed by an overnight incubation at room temperature with the monoclonal goat anti-beta2 antibody diluted 1:25. After washing with TBST, the membrane was incubated for 2 hours at room temperature with a 1:10,000 dilution of donkey anti-goat IgG-HRP labeled antibody (Amersham Biosciences). The remaining steps were the same as in the protocol described above.

Stripping Probed Membranes

Membranes were stripped of primary and secondary antibodies using Restore™ Western blot stripping buffer (Pierce, Rockford, IL). Briefly, the membrane was rinsed in TBST and incubated in the Restore™ Western blot stripping buffer for 15 minutes at room temperature, followed by a wash in TBST for 5 minutes at room temperature. The membrane was stored wet, wrapped in plastic wrap at 4º C until it was re-probed.

Cloning *cpb2* for Expression by *Escherichia coli*

The functional region of *cpb2* (the beta2 gene, minus the 90 base pair region that codes for the predicted signal sequence in *C. perfringens*), as described by Gibert et al. (1997), was cloned into a plasmid vector and expressed in *E. coli*. Western blotting assays showed that most of the recombinant beta2 protein toxin was in the cell culture supernatant versus the pellet of *E. coli*. Therefore, the cell culture supernatant of the recombinant *E. coli*, which included the recombinant beta2 protein toxin, was collected and concentrated for use in future experiments.

Preparation of the *cpb2* Insert

A 707 base pair region of the *cpb2* gene corresponding to the segment between base 124 and base 831 (figure 5) was amplified using PCR. During the PCR reaction a *Bam*H1 restriction enzyme site was added to the 5' end, and an *Eco*R1 restriction enzyme site was added to the 3' end to allow for directional insertion into the vector. These restriction enzyme sites, at these specific locations, were chosen because the vector that we selected has a *Bam*H1 and an *Eco*R1 site at a location that allowed for insertion of the gene segment of *cpb2* in the proper orientation within the vector (figure 6). Also, the region of *cpb2* that

was selected for insertion into the plasmid vector, did not contain either a *Bam*H1 or an *Eco*R1 site. To amplify the desired 707 base pair region of *cpb2* and to add the restriction enzyme sites, two sets of primers were used in the PCR reactions (table 6), one set designed by this laboratory and the other set designed by Bueschel et al. (2003) and Jost et al. (2005). For each PCR reaction, 5.0 μl of both the forward and reverse primers (10 pmol/μl) were mixed with 5.0 μl of 10X PCR buffer, 1.0 μl of 25 mM $MgCl_2$ (for a final concentration of 2 mM), 4.0 μl of dNTPs, 21 μl of H_20, 0.5 μl (2 units) of *Taq,* and 2.0 μl of *C. perfringens* genomic DNA (approximately 50 ng). The gene fragment, along with the flanking restriction enzyme sites, was amplified by denaturing for 2 minutes at 95º C, 10 cycles of: 30 seconds at 94º C, 30 seconds at 48º C, 1 minute at 72º C, 10 cycles of: 30 seconds at 94º C, 30 seconds at 54º C, 1 minute at 72º C, followed by 15 cycles of: 30 seconds at 94º C, 30 seconds at 60º C, 1 minute at 72º C and an additional period of extension for 10 minutes at 72º C. Two microliters of the PCR product was mixed with 1.0 μl of 6X loading dye, and 4.0 μl of H_2O, loaded onto a 1.5% agarose gel, and electrophoresed for 1 hour at 90 volts. The gel was stained using ethidium bromide and visualized with ultraviolet light. A negative control using H_2O in place of the sample DNA was processed with each batch of samples prepared for PCR. If only one band of the expected size was observed, the remaining PCR product was purified by spin dialysis through a Sepharose CL-6B (Sigma) column. Spin dialysis is a gel filtration chromatography technique used to separate small molecules from high molecular weight DNA. If multiple bands were observed in the PCR product, the

remaining 48 μl of PCR product was combined with 7.0 μl of 6X loading dye and electrophoresed in two adjacent wells of a 1.5% agarose gel at 100 volts for 1 hour. The band at the expected size was cut out of the gel and purified by electroelution using dialysis tubing with a MW cut-off of 12,000- 14,000. Quantitation and qualification of the PCR product DNA was performed as previously described under the section heading "Genomic DNA isolation from *C. perfringens* isolates" to determine the amount needed for use in the restriction enzyme digest.

Digestion of the *cpb2* amplicon with *Bam*H1 (Invitrogen) and *Eco*R1 (Invitrogen) was performed in one reaction mixture. Briefly, 10 μg of *cpb2* amplicon was mixed with 2.0 μl of 10X React® 3 buffer (supplied with the enzymes (Invitrogen)), 1.0 μl of *Bam*H1, 1.0 μl of *Eco*R1, and H_2O to bring the total volume to 20 μl. The reaction mixture was incubated at 35º C overnight. To verify that the amplicon was not digested by the restriction enzymes within the *cpb2* segment, and was of the appropriate size (716 base pair, including the added bases from the PCR), 2.0 μl of the reaction mixture was combined with 1.0 μl of 6X loading dye, and 4.0 μl of H_2O and electrophoresed through a 1.5% agarose gel for 1 hour at 90 volts. The gel was stained using ethidium bromide and visualized with ultraviolet light. The remaining reaction mixture was purified using spin dialysis through a Sepharose CL-6B column.

Ligation of the *cpb2* Insert with the pRSET Vector

We chose to use the pRSET A,B,C vector system (Invitrogen) because of the features of the vectors. The pRSET vector system comes with three vectors, designated A, B, C, with differences in their reading frames, allowing for selection of the recombinant vector that places the insert in the correct reading frame for expression. Each of the pRSET vectors has multiple restriction enzyme sites to allow for directional cloning, an ampicillin resistance gene (*bla*) for antibiotic selection of *E. coli* that contain the pRSET vector plasmid, and the T7 promoter which controls gene expression in an *E. coli* strain with *lac*I controlled expression of T7 RNA polymerase.

Depending on the primer set used to amplify the *cpb2* insert (table 6), either the pRSET A vector or the pRSET B vector was used. Vectors were digested with the same restrictions enzymes used to digest the inserts, following the same procedure described above.

Ligation of the vector with the insert used T4 DNA ligase (Promega). Briefly, a 1:3 ratio of vector to insert was mixed with 2.0 μl of 10X buffer (provided with the enzyme (Promega)), 1.0 μl of T4 DNA ligase, and H_2O to bring the total volume to 20 μl. This mixture was incubated at room temperature overnight. To verify that ligation reactions occurred, 2.0 μl of the reaction mixture was combined with 1.0 μl of 6X loading dye, and 4.0 μl of H_2O and electrophoresed through a 1.5% agarose gel for 1 hour at 90 volts. The gel was stained using ethidium bromide and visualized with ultraviolet light.

Top 10F' *E. coli* Transformation for Plasmid Maintenance

After ligation of the vector and *cpb2* insert, the ligation reaction mixture was used to transform Top10F' *E. coli* (see table 7 for a description of the genotype), using the protocol provided with the pRSET vector system. Briefly, 100 ng of the ligation mixture was added to 100 μl of competent Top10F' *E. coli*, and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42º C and then incubated on ice for 2 minutes, followed by the addition of 500 μl of SOC broth [20 grams of tryptone (Bacto Laboratories Pty, Ltd., Liverpool, United Kingdom), 5.0 grams of yeast extract (Bacto), 2.0 ml of 5 M NaCl (Sigma), 2.5 ml of 1 M KCl (Sigma), 10 ml of 1 M $MgCl_2$ (Sigma), 10 ml of 1 M $MgSO_4$ (Sigma), 20 ml of 1 M glucose (Bacto), bring the total volume to 1.0 liter with distilled H_2O and autoclave to sterilize]. The newly transformed Top10F' *E. coli* were then incubated at 35º C for 1 hour with shaking, plated onto Luria-Bertani (LB) agar [10 grams of tryptone (Bacto), 5.0 grams of yeast extract (Bacto), 10 grams of NaCl (Sigma), adjust pH to 7.5 using NaOH, add 15 grams of agar (Bacto), adjust the volume to 1.0 liter with distilled H_2O and heat to melt the agar into solution, autoclave to sterilize] containing 50 μg/ml of ampicillin (Sigma) and incubated at 35º C overnight. Colonies that grew on the LB agar plates with ampicillin were presumed to contain the pRSET vector, which carries an ampicillin resistance gene (*bla*), and individual colonies were selected to test for the presence of the *cpb2* insert in the pRSET plasmid vector.

Plasmid Isolation

The plasmids from colonies that grew on the LB agar with ampicillin plates after overnight incubation were isolated using the protocol for small-scale plasmid DNA isolation by alkaline lysis. A single colony was inoculated into 3.0 ml of LB broth containing 50 μg/ml of ampicillin and grown at 35º C with shaking overnight. The 3.0 ml overnight culture was centrifuged for 1 minute in a microcentrifuge at 14,000 x *g* to pellet the cells and the supernatant was discarded. The cell pellet was resuspended in 100 μl of a solution of 50 mM glucose, 10 mM ethylenediaminetetraacetic acid (EDTA), 25 mM Tris-HCl (pH 8.0), and 2 mg/ml of lysozyme (Sigma), and incubated for 5 minutes at room temperature. Next, 200 μl of a solution consisting of equal volumes of 0.4 N NaOH and 2% sodium dodecyl sulfate (SDS) was added to the viscous cell solution. The following step involved the addition of 150 μl of 3 M sodium acetate and incubation on ice for 5 minutes to allow formation of precipitate. The mixture was centrifuged for 15 minutes in a microcentrifuge at 14,000 x *g* to pellet the debris. The supernatant was transferred to a clean tube with 1.0 ml of cold 100% ethanol and incubated at -20º C for 1 hour. Next, the tube containing the plasmid DNA in ethanol was centrifuged for 20 minutes in a microcentrifuge at 14,000 x *g* to pellet the plasmid DNA. The ethanol was aspirated from the tube, 250 μl of 70% ethanol was added to wash the pellet, and the tube was centrifuged for 5 minutes in a microcentrifuge at 14,000 x *g*. The ethanol was removed by aspiration, and the pellet of plasmid DNA was allowed to air dry completely

before being resuspended in 40 μl of H_2O. After dissolving the pellet, 1.0 μl of RNase A solution (10 mg/ml) (Sigma) was added, and the tube was incubated at 35º C for 30 minutes. After this incubation, 10 μl of 5X stop mix (2% SDS, 25% glycerol, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added, and the tube was incubated at 70º C for 15 minutes to stop enzyme activity. The plasmid DNA was then purified by spin dialysis using a Sepharose CL-6B column.

The presence and orientation of the inserted *cpb2* in the plasmid was determined using the following methods. First, a cpb2PCR-1 simplex PCR reaction (described under the section heading "polymerase chain reaction: simplex PCR") was used to determine if a 567 base pair region of *cpb2* could be amplified from the plasmid, demonstrating the presence of *cpb2* as a part of the plasmid. Next, the plasmid was digested with *Hind*III restriction enzyme (Promega). Briefly, 10 μg of plasmid DNA, 2.0 μl of 10X buffer (supplied with the enzyme (Promega), 1.0 μl of *Hind*III enzyme and H_2O to a total volume of 20 μl, was incubated at 35º C for four hours. The entire digest reaction was mixed with 3.0 μl of 6X loading dye, and electrophoresed on a 1.5% agarose gel at 100 volts for 1 hour. The gel was stained with ethidium bromide and viewed under ultraviolet light. If the *cpb2* gene was successfully inserted into the plasmid in the correct orientation, two bands were expected with different band sizes dependent on whether the *cpb2* type was of the group-1 or group-2 type. If the *cpb2* gene inserted was of group-1, bands at 3415 base pair and 163 base pair were expected. If the *cpb2* gene inserted was of group-2, the bands expected were

2896 base pair and 673 base pair. The third method used nucleotide sequencing to verify the presence of the *cpb2* insert in the pRSET vector, as well as the orientation and reading frame of the *cpb2* insert, using a primer corresponding to the T7 promoter. These samples were sent for sequencing to the Indiana University School of Medicine's Biochemistry Biotechnology Facility, which utilizes a Perkin Elmer/Applied Biosystems 3100 Genetic Analyzer and Big Dye Terminator chemistry v3.1 (http://www.bbf.iu.edu/Getting_Started/HOME.htm, accessed June 16, 2005).

BL21(DE3)pLysS *E. coli* Transformation for Protein Expression

After isolation of the plasmid and verification of the *cpb2* insert in the pRSET vector, the plasmid was transformed into BL21(DE3)pLysS *E. coli* (see table 7 for a description of the genotype) for expression of the recombinant protein. We followed the protocol recommended by the supplier of the pRSET vectors. Briefly, 100 ng of the plasmid DNA was added to 100 μl of competent BL21(DE3)pLysS *E. coli*, and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42º C and then incubated on ice for 2 minutes, followed by the addition of 500 μl of SOC broth. The newly transformed BL21(DE3)pLysS *E. coli* cells were incubated at 35º C for 1 hour with shaking. Our initial experiments transforming BL21(DE3)pLysS *E. coli* with *cpb2* vectors

showed that cells plated onto LB agar plate containing chloramphenicol (34 μg/ml) (Sigma) and ampicillin (100 μg/ml) (Sigma) either grew very slowly or not at all. Because of this complication, we chose to follow an alternative protocol for toxic genes (genes of proteins that could potentially be toxic to the *E. coli* used to produce that protein) with minor modifications. Instead of inoculating agar plates with the mixture from the transformation reaction, 250 μl of the transformation reaction mixture was added to 10 ml of SOC broth containing chloramphenicol (34 μg/ml) and ampicillin (100 μg/ml) and incubated at 35º C overnight with agitation. BL21(DE3)pLysS *E. coli* cells that incorporated the plasmid vector will now carry the resistance genes for the antibiotics chloramphenicol and ampicillin. Next, 5 ml of the overnight growth was added to 50 ml of SOC broth containing chloramphenicol (34 μg/ml) and ampicillin (100 μg/ml). This culture was incubated at 35º C with agitation until the cells reached mid-log phase, determined using an optical density at 600 nm (OD_{600}) of 0.3. Isopropyl-β-D-thiogalactopyranosid (IPTG) at a final concentration of 1 mM was added to the mid-log phase culture to induce expression of the beta2 protein toxin via expression of the T7 RNA polymerase from the *lac*UV5 promoter. After the addition of IPTG, the cells were incubated an additional 2 hours at 35º C with agitation. Pilot experiments demonstrated that maximum secretion of the recombinant protein occurred after 2 hours of incubation at 35º C with agitation after addition of the IPTG and that the majority of the protein toxin was secreted in the cell culture supernatant. Therefore, after the 2 hour induction, the cells were pelleted and the proteins concentrated from the supernatant as described

above (refer to section: “isolation of culture supernatant proteins”). During the pilot experiments the cell culture supernatant was separated from the cell pellet and these were separately tested by Western blotting using the polyclonal anti-beta2 antibody, as well as the monoclonal anti-beta2 antibody to determine where the majority of the recombinant beta2 protein was located The *E. coli* of the cell pellet were lysed by heating cells to 100º C for 5 minutes, prior to performing the western blotting experiment. The Western blotting procedures were performed as previously described (refer to the section: “Western blotting”). These pilot experiments revealed that most of the recombinant beta2 protein was located in the cell culture supernatant of induced *E. coli* clones.

Protein Concentration Determination

The Coomassie Plus™ protein assay kit (Pierce, Rockford, IL) was used to determine the concentration of the total supernatant proteins collected after the *E. coli* was pelleted. Briefly, a series of bovine serum albumin (BSA) standards were made and assayed along with our unknown concentrations of recombinant proteins. Optical density (OD) values were obtained by measuring the BSA standards and recombinant proteins in a Molecular Devices (Sunnyvale, CA) Vmax Kinetic microplate reader, at 565 nm absorbance. A standard curve was prepared by plotting the average OD value for each BSA standard versus its

concentration (in μg/ml), and the estimated concentration for each recombinant protein was determined from the standard curve.

Cell Cytotoxicity Assays

To assess the activity of the recombinant beta2 protein toxin on live cells, we used cell cytotoxicity assays with caco-2 cells (ATCC, number HTB-37). Caco-2 cells are a cell line derived from a colorectal adenocarcinoma. They are epithelial by morphology, adhere to cell culture plates, and express characteristics of enterocyte differentiation. The gastrointestinal diseases of *C. perfringens* type A strains have severe effects in the colon; therefore, caco-2 cells were an appropriate cell line to use in our assays.

The propagation and subculturing conditions for caco-2 cells were performed according to the guidelines suggested by the supplier. The media used for growth of caco-2 cells was Dulbecco's Modified Eagle media with nutrient mixture F-12 (DMEM/F-12) (Gibco-Invitrogen Cell Culture, Carlsbad, CA); this is a minimum essential medium (Eagle) containing 2.0 mM L-glutamine and Earle's balanced salt solution with 1.5 grams/liter of sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate (80% of the total volume), plus 20% (of the total volume) fetal bovine serum. Cells were incubated at 35º C in a 5% CO_2 incubator, and the culture medium replaced every 2 to 3

days until cells were subcultured, which occurred once a week. To subculture cells, the growth medium was removed, and a solution of 0.25% trypsin and 0.53 mM EDTA (T-EDTA) added to the culture flask. The culture flask was incubated at 35º C until the caco-2 cells detached from the surface. The caco-2 cells in T-EDTA were poured into a 12 ml cell culture tube and centrifuged in a tabletop centrifuge for 5 minutes to gently pellet the cells. The T-EDTA was aspirated from the tube, and the cells resuspended in 1X phosphate buffered saline (PBS) (Gibco). Four fields were counted on a hemocytometer and averaged to determine the cell density; counting of additional fields did not significantly affect the final averaged cell density. The caco-2 cells in 1X PBS were diluted using the growth medium (pre-warmed to 35º C) to obtain a final concentration of 1.5 x 10^4 cells/ well and plated onto 48 well or 96 well flat-bottomed polystyrene cell culture plates (Corning Inc., Corning, NY) depending on the assay. One third of the cells were reserved for re-plating in a cell culture flask and grown as described above.

Upon the initial receipt of the cells, the caco-2 cells were grown and subcultured as described above with cells at each passage reserved and frozen in 95% growth medium + 5% DMSO, and stored at -80º C. It was noted that after five subculturings the caco-2 cells exhibited some morphological changes that suggested degeneration, including cell rounding and cell detachment. Therefore, for our experiments cells from passage five were grown and used within five subculturings, after which fresh passage five cells were pulled from storage.

Trypan Blue Exclusion Assay

Caco-2 cells were grown in 48-well flat bottomed plates until reaching 80% confluency (about 48 hrs of growth). Cells were rinsed once with pre-warmed (35º C) 1X PBS before addition of 1X PBS (used as a control) or recombinant protein dilutions. Each recombinant protein assayed was diluted in 1X PBS, in three dilutions of 100 μg/ml, 50 μg/ml, and 12.5 μg/ml. Four experimental wells for each dilution were set up, to allow for assaying at three time points of 2 hours, 5 hours, and 8 hours. The dilutions of recombinant proteins were added to the appropriate well after the cell monolayers were rinsed, and incubated at 35º C in a humid, 5% CO_2 incubator. To determine the relative percent of viable versus non-viable cells, we used the method of trypan blue exclusion (Freshney, 1994). At each time point, the recombinant protein dilution was aspirated from the cells, and 200 μl of 0.4% trypan blue solution (Sigma) (diluted 1:1 with 1X PBS) was added to the well. After incubation for 2 minutes, the trypan blue solution was aspirated from the well, and 100 μl of 1X PBS was added to the well to prevent the cells from drying. The individual wells were imaged using a Nikon Eclipse TS100-F inverted microscope (Nikon, Melville, NY), at 20x magnification. Images of two separate fields at 20x magnification were taken for each individual well (representing a single dilution at a single time point). The images were captured using a Nikon coolpix 4500 digital camera (Nikon) and converted to the tagged image file format (TIFF) using Adobe Photoshop® v. 7.0 (Adobe Systems Inc., San Jose, CA). Using the digital image, the number of viable unstained

cells versus non-viable stained cells was counted manually. The two images for each well (representing a single dilution at a single time point) were averaged together to obtain the final percentage of viable cells that was reported. The low number of replicates for the trypan blue exclusion assay precluded meaningful statistical comparison.

Lactate Dehydrogenase (LDH) Assay

To determine if the recombinant beta2 toxin caused membrane damage and cytolysis of caco-2 cells, we used the Lactate Dehydrogenase (LDH) assay (Oxford Biomedical Research, Oxford, MI). This method utilizes a colorimetric assay kit that measures the amount of LDH that is released by damaged or lysed cells (Korzeniewski and Callewaert, 1983). Measuring the amount of LDH released by damaged or lysed cells provided a method for comparison of the effect of different recombinant protein toxins, at different concentrations, on the caco-2 cells over a set period of time (8 hours). We followed the procedure for the chemotoxicity assay provided with the kit, with minor changes.

Caco-2 cells were grown in 96-well flat bottomed plates until reaching approximately 80% confluency (about 48 hours). Because the growth medium contained serum, cells were gently rinsed three times with serum free assay medium (1X PBS + 1% (w/v) BSA) before beginning the assay. Each

recombinant protein that was assessed was diluted in the assay medium at 100 μg/ml, 50 μg/ml, or 12.5 μg/ml. Experimental wells for each dilution of each recombinant protein were set up in quadruplicate, and control wells were set up in triplicate. Controls were used to measure the amount of LDH spontaneously released by the caco-2 cells (caco-2 cells exposed to assay medium only) and the maximum amount of LDH released by completely lysed caco-2 cells (using the lysing reagent provided with the kit). The dilutions of recombinant proteins were added to the test wells after the cell monolayers were rinsed, and were incubated at 35º C in a humid 5% CO_2 incubator for 8 hours. To assay for the amount of LDH released, the 100 μl of supernatant from the wells (controls, enzyme dilutions, and experimental), was transferred to a clean optically clear 96 well flat bottomed plate. Dilutions of 1:50, 1:100, 1:500, 1:1000, 1:1500, 1:2000, 1:2500, and 1:3000, were prepared with the LDH enzyme control provided with the kit and assayed along with the experimental wells. This series of dilutions was performed for each individual assay, and was used to generate the standard curve used to estimate the amount LDH released by the experimental wells. A total volume of 100 μl of fresh substrate mixture (consisting of buffer, substrate, color enhancer and color reagent; all provided with the kit) was added to each well for color development. Color was allowed to develop for 30 seconds in darkness, at which point 50 μl of 1N HCl was added to each well to stop further development of the color reaction. The absorbance for each well was measured at 490 nm in a Molecular Devices (Sunnyvale, CA) Vmax Kinetic microplate reader.

The amount of LDH released by the experimental caco-2 cells exposed to the varied dilutions of recombinant protein toxin was quantitated by plotting the average absorbance values for control dilutions against the concentration of LDH (in units/ml) at each dilution to produce a standard curve. A best-fit line was calculated for each standard curve, and the amount of LDH released for the experimental wells determined from the standard curve.

Statistical Analysis

Statistical analyses were performed using StatView 5.0.1 (SAS Institute Inc., Cary, NC). Chi square two-tailed analysis was used to determine the statistical difference between matched data sets which compared a diseased population set to the normal, or control group, population set. An analysis of variance (ANOVA) followed by Fisher's Post-hoc testing was used to determine the significant difference between the percentage cell damage (% CD) for the LDH assays. P-values <0.05 were considered significant.

RESULTS

Clostridium perfringens Isolates

Because *C. perfringens* type A is usually present in the intestinal microbiota of healthy humans, 100 well-characterized isolates from two fecal flora studies of healthy human volunteers were included in the present investigation. The majority were isolated from stool samples collected from 88 healthy males, with an age range of 21 to 51 years. The samples were collected between January 1985 and December 1985. The remaining 12 isolates were from stool samples from 1 female and 11 males, all of whom were over 18 years of age (exact age range is unknown), and were collected between November 1979 and September 1980. For purposes of this study, these 100 strains were designated as the population of *C. perfringens* isolates from healthy volunteers, and provided a control group for comparison with the other two groups of *C. perfringens* strains that were originally isolated from specimens collected from human patients with *C. perfringens* related diseases.

There are two gastrointestinal diseases associated with *C. perfringens* type A in humans, antibiotic associated diarrhea (AAD) and food-borne illness. Both diseases are associated with the presence of the enterotoxin. In this study, there were 144 isolates of *C. perfringens* from stool samples of patients

suspected of having AAD and 17 isolates from the stool samples of patients from two outbreaks of *C. perfringens* food-borne illness. Seventy-nine of the *C. perfringens* isolates were obtained from stool samples from patients who were suspected to have AAD between November 1994 and January 1995. The ages of these patients ranged from 17 months to 82 years of age, and 40 of the patients were male with the remaining 39 patients female. The remaining 65 *C. perfringens* isolates were collected from the stool samples of patients suspected of having AAD during January 1997 to December 1997. The ages and genders of these patients were not available. Finally, the remaining 17 isolates were from two separate outbreaks of *C. perfringens* food-borne illness that occurred in April and May of 1997 (10 isolates) and April of 1998 (7 isolates). The ages and genders of these patients were not available. This gave a total of 161 isolates in the population of *C. perfringens* isolates that were from patients with either *C. perfringens* food-borne illness or AAD.

Other diseases associated with *C. perfringens* can occur in almost any anatomic location of the human body. In most instances, the *C. perfringens* involved in these extraintestinal diseases are acquired endogenously, from the patient's own intestinal microbiota rather than from an exogenous source (Winn et al., 2006). Although the primary focus of this study was on *C. perfringens* gastrointestinal diseases, 59 isolates were included from extraintestinal patient specimens from which *C. perfringens* had been isolated and identified, to allow for comparisons between the *C. perfringens* fecal isolates and extraintestinal isolates from other infected sites. There was a wide range of sources for these

extraintestinal isolates including: blood from patients with bacteremia, infected soft-tissue wounds and abscesses, bile, peritoneal fluid, spleen, vaginal discharge material, and other wound sites and unknown sources. These isolates were from specimens that had been collected between January 1997 and August 2004, from 36 female and 23 male patients. The ages of these patients ranged from 3 months to 91 years. These 59 isolates are included together in the extraintestinal diseases group.

Genotype Results Determined by PCR

Multiplex PCR

A multiplex PCR method was used to determine the genotype of all of the *C. perfringens* isolates from the three populations described above. Six primer sets were used to produced amplicon bands corresponding to the size of the amplified a portion of a gene, if that gene was present in the genomic DNA purified from the *C. perfringens* isolate (table 3, figure 7). The four major lethal toxin genes produced amplicon bands of the following sizes: the alpha toxin gene (*cpa*), 324 base pair; the beta toxin gene (*cpb*), 196 base pair; the epsilon toxin gene (*etx*), 655 base pair; and the iota toxin gene (*iA*), 446 base pair. Additionally, if present in the genome of the isolate being analyzed, the

enterotoxin gene (*cpe*) was indicated by an amplicon band at 233 base pair, and the beta2 toxin gene (*cpb2*) was indicated by an amplicon band at 567 base pair.

ATCC and NCTC reference strains served as positive controls for the multiplex PCR procedure for each *C. perfringens* bio-type (figure 7). Additional ATCC and NCTC strains were included based on previous reports identifying them as *C. perfringens* strains positive for *cpb2* (i.e., *C. perfringens* ATCC8009, NCTC10719, and NCTC4964). All reference strains were positive for *cpa*, and for the appropriate major lethal toxin gene(s). Although other investigators reported ATCC8009 and NCTC4964 to carry *cpb2* (Garmory et al., 2000), the strains that we received were *cpb2* negative. In addition to the major lethal toxin gene(s), ATCC12917 was positive for *cpe*, the enterotoxin gene. ATCC27324, the type E strain, was positive for *cpe* and *cpb2*. A type C strain, NCTC10719 was also *cpb2* positive.

An H_2O blank was used as a negative control in all PCRs. Additionally, genomic DNA from a strain of *C. sporogenes* and a strain of *C. difficile* from the Anaerobe Laboratory stock culture collection, were tested to verify that other *Clostridium spp*. did not react with the primers used in the multiplex PCR, or any other PCRs that were performed. An image of a representative gel of multiplex PCR results for the genomic DNA from *C. perfringens* isolates from patients with *C. perfringens* gastrointestinal diseases is shown in figure 8.

All three populations of *C. perfringens* isolates tested were classified as type A, based on the presence of *cpa* only and the absence of the other three major lethal toxin genes. The majority of isolates, 61% of the total number of

isolates from all three populations, were positive only for *cpa* and were negative for *cpe* and *cpb2*. The percentage of isolates positive for only *cpa* varied between the three populations as follows: 88% of isolates from fecal samples from healthy human volunteers, 54% from fecal specimens from humans with gastrointestinal diseases, and 75% of the isolates from specimens received from humans with extraintestinal illnesses.

Isolates that were positive for *cpe* and/or *cpb2*, in addition to *cpa* were identified in each of the three populations. In the population of isolates from healthy human volunteers, 10% of isolates were positive for *cpb2* and *cpa*, 1% were positive for *cpe* and *cpa*, and 1% were positive for *cpb2*, *cpe*, and *cpa*. The results from the isolates from healthy volunteers were used as the control group in the statistical analysis of the results obtained from the other two populations of isolates.

Of the isolates from patients with *C. perfringens* related gastrointestinal-diseases, 24% were positive for *cpb2* and *cpa*, and 6% were positive for *cpb2*, *cpe*, and *cpa*. Therefore, 30% of these isolates were *cpb2* positive, which was significantly greater than the number of isolates that were positive for *cpb2* in the population of isolates from healthy volunteers ($p<0.01$). Of note, the number of *cpb2* positive gastrointestinal isolates was significantly higher ($p<0.01$) when compared to the population of extraintestinal disease isolates. A total of 16% of isolates from patients with *C. perfringens* related gastrointestinal-diseases were positive for *cpe* and cpa, in addition to the 6% that were positive for *cpb2*, *cpe*, and *cpa*. Compared to the 2% of isolates that were positive for *cpe* in the

population from healthy human volunteers, the 22% of *cpe* positive isolates was significantly higher ($p<0.01$). The 22% of *cpe* positive isolates in the gastrointestinal-diseases was also significantly higher ($p<0.05$) then the 3% of *cpe* isolates in the extraintestinal population of isolates. The difference in the percentages of isolates that were positive for *cpb2*, *cpe,* and *cpa*, between the patient population with *C. perfringens* related gastrointestinal-disease (6%) versus the population of isolates from healthy volunteers (1%) was also significant ($p<0.01$).

In the population from patients with *C. perfringens* related extraintestinal diseases, 20% of isolates were positive for *cpb2* and *cpa*. Although this was a higher percentage than the 10% of *cpb2* and *cpa* positive isolates in the population from healthy volunteers, it was not found to be a significant difference. Only 5% of the isolates from patients with *C. perfringens* related extraintestinal diseases were *cpe* and *cpa* positive, which was not significantly different from the 1% of *cpe* and *cpa* positive isolates observed in the population of isolates from healthy volunteers. No isolates in the population from *C. perfringens* related extraintestinal diseases were found to carry *cpb2*, *cpe*, and *cpa*.

The various combinations of genes identified in the three populations of *C. perfringens* isolates and the percentage of each combination are shown in table 8. Shown in figure 9 is a comparison of the percentages of the *cpa* positive, *cpa* and *cpb2* positive, and *cpa*, *cpb2* and *cpe* positive isolates from each of the three populations of *C. perfringens* isolates.

Simplex PCR

In approximately 50% of the multiplex PCR reactions performed on the DNA obtained from isolates positive for both *cpe* and *cpb2*, the band for *cpb2* was very faint when visualized using UV light. To confirm the presence of *cpb2* and *cpe* in an individual isolate's DNA, a simplex PCR, using a single set of primers for *cpb2*, was performed. We used the *cpb2* primer set from the multiplex PCR, without any of the other primer sets included in the reaction mixture; this simplex PCR is referred to as cpb2PCR-1 (figure 5 shows the location of the primers in the nucleotide sequence of *cpb2*). All isolates that were determined to be *cpb2* positive by multiplex PCR were confirmed as *cpb2* positive using cpb2PCR-1 (table 9). Isolates that were positive for *cpe*, but not *cpb2* by the multiplex PCR were also tested for the presence of *cpb2* by cpb2PCR-1, but none were found to be *cpb2* positive.

Two other simplex PCR reactions with primer sets designed to amplify different regions of *cpb2* were also performed. The purpose of these simplex PCR reactions was to further characterize the *cpb2* genes *C. perfringens* isolates from the three different populations that were positive for *cpb2* in the multiplex PCR. Herholz et al. (1999) described a set of primers for *cpb2* that amplified a 574 base pair region of *cpb2*, but which was 185 base pair further upstream of the region amplified by the primer set for *cpb2* used in the multiplex PCR and cpb2PCR-1; these primers were used in the simplex PCR cpb2PCR-2 (figure 5). Some isolates that were *cpb2* positive by cpb2PCR-1 did not produced a band to

indicate that the 574 base pair fragment of *cpb2* was amplified in the cpb2PCR-2 assay. In all three populations of *C. perfringens* isolates, 71 of the 320 isolates were *cpb2* positive by the multiplex PCR and confirmed using cpb2PCR-1. However, only 19 of the 71 *cpb2* positive *C. perfringens* isolates were positive for cpb2PCR-2. The highest percentage of positive cpb2PCR-2 isolates was in the population of isolates from healthy human volunteers, in which 9 of the 11 isolates (82%) were positive by cpb2PCR-2 (table 9). In the population of isolates from humans with *C. perfringens* gastrointestinal diseases, only 9 of the 48 (19%) *cpb2* positive isolates were positive by cpb2PCR-2; and only 1 of 12 (8%) isolates in the population from patients with extraintestinal *C. perfringens* diseases was cpb2PCR-2 positive (table 9).

A third simplex PCR used a set of primers designed by this laboratory to amplify the entire coding region of *cpb2* based on the *cpb2* sequence described by Shimizu et al. (2001; 2002a) from *C. perfringens* strain 13 (accession number AP003515), which is referred to here as CP13-*cpb2* (figure 5). This third simplex PCR is referred to as cpb2PCR-3. Only 5 of the 11 (45%) *cpb2* positive *C. perfringens* isolates from the population of healthy volunteers were positive using cpb2PCR-3 (table 9). None of the 48 *cpb2* positive *C. perfringens* isolates from patients with gastrointestinal diseases or the 12 *cpb2* positive isolates from patients with extraintestinal illnesses were positive by cpb2PCR-3.

Seventy-one *C. perfringens* isolates from all three populations of isolates were determined to be *cpb2* positive using the multiplex PCR, and confirmed as *cpb2* positive by cpb2PCR-1. However, only 19 of those isolates were positive

using cpb2PCR-2, and only 5 of those isolates were positive using cpb2PCR-2. This strongly suggested to us that there was some genetic variability between the *cpb2* genes carried by the *C. perfringens* isolates in our three populations.

Triplex PCR

Jost et al. (2005) designed a triplex PCR to separate *cpb2* identified in different isolates of *C. perfringens* into two groups, which they termed "consensus" or "atypical". We also determined that there were different versions of *cpb2* using DNA sequencing techniques. In the *cpb2* positive isolates of *C. perfringens* from our three populations, we found the *cpb2* version that Jost et al. (2005) termed atypical to be more common then their consensus *cpb2*. Therefore, our designation of group-2 *cpb2* corresponds to the atypical *cpb2* version described by Jost et al., and the designation group-1 *cpb2* corresponds to the consensus *cpb2* version described by Jost et al. (2005). We chose to use the triplex PCR to provide information to supplement the DNA sequencing results, which are described in the following section.

In the triplex PCR, a band of 304 base pair indicated that the *cpb2* was part of the group-1 *cpb2*, and a band at 741 base pair identified a *cpb2* gene as belonging to the group-2 *cpb2*. Both group-1 and group-2 *cpb2* were identified in all three of our study populations of *C. perfringens* isolates. The highest

percentage of group-1 *cpb2* was identified in the *C. perfringens* isolates from healthy human volunteers, with 5 of the 11 (46%) *cpb2* positive isolates positive for only group-1 *cpb2*, and 2 of the 11 (18%) positive for group-1 and group-2 *cpb2* genes. Four of the eleven (36%) *cpb2* positive isolates from the healthy human volunteers were positive for only group-2 *cpb2*. The majority of isolates from patients with *C. perfringens* related gastrointestinal disease carried only the group-2 *cpb2* gene; a total of 35 of the 48 (73%) *cpb2* positive isolates. In the remaining *cpb2* positive isolates from the population of patients with *C. perfringens* related gastrointestinal diseases, 5 of the 48 (10%) *cpb2* positive isolates carried only group-1 *cpb2*, and 8 of the 48 (17%) *cpb2* positive isolates carried both group-1 and group-2 *cpb2*. In the population of isolates from *C. perfringens* related extraintestinal disease patients, the majority of isolates were group-2 *cpb2*, with 9 of the 12 (75%) *cpb2* positive isolates positive for only group-2 *cpb2*. In the remaining isolates from the population from *C. perfringens* related extraintestinal diseases, only 1 of the 12 (8%) *cpb2* positive isolates was positive for only group-1 *cpb2*, and 2 of the 12 (17%) were positive for both group-1 and group-2 *cpb2* genes.

The percentages of *cpb2* isolates that were positive for group-1 *cpb2*, or group-2 *cpb2*, or both group-1 and group-2 *cpb2* as determined by the triplex PCR from all three populations of isolates are shown in table 10.

Nucleotide Sequences and Amino Acid Sequences Prediction

The sequence of the *cpb2* gene was published when the beta2 toxin was first described (accession number L77965, Gibert et al., 1997). This sequence was determined from a single isolate of *C. perfringens* CWC245, which is a type C strain isolated from a piglet with necrotic enteritis. This nucleotide sequence described by Gibert et al. (1997) will be referred to as CWC245-*cpb2* from this point forward. In 2001, Shimizu et al. published the entire genome of *C. perfringens* strain 13 (CP13), which was found to carry *cpb2* (accession number AP003515) on the plasmid. The *cpb2* sequence described by Shimizu et al. (2002a) will be termed CP13-*cpb2* from this point forward. An alignment of the sequences with ClustalW (Chenna et al., 2003) comparing CP13-*cpb2* to CWC245-*cpb2* found 95% identity between the two nucleotide sequences (table 11) within the open reading frame (ORF), and a 91% identity at the amino acid sequence level (table 12).

We were able to sequence 80 fragments of *cpb2* from 70 *cpb2* positive isolates from all three populations of isolates, using PCR products obtained from reactions with different primer sets (figure 5). Of all the *cpb2* positive *C. perfringens* isolates, only one did not have any portion of its *cpb2* sequenced. The complete 798 base pair *cpb2* coding sequence was determined from 7 of the 70 *cpb2* positive isolates that were sequenced from our three populations. The remaining *cpb2* sequences ranged in completeness from 54% to 96% of the total 798 base pair *cpb2* coding sequence. The number of bases or amino acids (for

the deduced protein sequences) for the *cpb2* nucleotide sequences are shown in tables 13 - 20, which detail the nucleotide sequence alignment scores that indicate the percentage of identity for each sequence.

Variations in the nucleotide sequence fragments of *cpb2* were found in *cpb2* positive *C. perfringens* isolates from the three populations of human sources, as well as in the *cpb2* positive reference isolates. The *cpb2* sequences were assigned to one of three groups based on their similarity to the published sequences CWC245-*cpb2* and CP13-*cpb2* and to other *cpb2* sequences within the group. Similarity was determined based on the ClustalW pairwise alignment scores, which was used to determine the percentage of identity between sequences. Based on nucleotide sequencing results, at least ten *cpb2* positive isolates, in addition to the type E reference strain ATCC 27324, were found to carry two versions of *cpb2*.

The complete *cpb2* sequence from NCTC 10719 had 98% identity with CWC245-*cpb2* and 95% identity with CP13-*cpb2* at the nucleotide level, and 96% identity with CWC245-*cpb2* and 93% identity with CP13-*cpb2* at the amino acid sequence level. Two versions of *cpb2* were identified for ATCC 27324, a type E reference strain. The first *cpb2* sequence of ATCC 27324 had 88% identity at the nucleotide level with CWC245-*cpb2* and 85% identity to CP13-*cpb2*, and at the amino acid sequence level had 86% identity with CWC245-*cpb2* and 81% identity with CP13-*cpb2*. The second *cpb2* sequence from ATCC 27324 had 73% identity to both CWC245-*cpb2* and CP13-*cpb2* at the nucleotide level and 62% identity to CWC245-*cpb2* and 63% identity with CP13-*cpb2* at the

amino acid sequence level. Alignment scores, representing the percentage of identity for the nucleotide sequences of *cpb2* from both reference isolates are shown in table 13, and scores for the amino acid sequences are in table 14.

Sixty of the eighty *cpb2* sequences differed from CWC245-*cpb2* by 24% to 36% in the ClustalW pairwise alignment scores, and from CP13-*cpb2* by 23% to 34% at the nucleotide level. However, these sequences aligned much more closely between each other, differing in their identity by 1% to 13% in the alignment scores at the nucleotide level. These sequences were identified as belonging to the group-2 *cpb2* sequences, and a consensus sequence based on the ClustalW alignment of the 58 *cpb2* sequences was derived. At the nucleotide level, the group-2 consensus sequence aligned with CWC245-*cpb2* and CP13-*cpb2* by 71% identity (table 11), but at the protein level by 63% and 64% identity, respectively (table 12). The complete nucleotide sequence of the group-2 *cpb2* consensus sequence is shown in alignment with CWC245-*cpb2* and CP13-*cpb2* in figure 10. The deduced protein sequence of the group-2 consensus sequence is shown in alignment with the protein sequences of CWC245-*cpb2* and CP13-*cpb2* in figure 11.

A subset of the group-2 *cpb2* sequences was identified. This subset consisted of four *cpb2* positive *C. perfringens* isolates, two from the population of patients with *C. perfringens* related gastrointestinal diseases and two from the patients with extraintestinal *C. perfringens* related diseases. These group-2 *cpb2* sequences, which we termed group-2T, were different from the group-2 consensus sequence (and the other *cpb2* sequences) in that there was a

deletion of 13 nucleotide base pair, from bases 106 to 119 (figure 10). The deduced amino acid sequence derived from this group-2T *cpb2* sequence with a 13 base pair deletion results in a premature stop codon, producing a peptide of only 26 amino acids in length instead of the 265 amino acid peptide produced by the *cpb2* sequences that do not have this 13 base pair deletion.

Group-1 *cpb2* sequences were identified in 13 *C. perfringens* isolates from the population of healthy human volunteers and humans with gastrointestinal disease. The group-1 *cpb2* sequences were similar at the nucleotide level to CWC245-*cpb2* between 85% and 97% identity, and with CP13-*cpb2* between 91% and 99% identity (table 15). At the protein level, group-1 *cpb2* sequences were similar to CWC245-*cpb2* between 78% and 91% identity, and with CP13-*cpb2* between 86% and 100% identity (table 16). A total of 58 *cpb2* sequences from *C. perfringens* isolates from all three populations of human sources belong to group-2, and had identity at the nucleotide level with CWC245-*cpb2* between 64% and 76%, and identity with CP13-*cpb2* between 66% and 77% (table 17). The deduced protein sequence for group-2 *cpb2* sequences were similar with CWC245-*cpb2* between 56% and 70% identity, and with CP13-*cpb2* between 57% and 71% identity (table 18).

The final grouping of *cpb2* sequences, group-3, is composed of six sequences; one from a *C. perfringens* isolate from a healthy human volunteer and the remaining five from humans with gastrointestinal diseases. Basic local alignment search tools (BLAST) analysis of the group-3 sequences determined that the closest genetic match was *cpb2* from the published sequences

CWC245-*cpb2* and CP13-*cpb2*, suggesting that these are variations of *cpb2* and not random PCR amplification of another gene. The six sequences in this group had less then 84% identity at the nucleotide level with the published sequences CWC245-*cpb2* and CP13-*cpb2*, and less then 78% identity at the protein level. Identity with the consensus sequence derived from group-2 *cpb2* sequences was lower, between 53% and 64% identity at the nucleotide level and between 47% and 61% identity at the protein level. Among group-3 *cpb2* sequences identity ranged from 62% to 96% at the nucleotide level, and from 53% to 96% identity at the protein level. Results for group-3 *cpb2* nucleotide sequences are shown in table 19, and protein sequences shown in table 20.

Ten *cpb2* positive isolates were found to carry two versions of *cpb2* using nucleotide sequencing techniques. These isolates were from the *C. perfringens* population from healthy volunteers and from the population with gastrointestinal diseases, in addition to the type E reference strain ATCC 27324. ATCC 27324, the *C. perfringens* type E reference strain, was found to carry both a group-1 and a group-2 version of *cpb2*. Of the ten *C. perfringens* isolates from our three populations that were found to carry two versions of *cpb2*, 6 carried group-1 and group-2 versions, 2 carried a group-1 and a group-2T version, and 2 carried a group-2 and a group-3 version (table 21).

Phenotype Results Determined by Western Blotting

Previous studies by Bueschel et al. (2003) and Waters et al. (2003) found variability in the phenotypic expression of beta2 toxin by *cpb2* positive isolates of *C. perfringens* from animal sources. Some isolates produced detectable amounts of toxin, while others did not produce detectable toxin. These investigations also found that the beta2 protein is secreted in the culture supernatant, and is not part of the cell content released upon cell lysis. To detect the beta2 protein in the cell culture supernatant of *cpb2* positive *C. perfringens* isolates from our three populations, we used Western blotting with a polyclonal anti-beta2 antibody that was kindly provided by Dr. M. R. Popoff. Images of representative Western blots are shown in figure 12; an immunoreactive signal at 28 kDa correlates to the expected size of the secreted form of the beta2 protein toxin. Due to a limited supply, Western blotting using a monoclonal anti-beta2 antibody was not performed on the culture supernatant fluid from *cpb2* positive *C. perfringens* isolates.

The percentage of *cpb2* positive *C. perfringens* isolates that expressed a detectable level of the beta2 toxin varied among the three populations. The highest percentage of isolates that expressed the beta2 protein toxin was from the population of humans with gastrointestinal disease, with 75% (36 of 48) of isolates producing a detectable level of beta2 protein toxin. In the population of isolates from healthy human volunteers, 55% (6 of 11) expressed a detectable level of the beta2 protein. Detectable levels of the beta2 protein toxin was

observed in only 42% (5 of 12) of the *C. perfringens* isolates from extraintestinal specimens.

We also observed variation in the production of a detectable level of the beta2 toxin by *C. perfringens* isolates that carried group-1, group-2, or group-3 versions of *cpb2*. Of the six *C. perfringens* isolates with only group-1 *cpb2*, only one was positive for the beta2 protein toxin (reference strain NTC 10719). In the group-2 *cpb2* positive *C. perfringens* isolates, 80% (33 of 41) were identified as producing the beta2 protein toxin by Western blotting with the polyclonal anti-beta2 antibody. A high percentage, 71% (10 of 14), of *C. perfringens* isolates carrying both group-1 and group-2 *cpb2* also produced the beta2 protein toxin. None of the four isolates that carried a group-2T *cpb2*, or the two isolates that also carried a group-1 *cpb2*, were found to produce the beta2 protein toxin. Of the group-3 *cpb2* isolates, 50% (2 of 4) were found to produce the beta2 protein toxin. One of the two *cpb2* positive *C. perfringens* isolates with both group-2 and group-3 *cpb2* was also found to produce a detectable level of the beta2 protein toxin. Table 21 displays the Western blotting results using the polyclonal anti-beta2 antibody, for isolates from each of the human populations of *C. perfringens* isolates divided into *cpb2* groupings, as well as results for the *cpb2* positive reference strains NCTC 10719 and ATCC 27324.

Recombinant *E. coli cpb2* Clones

We were able to successfully construct 18 recombinant *cpb2*-pRSET plasmids (table 22). A map of the plasmid, with a pRSETB vector and a group-2 *cpb2* insert is shown in figure 6. Each plasmid was initially selected by size, approximately 3.6 kilo base pair, which include the 2.9 kilo base pair size pRSET vector plus the 707 base pair *cpb2* insert. Subsequent testing was performed for each plasmid constructed to verify that a *cpb2* gene was inserted into the pRSET vector, and that it was in the correct orientation and reading frame. The simplex PCR, cpb2-PCR1, was performed for every plasmid constructed to verify the presence of a *cpb2* gene insert for each plasmid selected. Finally, nucleotide sequencing was performed for each plasmid by the Indiana University School of Medicine's Biochemistry Biotechnology Facility. This was to ensure that the *cpb2* insert was in the correct orientation and the correct reading frame.

One plasmid was constructed from *C. perfringens* type C reference strain NCTC 10719, which carried a *cpb2* gene that was similar by 99% to the CWC245-*cpb2* published *cpb2* sequence; this plasmid is designated as pHRD101. Four plasmids were constructed using *cpb2* from *C. perfringens* isolates from the population of healthy human volunteers, twelve plasmids were from isolates from the population with gastrointestinal disease, and one plasmid was constructed from an isolate of *C. perfringens* from a patient specimen from an extraintestinal disease source. Seven plasmids were constructed with *cpb2* from *C. perfringens* isolates that also carried *cpe*. Different versions of *cpb2*,

from each of the different groups were represented in the plasmids constructed. Three plasmids were constructed using group-1 *cpb2*; these are designated as plasmids pHRD102, pHRD103, and pHRD104. Ten plasmids were constructed with group-2 *cpb2*; these are the plasmids designated as pHRD201 to pHRD210. Two plasmids contained group-3 *cpb2* and were designated as pHRD301 and pHRD302. The remaining two plasmids had group-2T *cpb2* inserts, and were designated as pHRD401 and pHRD402.

After the successful construction of the plasmids containing *cpb2*, these plasmids were used to transform BL21(DE3)pLysS *E. coli* for expression of the recombinant beta2 protein. Additionally, a negative control was constructed using an *E. coli* transformed with an empty plasmid that consisted of a pRSETA vector without a *cpb2* insert. The pRSET plus *cpb2* plasmids also contained resistance genes to chloramphenicol and ampicillin, allowing for the selection of successfully transformed *E. coli*. Transformation was achieved for all 18 plasmids constructed.

Two separate Western blotting assays using the monoclonal anti-beta2 antibody and the polyclonal anti-beta2 antibody were performed on the culture supernatant fluid from each recombinant *cpb2 E. coli* clone (table 23). To verify production of a detectable level of the beta2 protein toxin, the Western blotting assays using the polyclonal anti-beta2 antibody was performed. This was the same Western blotting assay that was used to determine if the beta2 protein toxin was produced by *cpb2* positive *C. perfringens* isolates. We also chose to use our limited supply of the monoclonal anti-beta2 antibody for Western blotting

assays on cell culture supernatant on the recombinant *cpb2 E. coli* to help further characterize the recombinant beta2 protein toxins. With the monoclonal anti-beta2 antibody Western blotting assay, eleven of the eighteen recombinant *cpb2 E. coli* clones were positive for beta2 expression.

Activity of Recombinant Beta2 Protein

After expression of the recombinant beta2 toxin was induced by treatment with isopropyl-β-D-thiogalactopyranosid (IPTG), the culture supernatant fluid was collected and the proteins concentrated. The total amount of protein for the culture supernatant fluid from each clone was determined, and dilutions made to obtain final concentrations of 100 μg/μl, 50 μg/μl, and 12.5 μg/μl for use in both cell cytotoxicity assays. For all cell cytotoxicity assays, there was a negative control used, which was the cell culture supernatant from the *E. coli* that was transformed with the empty plasmid that consisted of the pRSETA vector, without a *cpb2* insert.

Trypan Blue Exclusion Assays

Results from the trypan blue exclusion caco-2 cytotoxicity assays demonstrate that the number of cells damaged in each assay is dependent on the recombinant *cpb2* protein used, the concentration of the recombinant *cpb2* protein, and the length of time the recombinant protein is exposed to the caco-2 cells. Representative images from clone pHRD203 of the trypan blue stained caco-2 cell images collected by digital photo microscopy and used to count cells to determine the percentage of non-viable caco-2 cells are shown in figure 13. The percentage of non-viable caco-2 cells counted in the digital images taken for each cell culture well, were plotted on a bar graph to determine if the recombinant beta2 protein caused an increase in the percentage of non-viable caco-2 cells, compared to the negative control. The cell culture supernatant from an *E. coli* transformed with an empty plasmid (a pRSETA vector without a *cpb2* insert) and grown and treated the same as the experimental *E. coli* that were transformed with the recombinant *cpb2* plasmids, served as the negative control in these experiments. The highest percentage of caco-2 cell damage that was observed with exposure to the cell culture supernatant fluid from the negative control is indicated on the bar graphs as the control upper limit. This control upper limit is included on all of the trypan blue exclusion assay bar graphs to allow for comparison to the negative control. The complete results of the negative control, labeled as BL21, are shown in figure 14.

The culture supernatant fluid containing recombinant beta2 protein from the pHRD101 *E. coli* clone caused an increase in the percentage of non-viable of caco-2 cells when the time of exposure was extended to 22 hours for all concentrations assayed (figure 14), but most notably at the highest concentration, 100 μg/μl. A slight increase in the percentage of non-viable caco-2 cells was also observed after 8 hours of exposure, but more then 60% of the caco-2 cells remained viable (figure 14) for each concentration of culture supernatant fluid recombinant beta2 protein from the pHRD101 clone.

The recombinant *cpb2* clones pHRD102, pHRD103, and pHRD104 with group-1 *cpb2* genes inserted into the plasmid, all caused an increase in the percentage of non-viable caco-2 cells. Clone pHRD102 increased the number of non-viable caco-2 cells from 20% to 99% during the 2 to 8 hours of incubation with the culture supernatant fluid proteins at a 100 μg/μl concentration (figure 15). Concentrations of 50 μg/μl and 12.5 μg/μl also caused an increase in non-viable cells after 5 to 8 hours of incubation (figure 15) though to a lesser degree than 100 μg/μl. An effect similar to clone pHRD102 was observed with the culture supernatant fluid from clone pHRD103, though with a higher percentage of non-viable cells for each concentration and length of time (figure 15). The remaining group-1 *cpb2* clone, pHRD104, caused an increase in non-viable cells between 44% to 98% after 5 to 8 hours of incubation for concentrations of 100 μg/μl and 50 μg/μl (figure 15). After 5 hours of incubation with culture supernatant fluid proteins from pHRD104, the highest percentage of non-viable cells was observed for the 12.5 μg/μl concentration of culture supernatant fluid

proteins, almost twice the number of non-viable cells observed with concentrations 50 μg/μl and 100 μg/μl (figure 15).

Culture supernatant fluid proteins from the ten group-2 *cpb2* clones were also tested using the trypan blue exclusion assay for their effect on caco-2 cells. Five of the ten group-2 clones: pHRD201, pHRD205, pHRD206, pHRD207, and pHRD208, had a negligible effect on the caco-2 cells (figure 16). The only increase above the control maximum was for pHRD205; at a concentration of 100 μg/μl, after 8 hours of incubation 29% of the caco-2 cells were non-viable (figure 16). The remaining five group-2 *cpb2* clones: pHRD202, pHRD203, pHRD204, pHRD209, and pHRD210, all had a more discernable effect on the caco-2 cells, though to varying degrees. The highest and most rapid effect observed was from clone pHRD203; culture supernatant from this clone killed 99% of caco-2 cells within 2 hours at both concentrations of 100 μg/μl and 50 μg/μl (figure 17). At a concentration of 12.5 μg/μl, culture supernatant fluid from clone pHRD203 had a minimal effect after 2 and 5 hours of incubation; but 99% of caco-2 cells were non-viable after 8 hours (figure 17). The culture supernatant fluid from clone pHRD202 had no clear effect after 2 hours at any concentration, but at 5 hours between 46% to 60% of cells were non-viable for the three concentrations, and after 8 hours between 75% and 88% of caco-2 cells were non-viable for all concentrations (figure 17). Although there was a minimal effect on the cells for culture supernatant fluid from pHRD205 at 2 and 5 hours, at 8 hours 98% of the caco-2 cells were killed at the 100μg/μl concentration (figure 17). Results for pHRD205 at concentrations of 50 μg/μl and 12.5 μg/μl were

difficult to understand; at both concentrations greater than 94% of caco-2 cells were non-viable after 2 hours, but only a minimal effect was observed at 5 hours and 8 hours (figure 17). Clone pHRD209 caused an increase in the percentage of non-viable caco-2 cells that increased with time and concentration; though the greatest effect was only 46% of non-viable cells after 8 hours for 100 μg/μl (figure 17). The remaining group-2 *cpb2* clone, pHRD210 showed a correlation between time and concentration after 8 hours, with 62% of caco-2 cells non-viable for 100 μg/μl (figure 17). However, at 2 and 5 hours the percentage of non-viable caco-2 cells was highest for the 50 μg/μl concentration of pHRD210 culture supernatant fluid, followed by the 12.5 μg/μl concentration, with the lowest effect observed for 100 μg/μl (figure 17).

The group-3 *cpb2*, is the group to which six *cpb2* sequences were assigned when they were found to have, at the nucleotide level, less then 84% identity to the published *cpb2* sequences and less then 64% with the group-2 *cpb2* consensus sequences. Identity within the group was also variable, between 62% to 96% identity at the nucleotide level, which did not allow for any reasonable consensus sequence to be derived. Two of the *cpb2* genes from this varied group of *cpb2* were randomly selected, and recombinant plasmids were constructed. The culture supernatant fluid from each of these group-3 clones was tested for an effect on caco-2 cells. At a concentration of 100 μg/μl, pHRD301 caused a gradual increase in the percentage of non-viable caco-2 cells, from 11% to 19% between 2 and 8 hours (figure 18). The opposite effect was observed for pHRD301 culture supernatant fluid at a concentration of 50

μg/μl, in which the percentage of non-viable cells decreased from 34% at 2 hours, to 6% at 8 hours (figure 18). At the lowest concentration, 12.5 μg/μl, pHRD301 had a minimal effect until after 8 hours, at which point 30% of caco-2 cells were non-viable (figure 18). Essentially no effect was observed for pHRD302, though a slight increase in the percentage of non-viable caco-2 cells above the control maximum was observed at 5 hours only for concentrations of 50 μg/μl (12%) and 12.5 μg/μl (14%) (figure 18).

Two clones with group-2T *cpb2* were tested for their effects on caco-2 cells measured by trypan blue exclusion. These clones were expected to have activity similar to that observed for the group-2 *cpb2* clones, because the region of the *cpb2* gene that was selected for insertion into the vector used for cloning was after the 13 base pair deletion observed in the group-2T *cpb2* nucleotide sequences. For clone pHRD401, only a slight increase in the percentage of non-viable caco-2 cells was observed at a concentration of 100 μg/μl after 5 and 8 hours incubation (figure 18). Clone pHRD402 had no effect above the negative control at any concentration or any time point measured in this assay (figure 18).

Lactate Dehydrogenase (LDH) Assay

With a colorimetric chemotoxicity assay kit, the amount of LDH released by caco-2 cells as an indicator of membrane damage was measured after

exposure to culture supernatant fluid from the recombinant *cpb2* clones. Because the assay kit only allowed for a limited number of assays, all assays were measured after an incubation time of 8 hours. Each concentration of culture supernatant fluid was assayed in quadruplicate, and the optical density readings measured by the spectrophotometer averaged and compared to the maximum LDH released by the controls according to the specifications in the protocol provided to determine the percentage of cell damage (% CD) for each concentration of each recombinant culture supernatant fluid. An analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) Post-hoc testing was used to verify statistical significance versus the BL21(DE3)pLysS culture supernatant fluid protein control for each concentration of recombinant beta2 protein, with a confidence level of 95%.

Recombinant beta2 protein in the culture supernatant fluid from pHRD101 increased the percentage of cell damage as compared to the BL21(DE3)pLysS culture supernatant fluid control. At 100 μg/μl and 12.5 μg/μl, the p-value was less than 0.001, and less than 0.05 at the 50 μg/μl concentration (figure 19). The *E. coli* BL21(DE3)pLysS used as a control had a 19% CD, 13% CD, and 12% CD (figure 19), respectively for concentrations of 100 μg/μl, 50 μg/μl, and 12.5 μg/μl of culture supernatant fluid.

For the recombinant *cpb2* clones with group-1 *cpb2* genes: pHRD102, pHRD103, and pHRD104, only pHRD102 had a significant increase in the % CD at all three concentrations (figure 20). Culture supernatant fluid from pHRD104

caused a small increase in the % CD ($p<0.05$) versus the concentration control at 12.5 μg/μl (figure 20), but not at 100 μg/μl or 50 μg/μl.

The culture supernatant fluid proteins from the ten group-2 *cpb2* clones were also tested for an effect on caco-2 cells using the LDH assay. The LDH assays of the group-2 clones differed from the results observed in the trypan blue exclusion assay; however, the presentation of data for the same clones is shown using the same clusters as from the trypan blue exclusion assay results (see figures 16 and 17). Culture supernatant fluid from pHRD201 increased the % CD at 100 μg/μl and 50 μg/μl (figure 21); though not a significant difference within results between the two concentrations, the % CD at 50 μg/μl was higher than at 100 μg/μl. The effect of pHRD205 culture supernatant fluid was observed as an increase in the % CD at 50 μg/μl and 12.5 μg/μl versus the concentration control; however, there was not a significant increase at 100 μg/μl (figure 21). No effect on the % CD was seen for pHRD206 at 100 μg/μl and 50 μg/μl, though a small increase was noted at 12.5 μg/μl compared to the concentration control (figure 21). A more dramatic effect was caused by pHRD207 culture supernatant fluid, with significant rises in the % CD observed for all three concentrations (figure 21). The culture supernatant fluid from pHRD208 did not have any significant effect on the % CD, at any concentration measured (figure 21). Both pHRD202 and pHRD203 significant increased the % CD at all three concentrations, with a noticeable decrease from the highest concentration to the lowest concentration assayed (figure 22). The effect on the % CD of pHRD204 culture supernatant fluid also decreased from the highest concentration to the lowest concentration,

though only the effect measured for 100 μg/μl was significant versus the concentration control (figure 22). The remaining group-2 *cpb2* clones, pHRD209 and pHRD210, had no significant effect on the % CD, except for pHRD209, which was slightly higher than the concentration control at 12.5 μg/μl (figure 22).

Two clones were constructed using group-3 *cpb2*, and culture supernatant fluid from each clone was tested for an effect on caco-2 cells using the LDH assay. At concentrations of 100 μg/μl and 50 μg/μl, pHRD301 significantly increased the % CD versus the concentration controls (figure 23), with a downward trend noted from the highest to the lowest concentration assayed. The culture supernatant fluid from pHRD302 caused a rise in the % CD at 50 μg/μl and 12.5 μg/μl versus the concentration controls (figure 23), though there was not a significant difference between the three concentrations assayed. Neither of the group-2T *cpb2* clones, pHRD401 and pHRD402 had significant effect in the % CD at any of the three concentrations assayed (figure 23).

DISCUSSION

The Beta2 Toxin Gene, *cpb2*, was Identified in the Genome of Type A, *C. perfringens* Isolates from Human Sources

At the inception of this investigation there were studies that had identified *cpb2* and the beta2 toxin in some *C. perfringens* isolates from animals with gastrointestinal diseases. However, there were no published studies regarding the recently described beta2 protein toxin and its possible relevance to *C. perfringens* related diseases in humans. We identified *cpb2* in the genome of *C. perfringens* isolates from humans; this served as an initial step to studying a possible role for the beta2 toxin from *C. perfringens* in gastrointestinal diseases in humans as well as *C. perfringens* extraintestinal diseases.

cpb2 was Found in a Significantly Higher Percentage of *C. perfringens* Isolated from Humans with Gastrointestinal Diseases

The results of this investigation revealed that the percentage of *C. perfringens* isolates from the population of patients with gastrointestinal diseases, which included patients with antibiotic-associated diarrhea (AAD) or food-borne

illness, had a significantly greater number of isolates that were positive for *cpb2* versus the control group (chi square analysis, $p<0.01$). The *C. perfringens* isolates from the population of patients with extraintestinal diseases (specimen sources included blood and other normally sterile body fluids, as well as aspirates from wounds and other soft tissue samples) carried *cpb2* at a greater frequency than the control population. However, this difference was not statistically significant. Because *C. perfringens* is commonly encountered in the normal fecal microbiota of healthy humans (Hentges, 1983), it was important to include a population of *C. perfringens* isolates from fecal samples from healthy human volunteers. This population served as a control group in our study to allow for comparison against *C. perfringens* isolates from humans with gastrointestinal diseases and from humans with extraintestinal diseases. The results of this investigation are in agreement with recently published studies that have also identified *cpb2* positive *C. perfringens* isolated from intestinal sources from humans with gastrointestinal diseases (Fisher et al., 2005; Miyamoto et al., 2006). However, our investigation is unique in that it also includes a comparably sized control population of isolates from healthy humans.

Because isolates can be a distinct type (type A to E), we also analyzed our *C. perfringens* isolates for the four major toxin genes: *cpa*, *cpb*, *etx*, and *iA*, and the enterotoxin gene *cpe*, that are used to type *C. perfringens*. This was in addition to our surveillance for the presence of *cpb2*. All 320 *C. perfringens* isolates from the three populations studied were classified as type A, indicating that the alpha toxin gene *cpa* was the only one of the four major toxin genes

present. These results are in agreement with previous studies that found type A to be the predominant type in the normal fecal microbiota of humans (Hatheway, 1990) and the type most often isolated from patients with *C. perfringens* related gastrointestinal diseases, including AAD (Modi and Wilcox, 2001). Type A strains are also associated with extraintestinal *C. perfringens* related diseases, including clostridial myonecrosis or gas-gangrene (Rood and Cole, 1991).

The Enterotoxin Gene was also Identified in the *C. perfringens* Isolates from All Populations

CPE, the *C. perfringens* enterotoxin, is a 35 kDa protein produced from the *cpe* gene, and it is strongly linked to food-borne *C. perfringens* disease in humans (McClane, 1992; Stark and Duncan, 1971). Further, enterotoxin positive *C. perfringens* have also been linked to non-food-borne gastrointestinal diseases, including AAD (McClane, 1994). There is good evidence that CPE plays a role in food-borne disease, sporadic diarrhea, and AAD; however, the enterotoxin is produced only during sporulation of *C. perfringens* and released upon lysis of the vegetative cell and release of the spore (Czeczulin et al., 1993). Therefore, conditions that will induce sporulation and subsequent lysis of the vegetative cells to release the spore are needed to implicate the *C. perfringens* enterotoxin as a possible cause for associated diseases.

For human fecal isolates, we grouped samples from *C. perfringens* AAD together with the samples from humans with *C. perfringens* food-borne diseases into one population identified as isolates from humans with *C. perfringens* related gastrointestinal diseases. This grouping allowed for more generalized comparisons to the *C. perfringens* isolates from healthy human volunteers and isolates from humans with extraintestinal *C. perfringens* diseases. While the focus of this investigation was not on the enterotoxin *per se*, we note that *cpe* was identified in 47% of the isolates from humans with *C. perfringens* food-borne illness, which was significantly higher than the 19% of *cpe* positive isolates from humans with *C. perfringens* related AAD ($p<0.01$, chi square analysis).

The total number of *cpb2* positive *C. perfringens* isolates from humans with gastrointestinal diseases was significantly higher than *cpb2* isolates from the control population from healthy volunteers. This result was not definitive, however, because isolates from humans with gastrointestinal diseases also had a significantly higher frequency of the enterotoxin gene, *cpe*. The occurrence of both *cpb2* and *cpe* was significantly greater in the population of isolates from humans with gastrointestinal diseases than in isolates from the control population. We did not identify *cpb2* and *cpe* in a single isolate in the *C. perfringens* isolates from humans with extraintestinal diseases.

Although *cpe* was identified in the genome of *C. perfringens* isolates from all three of our study populations, including isolates that were also *cpb2* positive, further study of *cpe* or production of the enterotoxin was beyond the scope of this investigation. A recent study by Fisher et al. (2005) suggested that the beta2

toxin could act as an accessory toxin in AAD caused by enterotoxin positive *C. perfringens* in humans. Future investigations in this area should include both individual and paired studies of *cpb2* and *cpe*. This will be necessary to aid in determining whether these two toxins contribute to *C. perfringens* diseases synergistically or are associated with different disease presentations. Such a study would also be useful for determining if the presence of both genes in a strain can impact the virulence potential of *C. perfringens* and its associated diseases.

Other PCR Assays Produced Results that were used to Further Characterize *cpb2* in the *C. perfringens* Isolates from All Populations

Different PCR techniques, including the multiplex PCR (Garmory et al., 2000) and simplex PCR (Herholz et al., 1999; the simplex cpb2PCR-2), as well as different primer sets were used in this study to amplify various regions of the *cpb2* gene. Of the 71 *cpb2* positive isolates of *C. perfringens*, only 19 were positive when tested with the simplex cpb2PCR-2 assay. Marks and Kather (2003) obtained similar results; they observed that the simplex cpb2PCR-2 assay produced fewer amplified *cpb2* bands from isolates than the multiplex PCR. This observation could be explained by differences in the primer sets used in the different PCR assays. For the simplex cpb2PCR-2, the primer set is for a 574

base pair region (bases 143 to 716), while the multiplex PCR primer set is for a 546 base pair region (bases 258 to 803, figure 5). The primer sets from these two PCR assays differ from the original published sequences (CWC245-*cpb2* and CP13-*cpb2*) by just two bases. For the multiplex PCR (which used the same *cpb2* primers that were used in the simplex PCR, cpb2PCR-1), there was one base difference in each of the forward and reverse primers. In contrast, for the simplex cpb2PCR-2, the sequences for the forward primer were identical, but two bases were different in the reverse primer (figure 5). The observation of fewer isolates as *cpb2* positive when the simplex PCR cpb2PCR-2 was used may be due to the base differences between the primer sequence and the *cpb2* sequences of the *C. perfringens* isolates. This would make primer annealing (and subsequent amplification) less likely.

An additional simplex PCR (cpb2PCR-3) was designed in an effort to amplify the entire coding region of *cpb2*. This new PCR assay used primers based on the CP13-*cpb2* sequence (Shimizu et al., 2001; 2002a). With this primer set, only five *cpb2* positive isolates (all from the population of healthy human volunteers) had *cpb2* that produced a band indicating amplification of *cpb2*. Results from these three PCR assays, the multiplex PCR (Garmory et al., 2000), the simplex cpb2PCR-2 (Herholz et al., 1999), and the simplex cpb2PCR-3, serve to emphasize that *cpb2* is a variable gene that is not highly conserved. The genetic variability of *cpb2* is important because this characteristic makes it unusual if not unique among the toxin genes of *C. perfringens*.

In addition to the multiplex PCR and the simplex PCR assays described above, a triplex PCR (Jost et al., 2005) has been used to differentiate *cpb2* into two types, group-1 or group-2 (table 24). Jost et al. (2005) designated the two types of *cpb2* as either "atypical" or "consensus". We chose to place our isolates in three groups based on the *cpb2* type that was found in an isolate. Our group-2 *cpb2* correlates with the "atypical" sequence described by Jost et al. (2005); however, the group-2 *cpb2* was more common in our work and therefore was not "atypical" (table 10). The "consensus" sequence referred to by Jost et al. (2005) correlates with our group-1 *cpb2*. There were no sequences described by Jost et al. (2005) that correlated with our group-3 *cpb2*. Group-2 *cpb2* was the version identified in the majority of *cpb2* positive *C. perfringens* isolates and was the most common version in the *C. perfringens* isolates from patients with gastrointestinal diseases (in 73% of isolates). Group-1 *cpb2* was identified in 46% of *C. perfringens* isolates from healthy human volunteers, although it was also observed in the other two populations of *C. perfringens* isolates. Additionally, we identified 12 *C. perfringens* isolates that were positive for both group-1 and group-2 *cpb2*, a phenomenon that was not described by Jost et al. (2005) and has not been described by others. This suggests that some strains of *C. perfringens* carry multiple versions of *cpb2*.

When we progressed to the next phase of our research involving the nucleotide sequencing of the *cpb2* genes in our *C. perfringens* isolates (described in the subsequent section), a number of flaws were noted with the triplex PCR as it applies to differentiation of *cpb2* into two groups. The

nucleotide sequencing results revealed multiple variations of *cpb2* and not simply two versions. The triplex PCR incorrectly identified four sequences as group-1 *cpb2* that had at most 79% identity to the CP13-*cpb2*. The nucleotide sequencing results indicated that these belonged to different groupings. Additionally, it was found that there was a subset of the group-2 *cpb2* that the triplex PCR cannot distinguish.

A Range of Genetic Variation was Identified in the *cpb2* Genes from the *C. perfringens* Isolates at the Nucleotide Level and in the Predicted Amino Acid Sequences

Variation in the *cpb2* genes and in the predicted protein sequence of the beta2 toxin could explain differences in virulence between isolates as well as differences in diseases and disease severity between different isolates of *C. perfringens*. Other investigators have demonstrated that sequences of most *C. perfringens* toxin genes tend to be highly conserved, with variants having different amino acids at only two or three positions (Hunter et al., 1992; Hunter et al., 1993; Sheedy et al., 2004). Given the expected conservative nature of *C. perfringens* toxin gene sequences, it was surprising to us that the two published *cpb2* sequences (Gibert et al., 1997; Shimizu et al., 2002a) aligned with only 95% identity at the nucleotide sequence level and with 91% identity at the amino

acid level (with 21 different amino acids out of the 265 amino acid sequence). Additional information was found in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html); there were *cpb2* sequences that were identified by the submitters as pseudogenes (accession numbers AJ537533-AJ537551), but these sequences were not described in the published literature. In order to develop a better understanding of the apparent variability in *cpb2* sequences, we performed nucleotide sequencing of portions of *cpb2* from *C. perfringens* isolates from all three populations.

Using PCR techniques with multiple primer sets, Jost et al. (2005) and Fisher et al. (2005) determined that there were genetic variants of *cpb2* and subdivided *cpb2* positive *C. perfringens* isolates into two groups. However, the nucleotide sequencing results from a total of 80 *cpb2* sequences in our study populations revealed a much wider range of variation in the nucleotide sequences of *cpb2*. All *cpb2* positive *C. perfringens* had at least a portion of *cpb2* sequenced (with one exception due to technical difficulties), and ten isolates had portions of two distinct versions of *cpb2* sequenced. In the *C. perfringens cpb2* positive isolates from all three study populations, we identified two distinct groups (group-1 and group-2) and one subgroup (group-2T) of *cpb2* sequences. A third group (group-3) was designated to identify *cpb2* sequences that were distinct from the other groups, but these sequences were also distinct from other sequences within the group. The *cpb2* sequences were assigned to different groupings to allow for comparison of the different versions of *cpb2* and their association with isolates from the different *C. perfringens* populations.

Many of the *C. perfringens* Isolates from Humans with Gastrointestinal Diseases Carried the Group-2 *cpb2*

Group-2 *cpb2* was the genetic variant most commonly found in *C. perfringens* isolates from humans with gastrointestinal diseases (including those associated with AAD and food-borne diseases), as well as in the control population and the population of *C. perfringens* isolates from humans with extraintestinal diseases. It is interesting to note that group-2 *cpb2* is more than 25% different in sequence identity compared to the original published sequences: CP13-*cpb2* and CWC245-*cpb2*. Also of note was a subset of sequences that had very high identity to the other group-2 sequences, but the sequences in this group-2T subset of group-2 had a 13 base pair deletion in the first 120 base pair of the ORF. This deletion results in a premature stop codon and, if expressed, a truncated form of the beta2 protein.

The *cpb2* positive *C. perfringens* isolates also contained a number of other variants of *cpb2*. Genes classified as group-1 *cpb2* were most similar to the published sequence CP13-*cpb2*. The group-1 *cpb2* includes 13 sequences that were similar by an average of 97% identity to the published sequence CP13-*cpb2* from a human type A strain (Shimizu et al., 2002a). The group-1 *cpb2* sequences were identified in *C. perfringens* isolates from all three populations. However, 17 of the 22 total group-1 *cpb2* were identified in *C. perfringens* isolates that also carried a group-2 or group2T *cpb2*.

The six *cpb2* group-3 sequences had similarity of less then 70% identity with the published sequences (CP13-*cpb2* and CWC245-*cpb2*) and the group-2 consensus sequence (that was derived from the 58 group-2 *cpb2* and the 4 group-2T *cpb2*). Five of these sequences were from *C. perfringens* isolates from patients with gastrointestinal diseases, and the remaining sequence was from an isolate from a healthy human volunteer. This grouping was based more on the observed differences from the published sequences and the group-2 consensus than on identity with other *cpb2* sequences within the grouping. Two of these six sequences were found in *C. perfringens* isolates that also carried a group-2 *cpb2*.

Many of the *C. perfringens* Isolates from Humans Carried Two Distinct Variants of *cpb2*

Nucleotide sequencing results, combined with results from the triplex PCR, provided evidence that 27% (19 of 71) of the *cpb2* positive *C. perfringens* isolates carried two distinct versions of *cpb2*. Six isolates that were shown to carry either a single group-1 or group-2 *cpb2* by triplex PCR were shown by nucleotide sequencing to carry multiple versions of *cpb2*. Two of these six isolates carried a group-2 *cpb2* and a group-3 *cpb2*; the remaining four carried a group-1 and group-2 *cpb2*. As shown by other researchers (Jost et al., 2005;

Fisher et al., 2005) and our results, there are multiple genetic variations of *cpb2*. However, our results also provide evidence that an isolate of *C. perfringens* can carry multiple versions of *cpb2*; other investigators have not described this phenomenon. Studies of the plasmid-borne toxin genes of *C. perfringens*, including *cpb2*, have shown that the toxin genes can be on multiple different plasmids and that a single isolate can carry multiple plasmids (Li et al., 2007; Sayeed et al., 2007). There is also evidence that the plasmids that carry many of the toxin genes of *C. perfringens* are able to undergo conjugative transfer (Brynestad et al., 2001; Fisher et al., 2005; Miyamoto et al., 2002), which would allow for the exchange of genetic material carried by *C. perfringens* strains on distinct plasmids. It appears reasonable that a *C. perfringens* strain could carry multiple different versions of *cpb2*. It is possible that the different versions of *cpb2* are associated with different plasmids, although additional work is needed to provide evidence to support this idea.

The Beta2 Protein was not Expressed by Every *cpb2* Positive *C. perfringens* Isolate from the Different Populations of Isolates

Variation in the expression or level of expression of the beta2 protein toxin is another factor that could explain differences in disease presentation, severity, and virulence of *C. perfringens* strains that cause diseases in humans. While

some investigators found a correlation between the presence of *cpb2* and production of the beta2 toxin by *C. perfringens* isolates from piglets (Waters et al., 2003), our analysis of beta2 toxin expression by *cpb2* positive *C. perfringens* isolates demonstrated that the mere presence of *cpb2* does not necessarily correlate with beta2 toxin production. Isolates positive for beta2 toxin expression were identified in all three populations, and the percentage of *cpb2* positive isolates that expressed beta2 toxin ranged from 42% to 79% for our different study populations (table 21). Isolates that carried only a group-1 *cpb2*, a group-2T *cpb2*, or both a group-1 and a group-2T *cpb2* were all negative for beta2 protein expression. Only three of the six isolates with a group-3 *cpb2* or a group-2 and group-3 *cpb2* produced the beta-2 protein. Other investigators have also described discrepancies in the presence of *cpb2* and production of the beta2 toxin. One such study found that 97% of *cpb2* positive isolates from pigs were positive for beta2 toxin production, while only 50% of isolates from other animal species (e.g. avian, bovine, canine, etc.) produced beta2 toxin (Bueschel et al., 2003). Results from this study demonstrate that the presence of group-2 *cpb2* is more likely to correlate with beta2 protein expression than merely the presence of any *cpb2*. This indicates that simply carrying a *cpb2* gene does not equate with the production of the beta2 toxin by a *C. perfringens* isolate. When considering only group-2 *cpb2* isolates, 79% were positive for beta2 toxin expression, and most of these isolates were from the *C. perfringens* collected from the fecal samples of humans with gastrointestinal diseases (including AAD and food-borne illness isolates).

The Group-2 *cpb2* Found in the Majority of *cpb2* Positive *C. perfringens* Isolates from Humans with Gastrointestinal Diseases were Most Likely to Produce the Beta2 Protein

The group-2 *cpb2* sequence was the most common sequence found in all populations in this study, including the control population of healthy human volunteers. The group-2 *cpb2* sequence was also the most common link between *C. perfringens* isolates that produced the beta2 toxin. In isolates that contained group-2 *cpb2* (either alone or in conjunction with a group-1 or group-3 *cpb2*), more than 78% (77 of 98) produced the beta2 toxin. In contrast, none of the isolates containing group-2T were found to produce the beta2 toxin. Of isolates that did not carry either group-2 or group-2T *cpb2*, only 30% (3 of 10) produced the beta2 toxin. These results are consistent with the results of Fisher et al. (2005) in which there was also a strong link between production of the beta2 toxin and the *cpb2*/*cpe* sequence in type A strains of *C. perfringens*. While the findings in the present study as well as those reported by Fisher et al. (2005) provide evidence that the beta2 toxin is a factor in AAD and other gastrointestinal diseases associated with *C. perfringens*, this deduction is not unequivocal because not all *cpb2* isolates in these studies produced the beta2 toxin.

A possible explanation as to why the beta2 toxin was not produced by all group-2 *cpb2* isolates may be related to the *cpb2* nucleotide sequence. It is possible that there were undetected mutations that could result in deletions or frame shift errors; this is a very real possibility for the *cpb2* sequences in the

present study for which the entire nucleotide coding sequence could not be determined. It is possible that some *C. perfringens* isolates that were negative for beta2 toxin production carried a group-2T *cpb2* instead of a complete group-2 *cpb2*, which we were only able to detect using nucleotide sequencing techniques. Other explanations are possible, however, because five isolates with group-2 *cpb2* that were negative for production of the beta2 toxin were definitely not group-2T *cpb2*; these were sequenced from the start codon of *cpb2* and did not have the 13-base pair deletion. Additional genetic factors that influence production of a protein include the presence of promoters within the nucleotide sequence, as well as other non-nucleotide signaling elements that trigger the cell to begin production of a protein. Because our results indicate that simply carrying a viable *cpb2* sequence does not equate with production of beta2 toxin by a *C. perfringens* isolate, further research that focuses on these other factors is needed.

Of the 22 isolates that carried a group-1 *cpb2* sequence, 11 were found by the Western blotting assay to produce the beta2 protein toxin. However, all of these 11 isolates also carried a group-2 *cpb2*. The assay we used did not distinguish between beta2 toxin from group-1 *cpb2* versus beta2 toxin from group-2 *cpb2*. None of the *C. perfringens* isolates from our three populations that carried only a group-1 *cpb2*, only a group-2T *cpb2*, or both group-1 and group-2T *cpb2* were found to produce the beta2 toxin.

The low number *C. perfringens* isolates in each of the groups of *cpb2* (further confounded by the various combinations when two versions of *cpb2* were

present) precluded meaningful statistical comparison of results from Western blotting. However, the results described here suggest that group-2 or possibly group-3 *cpb2* versions are more likely to play a role in *C. perfringens* diseases in humans because isolates with a *cpb2* from either of those groups had a higher percentage of beta2 toxin positive isolates than those isolates with a group-1 *cpb2*. Further study is needed to provide more information on the variations of the *cpb2* gene, as well as which versions are capable of producing a viable beta2 toxin.

There are many possible explanations for why *cpb2* positive isolates did not produce the beta2 protein. Reportedly most toxins of *C. perfringens* are expressed if the gene is present (Meer and Songer, 1997). However, silent genes (or cryptic genes) have been described in *C. perfringens*. Probably the most well-known examples are the type E strains of *C. perfringens* that are *cpe* positive but do not produce the enterotoxin, even under conditions that induce sporulation (Billington et al., 1998). It is also possible that a toxin was produced but not detected using our assay. This possibility seems unlikely, however, because culture supernatant fluid from only *C. perfringens cpb2* positive isolates was tested for the presence of the beta2 protein. This procedure was used following preliminary results that showed that the beta2 protein is secreted and does not remain associated with the vegetative cell. Results of Western blotting analysis by Fisher et al. (2005) and Waters et al. (2003) also support the beta2 toxin as secreted by vegetative *C. perfringens*. Furthermore, all cultures of *C. perfringens* in this study were grown to the log phase to ensure that there was

sufficient time to allow for expression and subsequent secretion of the beta2 toxin.

An additional possibility to explain why the beta2 toxin is not produced at a detectable level by some *C. perfringens cpb2* positive isolates could be in the regulation of expression, which can occur at multiple levels including during transcription and translation. In another investigation on *cpb2*/*cpe* positive *C. perfringens* isolates from humans, a quantitative Western blotting assay revealed that there was a ten-fold range of variation in the level of beta2 toxin produced (Fisher et al., 2005). It has also been shown using techniques to analyze mRNA that levels of the *cpb2* transcript could vary by as much as a 35-fold difference between *C. perfringens* isolates from pigs and horses (Water et al., 2005) and that the level of transcription had a positive correlation with the level of beta2 toxin production (Harrison et al., 2005). This is important because others have described a correlation between the level of toxin produced and the severity of the symptoms of *C. perfringens* diseases (Dupuy and Matamouros, 2006). It is interesting to note that a study describing group-1 and group-2 (table 24) *cpb2* in veterinary and human *C. perfringens* isolates found that only isolates with a group-1 *cpb2* produced a corresponding mRNA transcript and beta2 toxin while isolates with a group-2 *cpb2* did not produce *cpb2* mRNA or beta2 toxin (Jost et al., 2005).

Expression of the beta2 toxin appears to be positively regulated indirectly by VR-RNA (of the VirR/VirS two component regulatory system), with maximal production reached by the late log phase of growth (Ohtani et al., 2003). Current

evidence supports VirR/VirS elements as the primary mode of regulation for many of the virulence genes of *C. perfringens* (Lyristis et al., 1994; Shimizu et al., 1994). At present, the external stimuli that trigger activation of this system (and whether these are environmental or growth phase related) have not been identified for *C. perfringens*. The actual mechanism of gene regulation by VR-RNA has not yet been described, although it does not appear to regulate gene transcription by binding to complementary DNA or RNA of a promoter region (Shimizu et al., 2002b). Additional studies of the VirR protein have shown that it binds independently to two imperfect direct repeats (CCCAGTTNTNCAC) located upstream of the gene promoter (Cheung and Rood, 2000). Additional work has shown that the spatial organization of these repeats is important for transcription of the gene (Cheung et al., 2004). Future studies of the upstream region of *cpb2* in *C. perfringens* isolates are needed to determine if these imperfect direct repeats are at the proper upstream location. If the proper spatial organization is not shown, this could potentially explain the differences observed in production of the beta2 toxin by *cpb2* positive *C. perfringens* isolates. More studies on the mechanism of VR-RNA and the components involved in its mechanism of regulation are also needed. These could help identify additional areas of difference in the upstream and downstream regions of *cpb2* and determine whether these factors could subsequently affect regulation and expression of the beta2 toxin.

The anti-beta2 antibodies that were used in this study are an additional factor that may complicate the understanding of regulation and expression of the

beta2 toxin. The antibodies used were polyclonal anti-beta2 raised in rabbits but against the porcine *cpb2* sequence (Gibert et al., 1997), which was determined to be only 71% similar to the group-2 *cpb2*. Therefore, these antibodies might not have recognized epitopes of the beta2 protein toxin of some of our isolates (even if it was produced). Future studies using antibodies developed specifically against the beta2 toxins produced by the different *cpb2* groups will provide valuable information regarding which version of the beta2 toxin is produced by isolates that carry multiple version of *cpb2*. Such studies would also decrease the probability that epitopes of the beta2 protein toxin of some isolates would fail to be recognized.

E. coli Clones that Expressed Recombinant Beta2 Protein were Constructed and used to Study Potential Differences in the Activity Level of the Beta2 Protein from the Different Variants of *cpb2* on Cultured Caco-2 Cells

To study the different versions of *cpb2* that were discovered, we constructed 18 recombinant *cpb2* plasmids of representatives from each *cpb2* group. These plasmids were used to transform *E. coli*, which then expressed the recombinant beta2 protein. For all clones, including those with group-2T *cpb2*, the region of *cpb2* inserted into the vector began after the signal sequence. The signal sequence is the region of the 13 base pair deletion of group-2T; this

means that the group-2T clones should essentially be the same as the group-2 *cpb2* clones. Other investigators have cloned *cpb2* into *cpb2*/*cpe* negative type A *C. perfringens* strains for over-expression of the beta2 protein toxin (Fisher et al., 2005; Harrison et al., 2005). However, we chose to use an *E. coli* strain (BL21(DE3)pLysS) that was developed for expression of recombinant proteins to minimize the possibility that other *C. perfringens* toxins (such as the alpha toxin) or enzymes might affect the results of the cell cytotoxicity assays.

Expression of the beta2 toxin by each *cpb2 E. coli* clone was verified with the polyclonal anti-beta2 antibody (Gibert et al., 1997) and Western blotting techniques. This was the same anti-beta2 antibody that was used to determine production of the beta2 toxin by the *C. perfringens* isolates from the three different populations. However, when a monoclonal anti-beta2 antibody (Bueschel et al., 2003) was used, only 11 of the 18 recombinant *cpb2 E. coli* clones were positive (table 23). This monoclonal antibody was created against the original beta2 toxin (Gibert et al., 1997) that had the signal sequence removed (Bueschel et al., 2003). There was a limited supply of this monoclonal antibody, so we were unable to use it to assess all of the *C. perfringens* isolates from the three different populations. These results are difficult to interpret because the recombinant clones that were negative in this assay carried *cpb2* from all groups; as a result, we could not determine if the monoclonal anti-beta2 antibody reacted only with beta2 toxin from a specific *cpb2* group. There was no correlation between recombinant *cpb2 E. coli* clones that were negative in the monoclonal anti-beta2 assay and whether the corresponding *C. perfringens*

isolates were positive or negative in the assay that used the polyclonal anti-beta2 antibody. Four clones that were from *C. perfringens* isolates that were negative with the polyclonal anti-beta2 assay had the corresponding *cpb2* clones positive using the monoclonal anti-beta2 antibody. Conversely, three clones that were from *C. perfringens* isolates that were positive in the polyclonal anti-beta2 assay had a corresponding clone that was negative with the monoclonal anti-beta2 assay. A possible explanation for these contradictory results could be immunogenic differences between various beta2 toxins from the different groups of *cpb2*. Possible differences in immunogenicity could also potentially explain differences in the disease types and disease presentations for *C. perfringens* diseases in humans. Future studies with antibodies developed against beta2 proteins from the different groups of *cpb2* could provide valuable information regarding the immunogenic difference and potential cross-reactivity between the different beta2 proteins from the different groups of *cpb2*.

Beta2 Toxin from Clones with Either a Group-1 or a Group-2 *cpb2* (Found in the Majority of *C. perfringens* Isolates from Humans with Gastrointestinal Diseases) were Most Likely to be Cytotoxic to Caco-2 Enterocytes

The *cpb2* gene was cloned into *E. coli* to produce recombinant beta2 toxin. The results showed variability in the level of activity of the recombinant

beta2 toxin on caco-2 cells between clones from the different groups of *cpb2*. These assays using caco-2 cells were performed because expression of beta2 toxin by *C. perfringens* isolates is not sufficient evidence to link the beta2 toxin to diseases in humans. It must also be demonstrated that the protein can function as an active toxin capable of producing damage to enterocytes or other cell types (i.e., cytotoxicity). Two separate cell cytotoxicity assays, both of which used caco-2 enterocytes, were used to test cell culture supernatant fluid containing the recombinant beta2 protein from the *E. coli* clones. When *cpb2* was first described, purified beta2 protein was shown to be cytotoxic to CHO (Chinese hamster ovary) cells and intestinal I407 cells as well as lethal to mice (Jolivet-Reynaud et al., 1986; Gibert et al. 1997). These findings established the beta2 protein as a putative toxin. We chose the caco-2 cultured cell line because the gastrointestinal diseases of type A strains of *C. perfringens* can potentially have severe effects in the human colon, making caco-2 cells (which are derived from a colorectal adenocarcinoma and express features of polarized enterocyte differentiation) appropriate cells to use.

Our cell cytotoxicity assays showed a wide range in the level of activity on caco-2 cells of the cell culture supernatant fluid that contained the recombinant beta2 toxin. The recombinant beta2 toxin was shown to have the ability to damage and kill enterocytes using the trypan blue exclusion assay. The second cell cytotoxicity assay showed that the recombinant beta2 cell culture supernatant proteins were damaging the caco-2 cells, as determined by measuring the amount of lactate dehydrogenase (LDH) in the extracellular assay

buffer. LDH is a cytosolic component released upon cell lysis and considered to be an indicator of membrane integrity. It is often used as an indicator of cytolysis for caco-2 cells as well as other cell culture lines (Araki et al., 2001). The advantage of the LDH assay versus the trypan blue exclusion assay is that it identifies when a substances is damaging to the cell membrane instead of simply indicating that a cell is damaged or dead.

Both cytotoxicity assays showed that the recombinant beta2 protein from clones with group-1, group-2, or group-3 *cpb2* all have the potential to cause cell death of enterocytes. Clones expressing group-2T beta2 recombinant proteins did not cause a significant increase in the percent of dead caco-2 cells in either assay (Figures 11 and 16). Results of the trypan blue exclusion assay did not always correlate with results for the LDH assay for clones with group-1, group-2, or group-3 *cpb2*. However, the LDH assay measures a specific cytosolic component that is released upon cell lysis while the trypan blue exclusion assay reflects changes in the permeability of the cell membrane that allows the trypan blue dye to enter the damaged cell. Additionally, we chose the 8 hour end point for the LDH measurement in order to keep exposure times similar to the trypan blue exclusion assay. Therefore, although the caco-2 cells may have been damaged, cell lysis might not have occurred at the time (8 hours) the assay buffer was removed and assayed. Degradation of the recombinant beta2 protein over time may have affected the assay; this would suggest that the stability of the beta2 protein might vary between the different groups of *cpb2*, as has been described by others (Gibert et al., 1997; Vilei et al., 2005). There are other

additional technical factors that could have impacted our results. Because it can be technically challenging and time consuming, we chose not to purify the recombinant beta2 toxin; therefore, other proteins produced by the *E. coli* could have affected the stability of the recombinant beta2 toxin. Additionally, the 6x-HIS tag that precedes the recombinant beta2 toxin was also not removed, and this could have influenced the protein structure of the recombinant beta2 toxin, thereby affecting its activity level with regards to the caco-2 cells.

Despite the lack of a clear correlation between results from the trypan blue exclusion assay and the LDH assay, we were able to make a number of important observations regarding the activity of recombinant beta2 proteins. First, recombinant beta2 proteins have the ability to damage enterocytes and cause cell death (with the exception of group-2T beta2 proteins). Second, an increase in concentration of the recombinant beta2 proteins often resulted in a corresponding increase in the percent of dead cells or damaged cells. Third, an increase in the length of time the recombinant beta2 protein was exposed to caco-2 cells typically produced an increase in the percent of cell damage. This observation was noted for increases in the length of beta2 exposure to caco-2 cells at different concentrations of the recombinant beta2 proteins.

The authors who originally described *cpb2* also noted that beta2 protein was cytotoxic for CHO (Chinese hamster ovary) cells and intestinal I407 cells (which ATCC now considers to be HeLa cells; Jolivet-Reynaud et al., 1986; Gibert et al. 1997). To date, there has been only one publication by others addressing the effect of the beta2 toxin on caco-2 cells (Fisher et al., 2005).

Fisher and colleagues (2005) described cell damage (as measured by an assessment of cell morphologic changes) after 5 hours of exposure to cell culture supernatant fluid from a type A *cpb2*+/*cpe*+ strain (vegetative, not sporulating cells). These same investigators also cloned a group-1 *cpb2* (which they termed CPB2h2, table 24) and a group-2 *cpb2* (which they termed CPB2h1, table 24) into *cpb2*/*cpe* negative type A strains of *C. perfringens*. The beta2 toxin was purified from the cell culture supernatant fluid from each of these clones. It was discovered that a 13-fold higher concentration of the purified beta2 from the group-1 *cpb2* was required to damage 50% of the caco-2 cells in the assay versus the group-2 *cpb2* after 2 hours of exposure to the caco-2 cells (Fisher et al., 2005). These investigators used a modified version of the purification technique of Gibert et al. (1997) but did not note if there was any degradation or instability of their purified beta2 toxin. The results described by Fisher et al. (2005) as well as the results we described here indicate that variable concentrations of the beta2 toxin *in vivo* could explain differences in disease severity and presentation by different *C. perfringens* in human gastrointestinal diseases.

Determining the actual role of *cpb2* and the beta2 toxin in diseases caused by *C. perfringens* in humans, especially gastrointestinal diseases such as AAD and food-borne illnesses, awaits further study. In particular, these studies will need to define the mechanism of regulation of beta2 toxin, determine the specific activity of the beta2 toxin, and identify the location of that activity in human tissue. Questions on the stability of beta2 toxin both in host tissue and in

different culture media also need to be addressed. It is hoped that the results of the experiments described in this investigation will contribute to understanding the potential contribution of the beta2 toxin to the virulence of *C. perfringens* and the many different types diseases this organism can cause in humans.

SUMMARY OF CONCLUSIONS

The Beta2 Protein Toxin Gene, *cpb2*, was Identified in the Genome of Type A *C. perfringens* Isolates from Human Sources

Although *cpb2* was identified in all three of our study populations, chi-square statistical analysis showed that *cpb2* is found in a significantly higher percentage of isolates from humans with *C. perfringens* related gastrointestinal diseases than in *C. perfringens* isolates from healthy human volunteers. Though a higher percentage of isolates from humans with extraintestinal *C. perfringens* diseases carried *cpb2* compared to the healthy human volunteer isolates, this difference was not statistically significant. Other PCR assays produced results that were used to further characterize *cpb2* and provided information regarding the possible genetic differences of *cpb2* in the *C. perfringens* isolates from all the populations that were studied in this investigation.

A Range of Genetic Variation was Identified in the *cpb2* Genes from the *C. perfringens* Isolates at the Nucleotide Level as well as in the Predicted Amino Acid Sequences

Genetic variation in the *cpb2* genes and the predicted protein sequence of the beta2 protein toxin in isolates of *C. perfringens* could explain differences in virulence between isolates, presentation, disease, and disease severity. In the *C. perfringens* isolates from humans with gastrointestinal diseases, including those with an AAD association and those from food borne disease isolates, group-2 *cpb2* (which varied by more then 30% identity to the currently published *cpb2* sequences, CP13-*cpb2* and CWC245-*cpb2*) was the genetic variant most commonly found. A number of other variants of *cpb2* were also identified in the *cpb2* positive isolates, although how these differences might affect disease presentation, disease severity, and virulence remains unclear. Finally, a number of isolates were found to carry two distinct versions of *cpb2*, each from a different group.

The Beta2 Protein was not Expressed by Every *cpb2* Positive *C. perfringens* Isolate from the Different Populations of Isolates

Difference in the expression or level of expression of the beta2 protein toxin is another factor that could explain differences in disease presentation, disease severity, and virulence of *C. perfringens* strains that cause diseases in humans. Seventy-nine percent of group-2 *cpb2 C. perfringens* isolates (including isolates that carried both a group-1 and group-2 *cpb2*) were positive for beta2 protein expression; most of these isolates were from the *C. perfringens* collected from the fecal samples of humans with gastrointestinal diseases, including patients with AAD or food-borne outbreak isolates. Isolates that carried only a group-1 *cpb2*, a group-2T *cpb2*, or both a group-1 and a group-2T *cpb2* were all negative for beta2 protein expression. Only 50% of isolates with a group-3 *cpb2* or a group-2 and group-3 *cpb2* produced the beta-2 protein. There are many different possible reasons for why some *cpb2* positive *C. perfringens* isolates failed to express the beta2 toxin.

E. coli Clones that Expressed Recombinant Beta2 Protein were Constructed and used to Study Potential Differences in the Activity Level of the Beta2 Protein from the Different Variants of *cpb2* on Cultured Caco-2 Cells

The recombinant beta2 proteins were able to cause a range of cellular damage to the caco-2 cultured cells at different concentrations of the protein. It was also noted that the two group-2T clones, despite having the entire coding region, had no effect on caco-2 cells as measured by either assay. It appeared that recombinant beta2 toxin from clones with either a group-1 or a group-2 *cpb2* (found in the majority of *C. perfringens* isolates from humans with gastrointestinal diseases) were most likely to be cytotoxic to caco-2 enterocytes. However, there were no obvious differences in the level of activity between the recombinant beta2 protein from these different *cpb2* group clones using either the trypan blue exclusion assay or the lactate dehydrogenase (LDH) assay.

Table 1. Traditional biochemical test results and short-chain fatty acids detected by GLC for *C. perfringens* and selected other clinically significant clostridia.

	Carbohydrate Fermentations[a]												
	Glucose	Lactose	Maltose	Mannitol	Mannose	Salicin	Sucrose	Esculin Hydolysis	Indole	Nitrate	Gelatin	Milk[b]	Short-chain Fatty Acids (GLC)[c]
C. perfringens	+	+	+	-	+	-	+(-)	+/-	-	+/-	+	d&c	A,B,L (p,s)[d]
C. difficile	+	-	-	w	v	-	-	+	-	-	+	-	B,A,ic,iv,ib (v,l)
C. septicum	+	-	-	-	-	v	-	+	-	v	+	c(d)	B,A (p,l)
C. bifermentans	+	-	w(-)	-	-(w)	-	-	+	+	-	+	d	A (iv,ic,p,ib,b,l,s)
C. botulinum	+	-	-(v)	-	-(v)	-	-	+/-	-	-	+	d/c	variable, depends on subtype
C. tetani	+	-	-	-	-	-	-	-	v	-	+	d	A,B,p (l,s)

[a] Carbohydrate fermentation reactions: +: positive; -: negative; +(-): most strains positive, some negative; -(+): most strains negative, some positive;+/-: some strains positive, some strains negative; w: weakly positive; v: variable results.

[b] Milk reactions: d: digest; c= clot (or curd); d&c: digest & clot; d/c: some strains digest, some strains clot; c(d): most strains clot, some strains digest; -: negative.

[c] Short-chain fatty acids (GLC): Capital letters indicate major products, lower case letters indicate minor products, parentheses indicate variable reactions.

[d] A= acetic; P= propionic; IB= isobutyric; B= butyric; IV= isovaleric; V= valeric; IC= isocaproic, C= caproic, L= lactic, S= succinic.

Table 2. The five types of *C. perfringens* and the major toxins produced by each type.

	Toxin(s) Produced			
Type	Alpha	Beta	Epsilon	Iota
A	X			
B	X	X	X	
C	X	X		
D	X		X	
E	X			X

Table 3. Multiplex PCR primer sets.

Gene	Primer	Primer Sequence (5' to 3')	Amplicon size (bp)	Reference
Alpha toxin gene *(cpa)*	cpa-F[a]-1438	GCTAATGTTACTGCCGTTGA	324	Titball et al. 1989
	cpa-R[b]-1743	CCTCTGATACATCGTGTAAA		
Beta toxin gene *(cpb)*	cpb-F-871	GCGAATATGCTGAATCATCTA	196	Hunter et al. 1993
	cpb-R-1046	GCAGGAACATTAGTATATCTTC		
Iota toxin gene *(iA)*	iA-F-275	ACTACTCTCAGACAAGACAG	446	Perelle et al. 1993
	iA-R-701	CTTTCCTTCTATTACTATACG		
Epsilon toxin gene *(etx)*	etx-F-227	GCGGTGATATCCATCTATTC	655	Hunter et al. 1993
	etx-R-862	CCACTTACTTGTCCTACTAAC		
Beta2 toxin gene *(cpb2)*	cpb2-F-492	AGATTTTAAATATGATCCTAACC	567	Gibert et al. 1997
	cpb2-R-1058	CAATACCCTTCACCAAATACTC		
Enterotoxin gene *(cpe)*	cpe-F-439	GGAGATGGTTGGATATTAGG	233	Czeczulin et al. 1993
	cpe-R-650	GGACCAGCAGTTGTAGATAC		

[a]F=forward primer.

[b]R=reverse primer.

Table 4. Three simplex PCR reactions used to confirm and characterize *cpb2* from *C. perfringens* isolates.

Simplex PCR	Primer name	Primer 5' to 3'	Amplicon size (bp)	Annealing temperature (°C)	Reference
cpb2PCR-1	cpb2-F[a]-492 / cpb2-R[b]-1058	Refer to Table 3.	567	55	Garmory et al. 2000
cpb2PCR-2	hb2F-377	GAAAGGTAATGGAGAATTATCTTAATGC	574	48	Herholz et al. 1999
	hb2R-950	GCAGAATCAGGATTTTGACCATATACC			
cpb2PCR-3	myb2F-33	GAATTGTAAAAAAAATTTCAGGG	847	50	This research
	myb2R+22	CACTTCATATTTTTTCTATGC			

[a]F=forward primer.

[b]R=reverse primer.

Table 5. The two forward primers, and single reverse primer used in the triplex PCR.

Primer	Sequence (5' - 3')
Grp1cpb2F[a]	ATTATGTTTAGGAATACAGTTA
Grp2cpb2F	CAATTGGGGGAGTTTATCCACAA
cpb2R-1058[b]	CAATACCCTTCACCAAATACTC

[a]F=forward primer

[b]R=reverse primer

Table 6. Primers used to amplify *cpb2* and add restriction enzyme cut sites, to allow for insertion of the *cpb2* sequence into the pRSET A or B vector.

Primer Name	Primer sequence (5' - 3')	Compatable pRSET vector	Reference
b2clnBamH1-F[a]	ATGCG|GATCCAATGAAGTGAATAAATACC		This research
13b2clnBamH1-F	AAATG|GATCCAAGGAAATCGACGCTTAT	B	This research
b2clnEcoR1-R[b]	ATGCG|AATTCCTATGCACAATATCCTTC		This research
AtypBamH1B2-F	CCTAATACAGTG|GATCCAAATGAAGTG		Jost et al. 2005
AtypEcoR1B2-R	TATAAATAAATAG|AATTCTCTAAAACC	A	Jost et al. 2005
ConEcoR1B2-R	GTCACTTCAG|AATTCTTTCTATGCAC		Bueshel et al. 2003

[a] F=forward primer

[b] R=reverse primer

| Designates location of restriction enzyme cut site.

*Eco*R1 Restriction enzyme recognition sequence is highlighted in yellow.

*Bam*H1 Restriction enzyme recognition sequence is highlighted in green.

Table 7. The *E. coli* strains used in cloning *cpb2,*and a description of their genotypes as listed by the supplier (Invitrogen).

E. coli strain	Genotype description	Purpose
TOP 10F'	F' {*lac*I^{q}, Tn*10*(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G	Propagation and maintenance
BL21(DE3)pLysS	F^{-}, *omp*T *hsd*SB ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS (Cam^{R})	Expression of protein

Table 8. Multiplex PCR genotype results for the *C. perfringens* isolates that have been divided into three populations, isolates from a group of healthy human volunteers, isolates from patients with *C. perfringens* related gastrointestinal diseases, and isolates from patients with *C. perfringens* related extraintestinal diseases.

	Isolate Population		
Positive gene(s)	Healthy (n=100)	Gastrointestinal Diseases (n=161)	Extraintestinal Disease (n=59)
cpa	88 (88%)	88 (54%)	44 (75%)
cpa, cpb2	10 (10%)	38 (24%)	12 (20%)
cpa, cpe	1 (1%)	25 (16%)	3 (5%)
cpa, cpb2, cpe	1 (1%)	10 (6%)	0

Table 9. Results from the three separate simplex PCRs that were perfomed to further characterize and classify the *cpb2* genotypes identified in the three different populations of *C. perfringens* isolates.

	Isolate Population		
Simplex PCR	Healthy (n= 11)	Gastrointestinal Disease (n= 48)	Extraintestinal Disease (n= 12)
cpb2PCR-1	11 (100%)	48 (100%)	12 (100%)
cpb2PCR-2	9 (82%)	9 (19%)	1 (8%)
cpb2PCR-3	5 (45%)	0	0

Table 10. Results of the triplex PCR that was used to determine the *cpb2* group for a *C. perfringens* isolate, with the isolates divided into the three different populations. Most *C. perfringens* isolates carried *cpb2* from one group, however, isolates were identified from all three populations of *C. perfringens* isolates that carried at least two versions of c*pb2*, one from each group.

	Isolate Population		
	Healthy	Gastrointestinal Disease	Extraintestinal Disease
Triplex PCR	(n= 11)	(n= 48)	(n= 12)
Group 1 (consensusl)	5 (46%)	5 (10%)	1 (8%)
Group 2 (atypical)	4 (36%)	35 (73%)	9 (75%)
Group 1 and Group 2	2 (18%)	8 (17%)	2 (17%)

Table 11. ClustalW pairwise alignment scores (indicating percent similarity) of the published sequences of *cpb2*, CWC245 and CP13, and the Group-2 (Grp2) *cpb2* consensus sequence determined in this studv.

Bio Type	*cpb2* Sequence	*cpb2* Sequence		
		CWC245	CP13	Grp 2
C	CWC245	--	95	71
A	CP13	95	--	71
A	Grp 2	71	71	--

Table 12. ClustalW pairwise alignment scores (indicating percent similarity) of the deduced protein sequences of the beta2 toxin from the published sequences of *cpb2* from CWC245 and CP13, and the deduced protein sequence from the Group-2 (Grp2) *cpb2* consensus sequence determined in this study.

Bio Type	*cpb2* Sequence	*cpb2* Sequence		
		CWC245	CP13	Grp 2
C	CWC245	--	91	63
A	CP13	91	--	64
A	Grp 2	63	64	--

Table 13. ClustalW pairwise alignment scores (indicating percent identity) of the *cpb2* sequences from *cpb2* positive reference strains versus the published sequences of *cpb2*, CWC245 and CP13, and the Group-2 (Grp2) *cpb2* consensus sequence.

			Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
Bio Type	Reference Strain	Number of Base Pairs	CWC 245	CP13	Grp 2
C	10719	816	98	95	72
E	27324(1)[a]	618	88	85	69
	27324(2)	560	73	73	92

[a] Parantheses next to the strain number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 14. ClustalW pairwise alignment scores (indicating percent identity) of the deduced protein sequences of the beta2 toxin from *cpb2* positive reference strains versus the published sequences CWC245 and CP13, and the deduced protein sequence from the Group-2 (Grp2) *cpb2* consensus sequence.

Bio Type	Reference Strain	Number of Amino Acids	Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
			CWC 245	CP13	Grp 2
C	10719	265	96	93	63
E	27324(1)[a]	215	86	81	70
	27324(2)	186	62	63	86

[a] Parantheses next to the strain number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 15. ClustalW pairwise alignment scores (indicating percent identity) of Group-1 *cpb2* sequences versus the published *cpb2* sequences, CWC245 and CP13, and the Group-2 (Grp2) consensus *cpb2* sequence.

Population Source[a]	Research Isolate Number	Number of Base Pairs	Clustal W Pairwise Alignment score versus *cpb2* Sequence: CWC245	CP13	Grp 2
HV	1009	843	93	98	67
HV	1010	675	92	98	73
HV	1014	897	94	99	71
HV	1017	822	94	99	72
HV	1066(1)[b]	543	85	93	67
HV	1067(1)	570	92	99	72
HV	1068(1)	584	91	97	70
HV	1069(1)	567	92	97	71
GI	2109(1)	574	92	98	72
GI	2116(1)	584	97	93	72
GI	2120	846	94	99	71
GI	2121(1)	908	90	95	67
GI	2122(1)	599	92	91	69

[a] Population source indicates which population of human sources the isolate is from. HV: Healthy volunteers, GI: Gastrointestinal diseases.

[b] Parantheses next to the research isolate number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 16. ClustalW pairwise alignment scores (indicating percent identity) of the deduced protein sequences of the beta2 toxin from Group-1 *cpb2* sequences versus the deduced beta2 toxin sequence from published *cpb2* sequences, CWC245 and CP13, and the Group-2 (Grp2) consensus *cpb2* sequence.

			Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
Population Source[a]	Research Isolate Number	Number of Amino Acids	CWC245	CP13	Grp 2
HV	1009	265	89	98	62
HV	1010	225	88	96	66
HV	1014	265	90	98	64
HV	1017	265	90	99	63
HV	1066(1)[b]	182	78	87	60
HV	1067(1)	189	84	94	65
HV	1068(1)	194	87	95	64
HV	1069(1)	188	86	96	67
GI	2109(1)	190	88	97	66
GI	2116(1)	194	93	86	65
GI	2120	265	91	100	64
GI	2121(1)	265	90	98	62
GI	2122(1)	199	89	86	65

[a] Population source indicates which population of human sources the isolate is from. HV: Healthy volunteers, GI: Gastrointestinal diseases.

[b] Parantheses next to the research isolate number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 17. ClustalW pairwise alignment scores (indicating percent identity) of Group-2 *cpb2* sequences versus the published *cpb2* sequences, CWC245 and CP13, and the Group-2 (Grp2) consensus *cpb2* sequence. Isolates shadowed in gray, belong to Grp2T, and carry a 13 base pair deletion in the open reading frame of *cpb2.*

Population Source[a]	Research Isolate Number	Number of Base Pairs	Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
			CWC245	CP13	Grp 2
HV	1008	810	71	71	97
HV	1066(2)[b]	819	72	73	98
HV	1067(2)	793	69	70	97
HV	1068(2)	781	70	70	96
HV	1069(2)	824	71	72	98
HV	1093	867	66	68	93
GI	2010	543	74	74	97
GI	2012	784	69	71	96
GI	2016	564	74	74	91
GI	2023	807	70	70	96
GI	2048	558	75	75	95
GI	2049	786	69	69	95
GI	2050(2)	801	71	71	96
GI	2051	785	72	72	98
GI	2052	811	73	73	98
GI	2061	813	70	70	96
GI	2062	538	73	73	99
GI	2063	818	71	71	99
GI	2064	826	72	72	98
GI	2065	560	75	75	98
GI	2066	813	72	73	98
GI	2067	809	72	72	98

GI	2068	485	75	74	98
GI	2069	774	70	71	96
GI	2070	777	68	68	95
GI	2071	775	69	69	96
GI	2073	792	69	69	97
GI	2074	806	70	70	97
GI	2076	823	74	74	94
GI	2077	545	69	70	95
GI	2082	538	71	71	97
GI	2093	484	64	67	87
GI	2107	541	72	72	97
GI	2108(2)	783	69	69	96
GI	2109(2)	488	66	66	87
GI	2110	561	70	70	88
GI	2115	798	70	70	98
GI	2116(2)	775	68	68	97
GI	2121(2)	815	72	72	98
GI	2122(2)	699	67	67	95
GI	2124	522	69	69	92
GI	2125	523	74	74	90
GI	2128	771	67	68	95
GI	2132	782	68	68	96
GI	2133	542	73	73	96
GI	2134	780	68	68	96
GI	2137	778	68	68	95
GI	2139	548	74	74	95
GI	2140	513	68	68	89
EI	3002	805	72	72	98
EI	3008	454	74	73	99

EI	3017	558	75	75	98
EI	3023	545	73	71	94
EI	3032	557	75	75	98
EI	3033	563	76	77	97
EI	3037	789	71	71	99
EI	3040	790	71	72	99
EI	3042	777	72	72	98
EI	3044	786	70	71	98
EI	3052	794	71	71	98

[a] Population source indicates which population of human sources the isolate is from. HV: Healthy volunteers, GI: Gastrointestinal diseases, EI: Extra-intestinal diseases.

[b] Parantheses next to the research isolate number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 18. ClustalW pairwise alignment scores (indicating percent identity) of the deduced protein sequences of the beta2 toxin from Group-1 *cpb2* sequences versus the deduced beta2 toxin sequence from published *cpb2* sequences, CWC245 and CP13, and the Group-2 (Grp2) consensus deduced beta2 toxin sequence. Isolates shadowed in gray belong to Grp2T, and carry a 13bp deletion in the open reading from of *cpb2* resulting in a premature stop codon, which does not allow for a complete amino acid sequence to be deduced.

			Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
Population Source[a]	Research Isolate number	Number of Amino Acids	CWC245	CP13	Grp 2
HV	1008	261	62	62	96
HV	1066(2)[b]	262	62	63	97
HV	1067(2)	261	59	60	93
HV	1068(2)	252	59	60	96
HV	1069(2)	264	62	63	98
HV	1093	255	61	62	98
GI	2010	181	67	67	96
GI	2012				
GI	2016	185	66	67	85
GI	2023	256	60	61	95
GI	2048	285	69	69	92
GI	2049	256	58	59	94
GI	2050(2)	256	60	60	92
GI	2051	261	63	63	98
GI	2052				
GI	2061	263	58	60	92
GI	2062	179	67	67	97
GI	2063	261	62	62	97
GI	2064	265	62	63	97

GI	2065	186	69	69	97
GI	2066	262	62	62	98
GI	2067	261	62	63	98
GI	2068	181	66	66	96
GI	2069	255	59	60	96
GI	2070	256	59	60	96
GI	2071	256	60	61	97
GI	2073	262	61	61	97
GI	2074	262	59	59	95
GI	2076	263	65	66	92
GI	2077	181	64	64	93
GI	2082	178	67	67	97
GI	2093	161	59	58	81
GI	2107	180	65	65	93
GI	2108(2)	260	57	57	91
GI	2109(2)	162	58	58	82
GI	2110	187	60	60	83
GI	2115	262	61	62	98
GI	2116(2)	256	60	61	97
GI	2121(2)	261	62	63	96
GI	2122(2)	229	61	62	96
GI	2124	173	64	64	90
GI	2125	154	65	65	92
GI	2128	256	56	57	92
GI	2132	260	57	58	93
GI	2133	180	66	66	93
GI	2134	259	56	57	93
GI	2137	258	56	57	92
GI	2139	182	67	67	91

GI	2140	170	56	57	78
EI	3002				
EI	3008	151	66	65	98
EI	3017	185	69	69	95
EI	3023	181	66	67	91
EI	3032	185	67	67	96
EI	3033	187	70	71	93
EI	3037	256	61	62	98
EI	3040	256	62	62	98
EI	3042				
EI	3044	255	60	61	97
EI	3052	256	61	62	98

[a] Population source indicates which population of human sources the isolate is from. HV: Healthy volunteers, GI: Gastrointestinal diseases, EI: Extra-intestinal diseases.

[b] Parentheses next to the research isolate number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 19. ClustalW pairwise alignment scores (indicating percent identity) of Group-3 *cpb2* sequences versus the published *cpb2* sequences, CWC245 and CP13, the Group-2 (Grp2) consensus *cpb2* sequence.

Population Source[a]	Research Isolate number	Number of Base Pairs	Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
			CWC 245	CP13	Grp 2
HV	1028	554	70	71	53
GI	2152	598	78	79	67
GI	2153	579	71	77	67
GI	2096	574	72	74	63
GI	2108(3)[b]	530	81	84	62
GI	2050(3)	553	80	83	64

[a] Population source indicates which population of human sources the isolate is from. HV: Healthy volunteers, GI: Gastrointestinal diseases.

[b] Parentheses next to the research isolate number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 20. ClustalW pairwise alignment scores (indicating percent identity) of the deduced protein sequences of the beta2 toxin from Group-3 *cpb2* sequences versus the deduced beta2 toxin sequence from published *cpb2* sequences, CWC245 and CP13, and the Group-2 (Grp2) consensus dedueced beta2 toxin sequence.

Population Source[a]	Research Isolate Number	Number of Amino Acids	Clustal W Pairwise Alignment score versus *cpb2* Sequence:		
			CWC 245	CP13	Grp 2
HV	1028	184	56	58	47
GI	2152	200	69	72	61
GI	2153	192	67	70	61
GI	2096	191	66	67	56
GI	2108(3)[b]	176	69	72	53
GI	2050(3)	183	72	78	57

a Population source indicates which population of human sources the isolate is from. HV: Healthy volunteers, GI: Gastrointestinal diseases.

b Parentheses next to the research isolate number indicates that more then one version of *cpb2* was identified for that isolate. Alignment scores shown in that row, are only for that version of *cpb2*.

Table 21. Western blotting results of cell culture supernatant from *cpb2* positive *C. perfringens* reference strains and from *C. perfringens* isolates from human sources using a polyclonal anti-beta2 antibody. The *cpb2* group(s) are based on sequencing and triplex PCR results.

Reference Strain	*cpb2* Group(s)	Result	
		Positive	Negative
10719 (n=1)	CWC245	1	-
27324 (n=1)	1 & 2	-	1

Isolate Population[a]	*cpb2* Group(s)		Result	
			Positive	Negative
GI (n=48)	1	(n=1)	-	1
	2	(n=31)	27	4
	2T	(n=1)	-	1
	3	(n=3)	2	1
	1 & 2	(n=9)	6	3
	1 & 2T	(n=1)	-	1
	2 & 3	(n=2)	1	1
HV (n=11)	1	(n=4)	-	4
	2	(n=2)	2	-
	3	(n=1)	-	1
	1 & 2	(n=4)	4	-
EI (n=12)	2	(n=8)	4	4
	2T	(n=1)	-	1
	1 & 2	(n=2)	1	1
	1 & 2T	(n=1)	-	1

[a] GI: Isolates from humans with *C. perfringens* related gastrointestinal diseases, HV: Isolates from healthy human volunteers, EI: Isolates from humans with extra-intestinal *C. perfringens* related diseases.

Table 22. Recombinant *cpb2* plasmids constructed for cloning into *E. coli* BL21(DE3)pLysS for expression.

Population of Isolate[a]	Research Number of *C. perfringens* Isolate	Toxin Genes of the *C. perfringens* Isolate	*cpb2* genes[b]	Recombinant Plasmid Designation
Reference strain	NCTC 10719	*cpa, cpb, cpb2*	CWC245	pHRD101
HV	1009	*cpa, cpb2*	1	pHRD102
GI	2120	*cpa, cpb2, cpe*	1 &	pHRD103
			2	pHRD203
GI	2121	*cpa, cpb2*	1 & (2)	pHRD104
HV	1008	*cpa, cpb2*	2	pHRD201
GI	2109	*cpa, cpb2*	(1) & 2	pHRD202
GI	2140	*cpa, cpb2*	2	pHRD204
EI	3037	*cpa, cpb2*	2	pHRD205
GI	2074	*cpa, cpb2, cpe*	2	pHRD206
GI	2076	*cpa, cpb2, cpe*	2	pHRD207
HV	1093	*cpa, cpb2*	2	pHRD208
GI	2066	*cpa, cpb2, cpe*	2	pHRD209
GI	2049	*cpa, cpb2*	2	pHRD210
GI	2152	*cpa, cpb2, cpe*	3	pHRD301
HV	1028	*cpa, cpb2, cpe*	3	pHRD302
GI	2052	*cpa, cpb2*	2T	pHRD401
GI	2012	*cpa, cpb2, cpe*	(1) & 2T	pHRD402

[a] GI: Isolates from humans with *C. perfringens* related gastrointestinal diseases, HV: Isolates from healthy human volunteers, EI: Isolates from humans with extra-intestinal *C. perfringens* related diseases.

[b] The number indicates the group to which the version of *cpb2* carried by a *C. perfringens* isolate belongs. Isolates with two versions of *cpb2* have both versions listed. The group number not in parentheses was the *cpb2* gene used to construct the plasmid.

Table 23. Results from two separate Western blotting assays using either monoclonal (mAb) or polyclonal (pAb) anti-beta2 antibody, on the cell culture supernatant fluid (CSF) from the recombinant *E. coli cpb2* clones.

Recombinant Plasmid number	*cpb2* group	Recombinant *E. coli* - CSF	
		mAb	pAb
pHRD101	CWC245	P[a]	P
pHRD102	1	P	P
pHRD103	1	N	P
pHRD104	1	P	P
pHRD201	2	P	P
pHRD202	2	P	P
pHRD203	2	P	P
pHRD204	2	P	P
pHRD205	2	N	P
pHRD206	2	P	P
pHRD207	2	N	P
pHRD208	2	N	P
pHRD209	2	N	P
pHRD210	2	P	P
pHRD301	3	N	P
pHRD302	3	P	P
pHRD401	2T	P	P
pHRD402	2T	N	P

[a] P: Positive result. N: Negative result.

Table 24. Comparison of sequence variants of *cpb2* described in this investigation, in the current literature, and GenBank.

Reference	Name of the cpb2 sequence:		Similarity(% identity) of the sequence compared to:		Comparison to other *cpb2* groups described in this study	GenBank accession number	Source of *C. perfringens* isolate(s)
	in this investigation	in the original publication	the original *cpb2* sequence (CWC245-cpb2)[a]	the group-2 *cpb2* consensus sequence			
This study	Group-1	-	92%	70%	-	-	Human fecal samples, and other extraintestinal human specimens
	Group-2		71%	96%	-	-	
	Group-3		75%	63%	-	-	
Gibert et al. (1997)	CWC245-*cpb2*	*cpb2*	-	71%	92% identity to group-1	L77965	*C. perfringens* type C strain CWC245, piglet with necrotizing enterocolitis
Shimizu et al. (2001, 2002)	CP13-*cpb2*	*cpb2*	95%	71%	97% identity to group-1	AP003515	Strain 13, a *C. Perfringens* type A, human isolate

Jost et al. (2005, 2006)	Same as the CWC245-*cpb2*	Consensus *cpb2*	-	71%	92% identity to group-1	-	Reference: Gibert et al., 1997
	Similar to group-2 consensus sequence	Atypical *cpb2*	71%	98%	98% identity to the group-2 consensus sequence	AY609161 - AY609183[b]	*C. perfringens* stains, types A -E from animal sources from the CEDU[c]
Fisher et al. (2005)	97% identity to CP13-*cpb2*	CPB2h2	94%	72%	97% identity to group-1	AY730632 - AY730634, AY730636	AAD or SD[d] *C. perfringens* isolates from humans
	Similar to group-2 consensus sequence	CPB2h1	71%	99%	99% identity to the group-2 consensus sequence	AY730630 - AY730631, AY730635	

[a] Similarity is based on the average of the ClustalW alignment scores for the group being compared.

[b] Accession numbers AY609164 and AY609183 have the same 13 base-pair deletion seen in the group-2T *cpb2.* Accession numbers AY6097178 - AY609182 have a single deletion of a thymine base 220 base-pairs after the start codon that would result in a truncated protein of only 73 amino acids.

[c] CEDU: Clostridial Enteric Disease Unit, at the University of Arizona

[d] AAD: antibiotic associated diarrhea, SD: sporadic diarrhea.

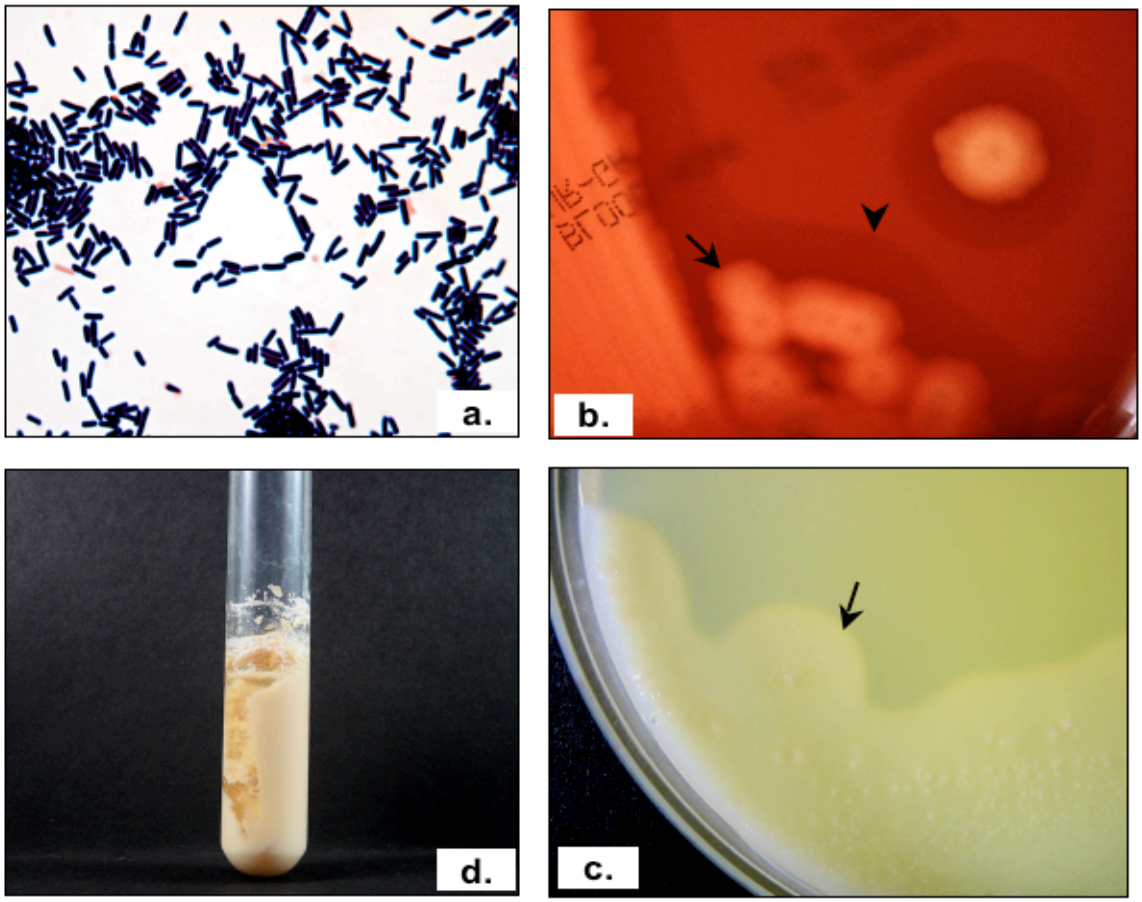


Figure 1. Traditional methods for identification of *C. perfringens* isolates in the clinical microbiology laboratory.

Image a: Gram's stain of *C. perfringens* showing the typical appearance of large, boxcar-shaped Gram-positive rods.

Image b: Colonies of *C. perfringens* on an anaerobe blood agar (prepared with sheep blood). The arrow indicates the inner zone of hemolysis. The arrow-head indicates the outer zone of hemolysis.

Image c: Growth of *C. perfringens* on egg yolk agar. The creamy white precipitate within the agar (arrow) indicates a positive lecithinase reaction.

Image d: Milk proteolysis test with abundant gas production, curdle and digestion typical of *C.perfringens*.

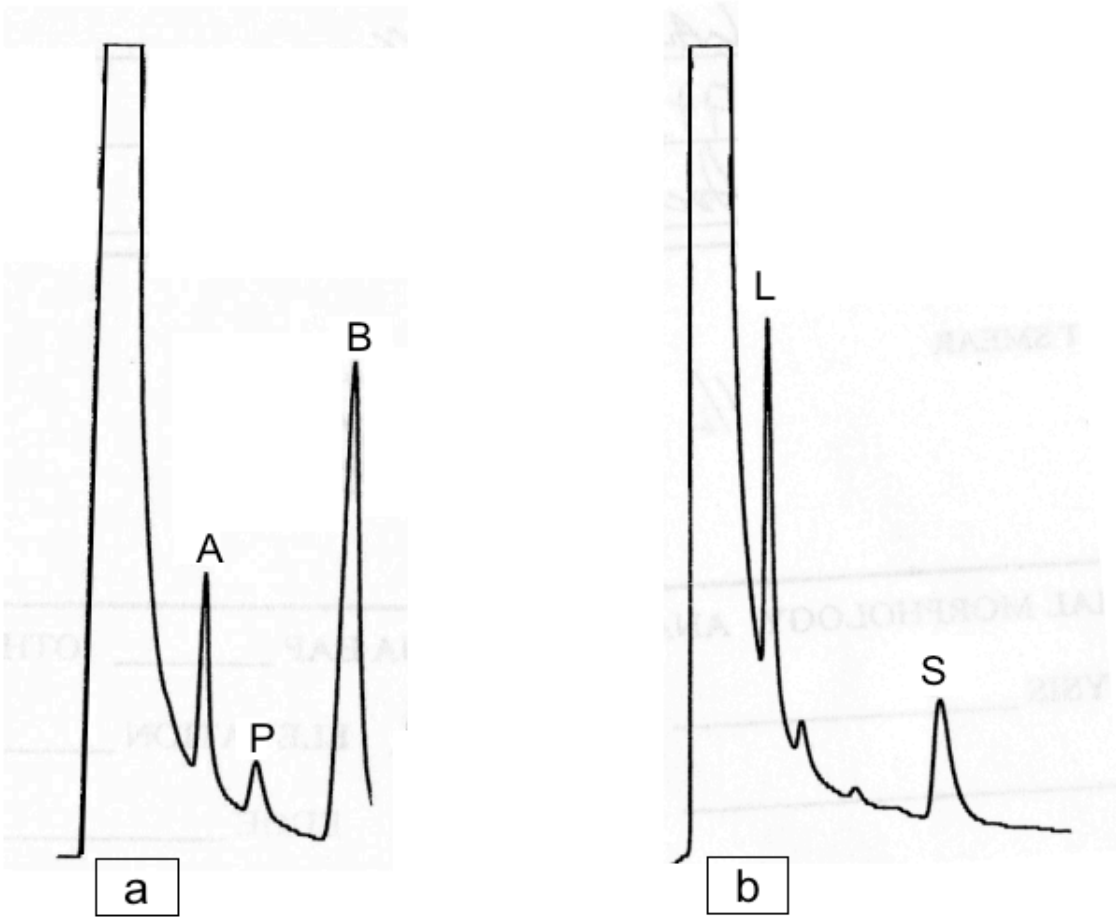


Figure 2. Typical results for *C. perfringens* short-chain fatty acid analysis using gas-liquid chromatography.

Image a: Volatile short-chain fatty acid profile.

Image b: Non-volatile short-chain fatty acid profile.

A: acetic acid, P: propionic acid, B: butyric acid, L: lactic acid, S: succinic acid.

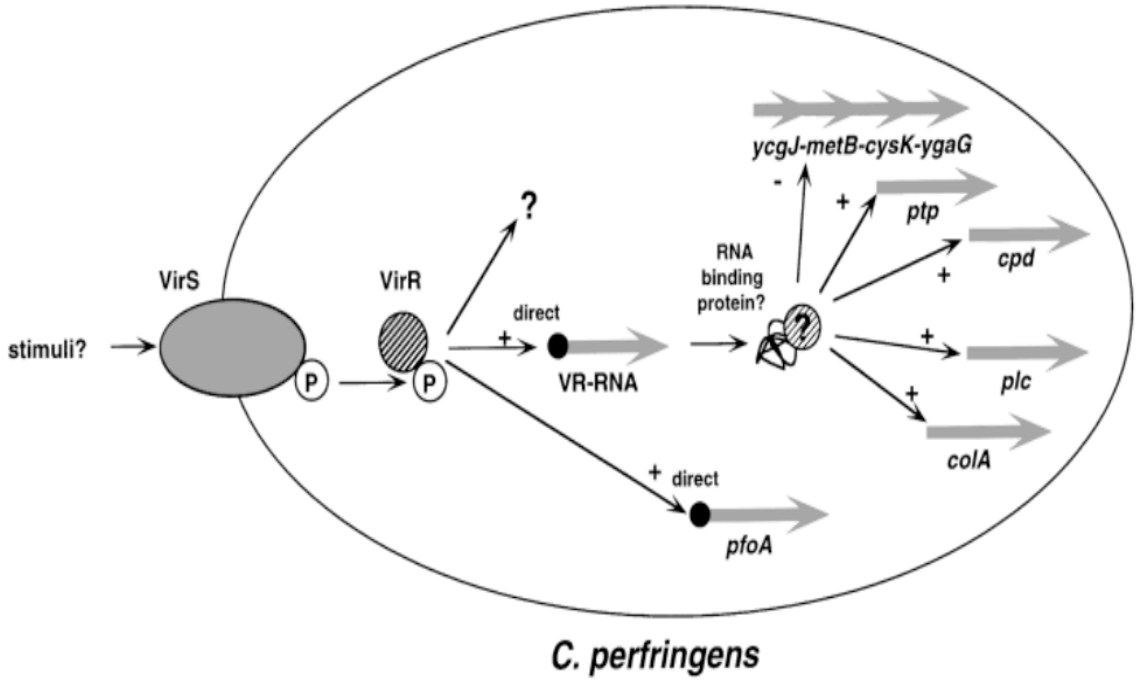
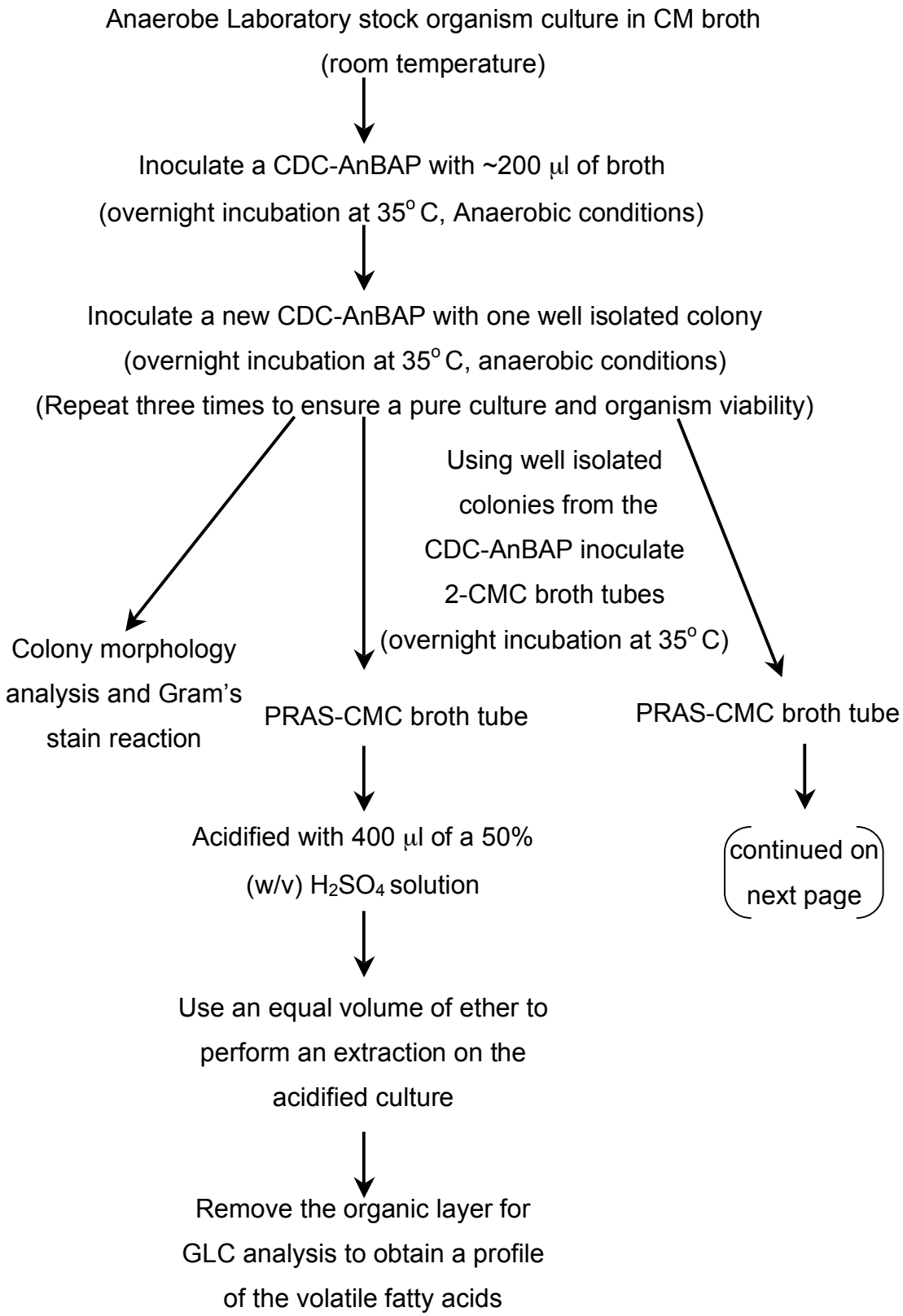


Figure 3. The VirS/VirR- VR-RNA regulatory cascade, which controls many of the virulence genes of *C. perfringens*. In response to some unknown stimuli, VirS undergoes autophosphorylation, and subsequently phosphorylates VirR, located in the cell cytoplasm. After phosphorylation VirR can either directly regulate transcription by binding to specific promoters in the DNA, or VirR can bind to the promoter region for VR-RNA (VirR- regulated RNA). The increase in VR-RNA in the cell cytoplasm, results in both positive and negative regulation of a number of toxin genes in *C. perfringens* (Image from Shimizu et al., 2002).


Anaerobe Laboratory stock organism culture in CM broth
(room temperature)
Inoculate a CDC-AnBAP with ~200 μl of broth
(overnight incubation at 35° C, Anaerobic conditions)
Inoculate a new CDC-AnBAP with one well isolated colony
(overnight incubation at 35° C, anaerobic conditions)
(Repeat three times to ensure a pure culture and organism viability)
Using well isolated colonies from the CDC-AnBAP inoculate 2-CMC broth tubes (overnight incubation at 35° C)
Colony morphology analysis and Gram's stain reaction
PRAS-CMC broth tube
PRAS-CMC broth tube
Acidified with 400 μl of a 50% (w/v) H_2SO_4 solution
continued on next page
Use an equal volume of ether to perform an extraction on the acidified culture
Remove the organic layer for GLC analysis to obtain a profile of the volatile fatty acids

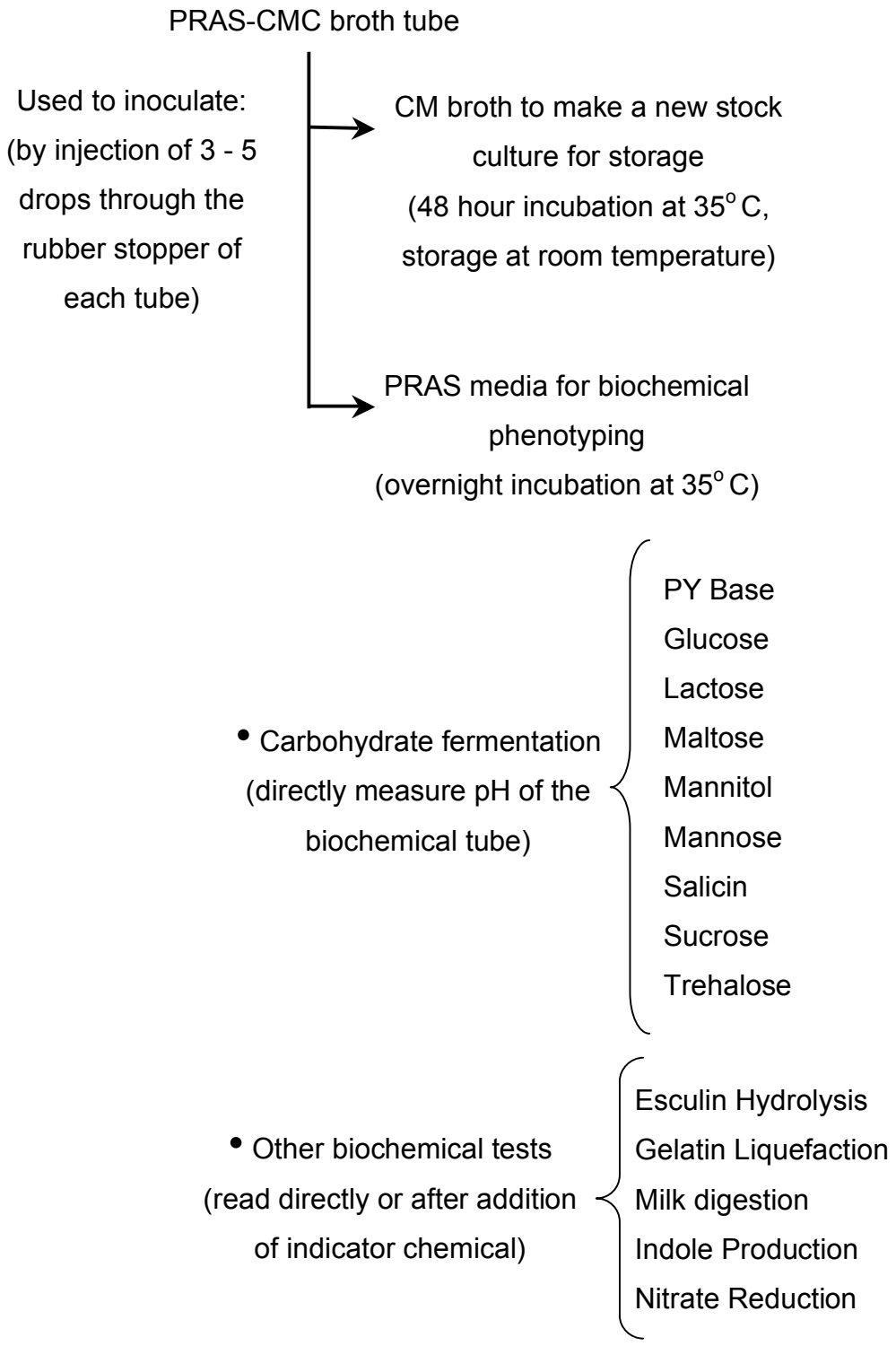
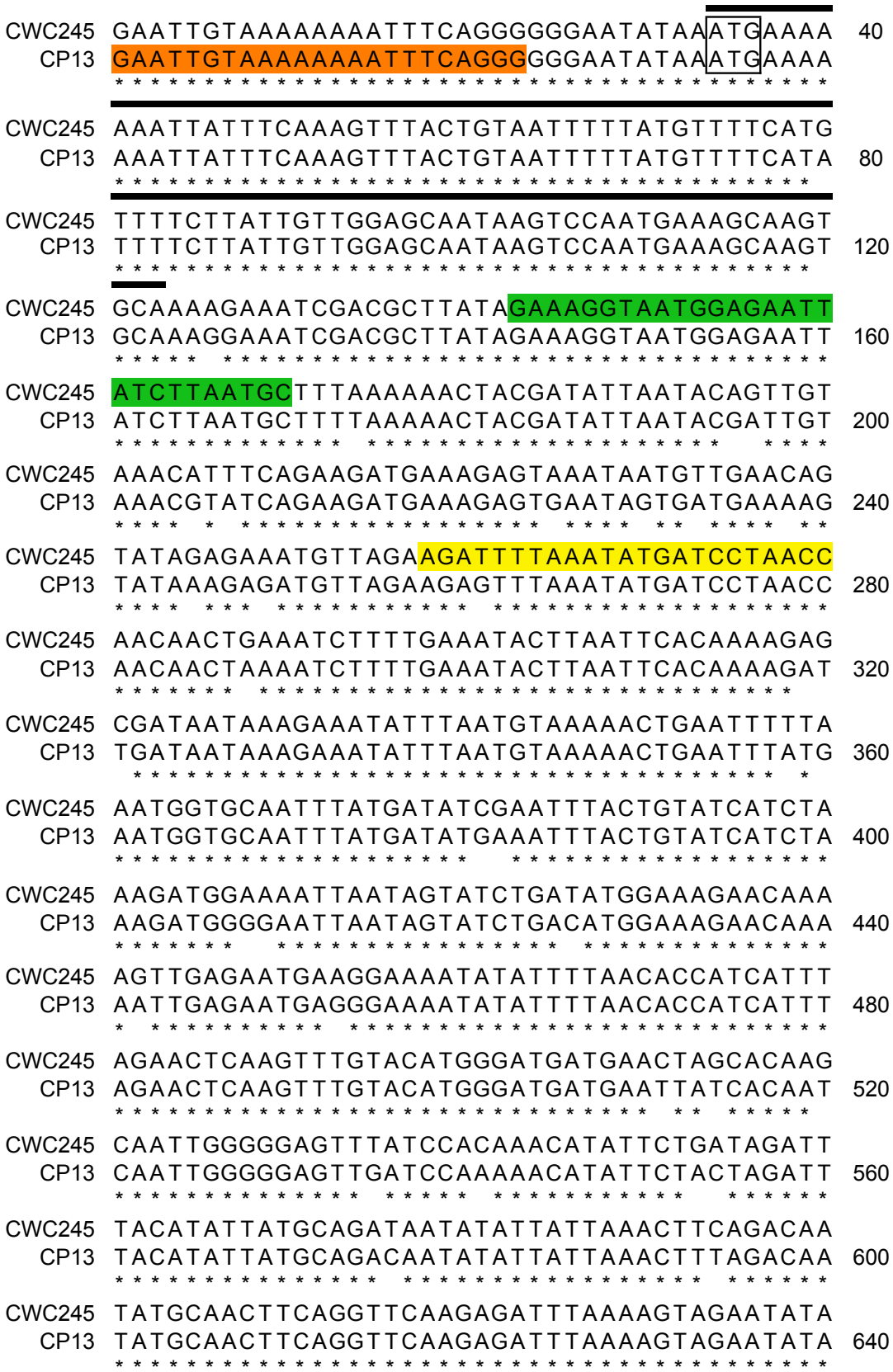


Figure 4. Flow chart used for culture and identification of *C. perfringens* isolates.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CP13 GAATTGTAAAAAAAATTTCAGGGGGGAATATAAATGAAAA
CWC245 AAATTATTTCAAAGTTTACTGTAATTTTTATGTTTTCATG
CP13 AAATTATTTCAAAGTTTACTGTAATTTTTATGTTTTCATA 80
CWC245 TTTTCTTATTGTTGGAGCAATAAGTCCAATGAAAGCAAGT
CP13 TTTTCTTATTGTTGGAGCAATAAGTCCAATGAAAGCAAGT 120
CWC245 GCAAAAGAAATCGACGCTTATAGAAAGGTAATGGAGAATT
CP13 GCAAAGGAAATCGACGCTTATAGAAAGGTAATGGAGAATT 160
CWC245 ATCTTAATGCTTTAAAAAACTACGATATTAATACAGTTGT
CP13 ATCTTAATGCTTTTAAAAACTACGATATTAATACGATTGT 200
CWC245 AAACATTTCAGAAGATGAAAGAGTAAATAATGTTGAACAG
CP13 AAACGTATCAGAAGATGAAAGAGTGAATAGTGATGAAAAG 240
CWC245 TATAGAGAAATGTTAGAAGATTTTAAATATGATCCTAACC
CP13 TATAAAGAGATGTTAGAAGAGTTTAAATATGATCCTAACC 280
CWC245 AACAACTGAAATCTTTTGAAATACTTAATTCACAAAAGAG
CP13 AACAACTAAAATCTTTTGAAATACTTAATTCACAAAAGAT 320
CWC245 CGATAATAAAGAAATATTTAATGTAAAAACTGAATTTTTA
CP13 TGATAATAAAGAAATATTTAATGTAAAAACTGAATTTATG 360
CWC245 AATGGTGCAATTTATGATATCGAATTTACTGTATCATCTA
CP13 AATGGTGCAATTTATGATATGAAATTTACTGTATCATCTA 400
CWC245 AAGATGGAAAATTAATAGTATCTGATATGGAAAGAACAAA
CP13 AAGATGGGGAATTAATAGTATCTGACATGGAAAGAACAAA 440
CWC245 AGTTGAGAATGAAGGAAAATATATTTTAACACCATCATTT
CP13 AATTGAGAATGAGGGAAAATATATTTTAACACCATCATTT 480
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CP13 AGAACTCAAGTTTGTACATGGGATGATGAATTATCACAAT 520
CWC245 CAATTGGGGGAGTTTATCCACAAACATATTCTGATAGATT
CP13 CAATTGGGGGAGTTGATCCAAAAACATATTCTACTAGATT 560
CWC245 TACATATTATGCAGATAATATATTATTAAACTTCAGACAA
CP13 TACATATTATGCAGACAATATATTATTAAACTTTAGACAA 600
CWC245 TATGCAACTTCAGGTTCAAGAGATTTAAAAGTAGAATATA
CP13 TATGCAACTTCAGGTTCAAGAGATTTAAAAGTAGAATATA 640

Figure 5. Locations of the three sets of primers that were used in our attempts to sequence *cpb2* from our *cpb2* positive *C. perfringens* isolates, as well as for the three simplex PCRs. CWC245 is *a C. perfringens* type C strain (Gibert et al., 1997), and CP13 is a type A *C. perfringens* strain (Shimizu et al., 2002). The start and stop codons of the ORF are indicated by a box around the bases. The black bar represents the signal sequences that are removed when the protein is secreted. * Below the sequences indicates a match in the base-pairs between the sequences. The bases highlighted in yellow indicate the location of the primers used in the multiplex PCR and cpb2PCR-1. The bases that are highlighted in green indicate the location of the primers designated by Herholz et al. (1999) that were used in cpb2PCR-2. The bases that are highlighted in orange indicate the location of the primers that were designed by this laboratory and used for cpb2PCR-2.

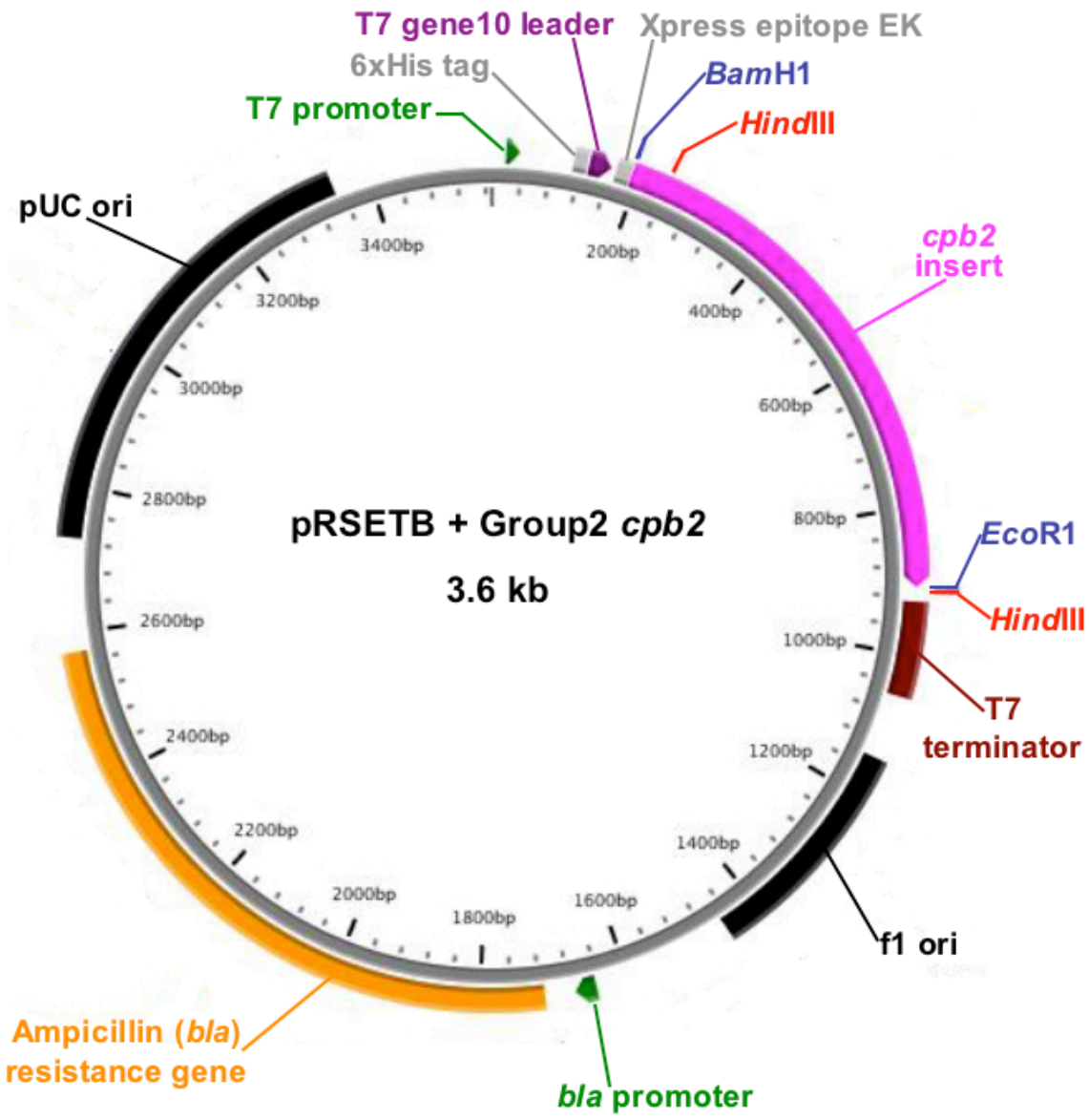


Figure 6. Plasmid map of a group-2 *cpb2* inserted into the plasmid pRSETB. f1 ori: bacteriophage f1 origin of replication. pUC ori: bacterial pUC origin of replication. Ampicillin (*bla*) resistance gene, a beta-lactamase to confer ampicillin resistance. *Bam*H1: *Bam*H1 restriction enzyme site. *Hind*III: *Hind*III restriction enzyme site. *Eco*R1: *Eco*R1 restriction enzyme site. Generated using PlasMapper (Dong et al., 2004).

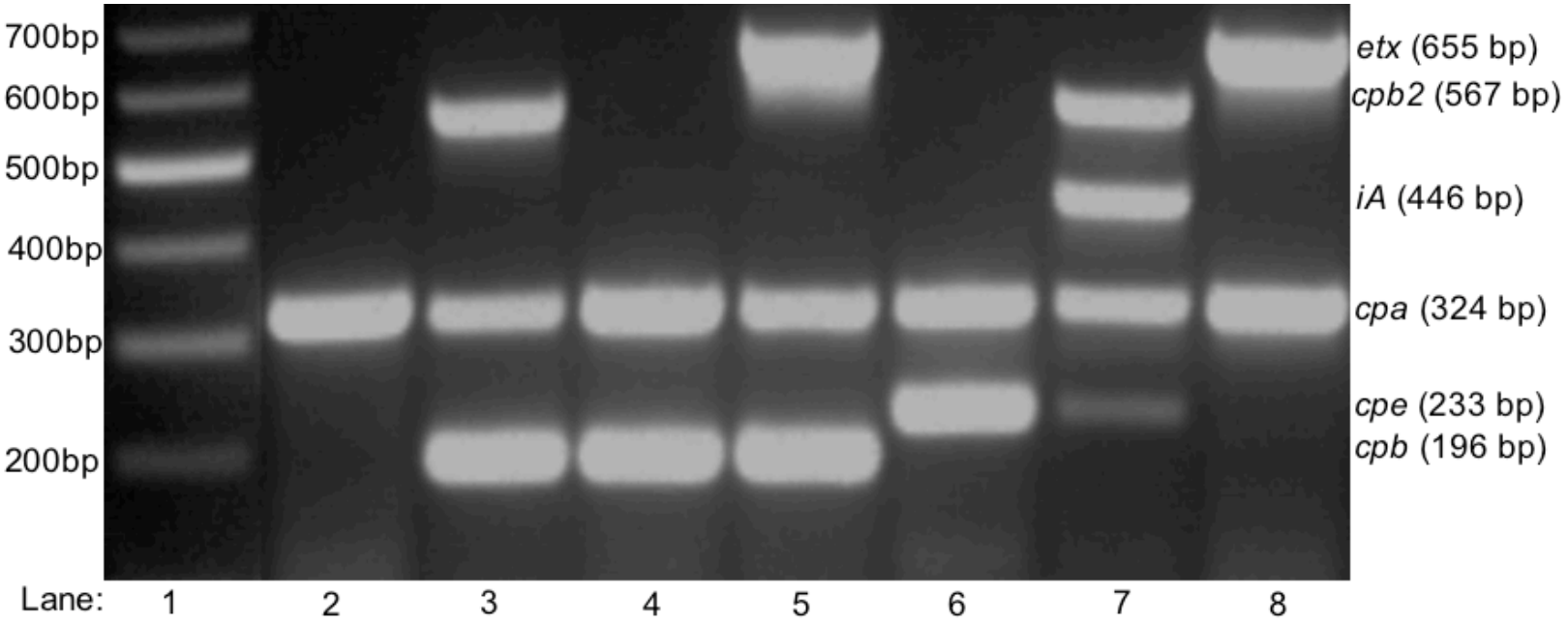


Figure 7. Multiplex PCR image of *C. perfringens* reference strains used as positive controls for the six genes, *cpa*, *cpb*, *etx*, *iA*, *cpe*, and *cpb2*, that were amplified if that gene was present in the genomic DNA of the *C. perfringens* strain being tested. Each of the six gene fragments that were amplified produced a band of a specific size, shown to the right of the image (bp: base pairs). Lane 1: 100 base pair DNA ladder, the size of each band is shown to the left of the image. Lane 2: ATCC 13124, type A, *cpa* positive. Lane 3: ATCC 10719, type C, *cpa*, *cpb,* and *cpb2* positive. Lane 4: NCTC 3227, type C, *cpa* and *cpb* positive. Lane 5: NCTC 4964, type B, *cpa*, *cpb*, and *etx* positive. Lane 6: ATCC 12917, type A, *cpa* and *cpe* positive. Lane 7: ATCC 27324, type E, *cpa*, *iA*, *cpe*, and *cpb2* positive. Lane 8: NCTC 8503, type D, *cpa* and *etx* positive.

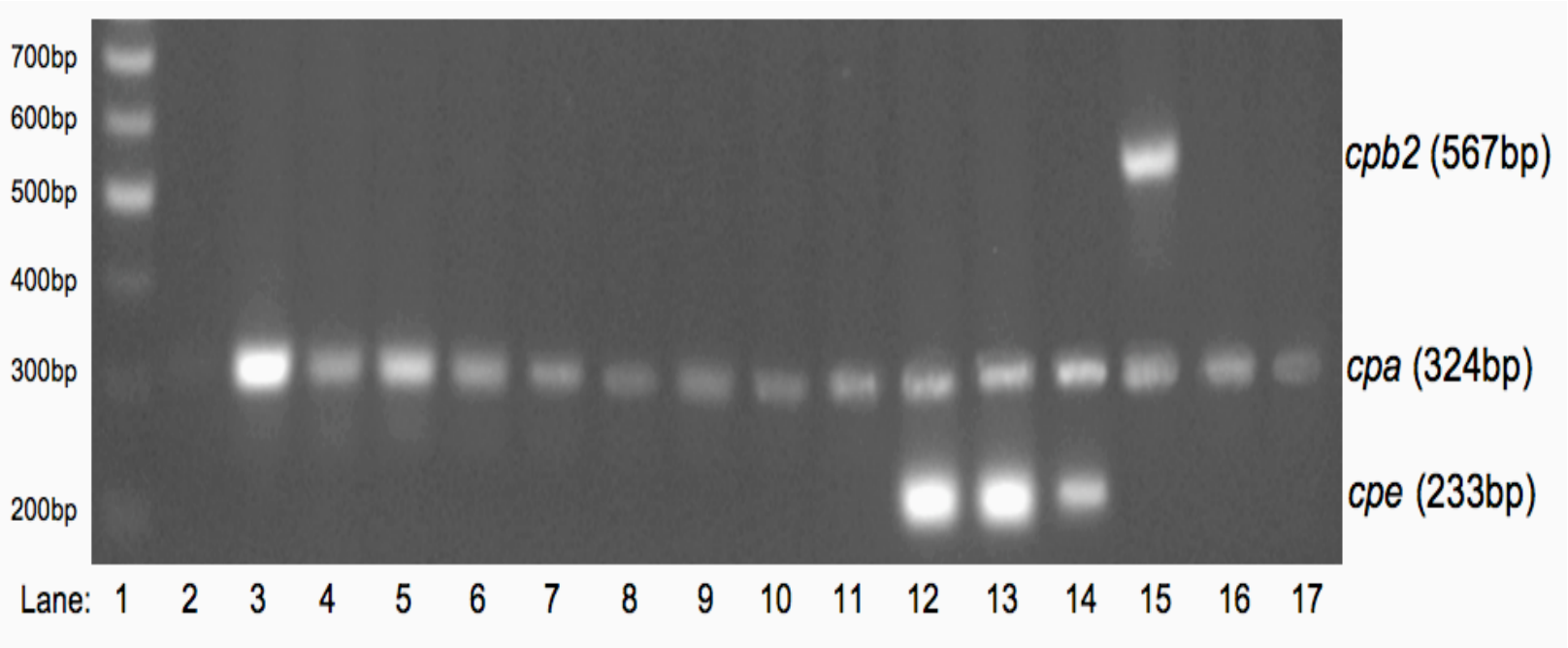


Figure 8. Multiplex PCR analysis of genomic DNA isolated from *C. perfringens* from humans with gastrointestinal disease. Refer to figure 7 for an image of the multiplex PCR C perfringens reference strains used as positive controls. The three gene fragments that were amplified in these samples produced a band of a specific size, shown to the right of the image (bp: base pairs). Lane 1: 100 base pair DNA ladder. Lane 2: H_2O blank. Lanes 3 - 11, and 16 - 17: *cpa* positive isolates. Lanes 12 - 14: *cpa* and *cpe* positive isolates. Lane 15: *cpa* and *cpb2* positive isolates.

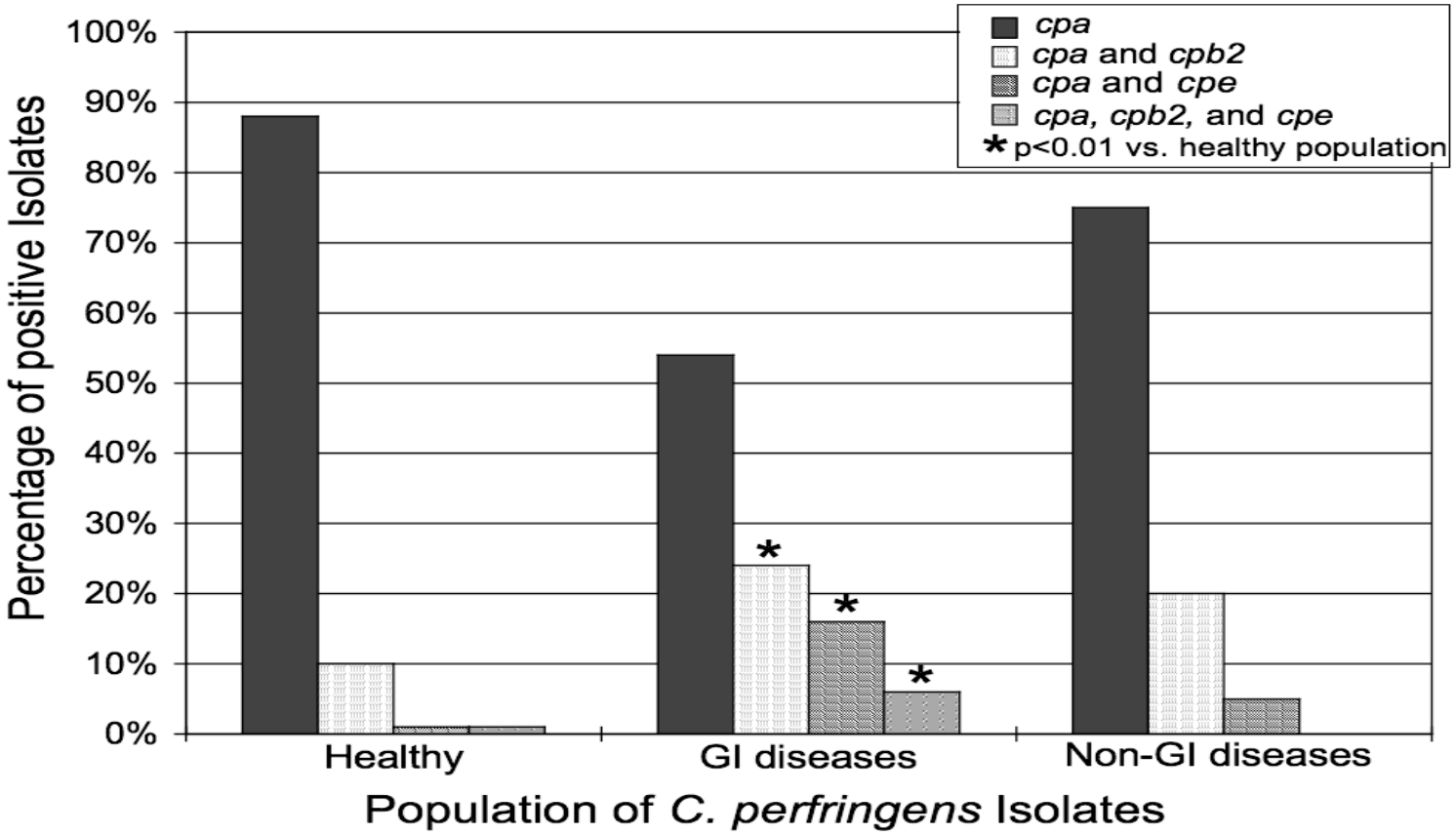
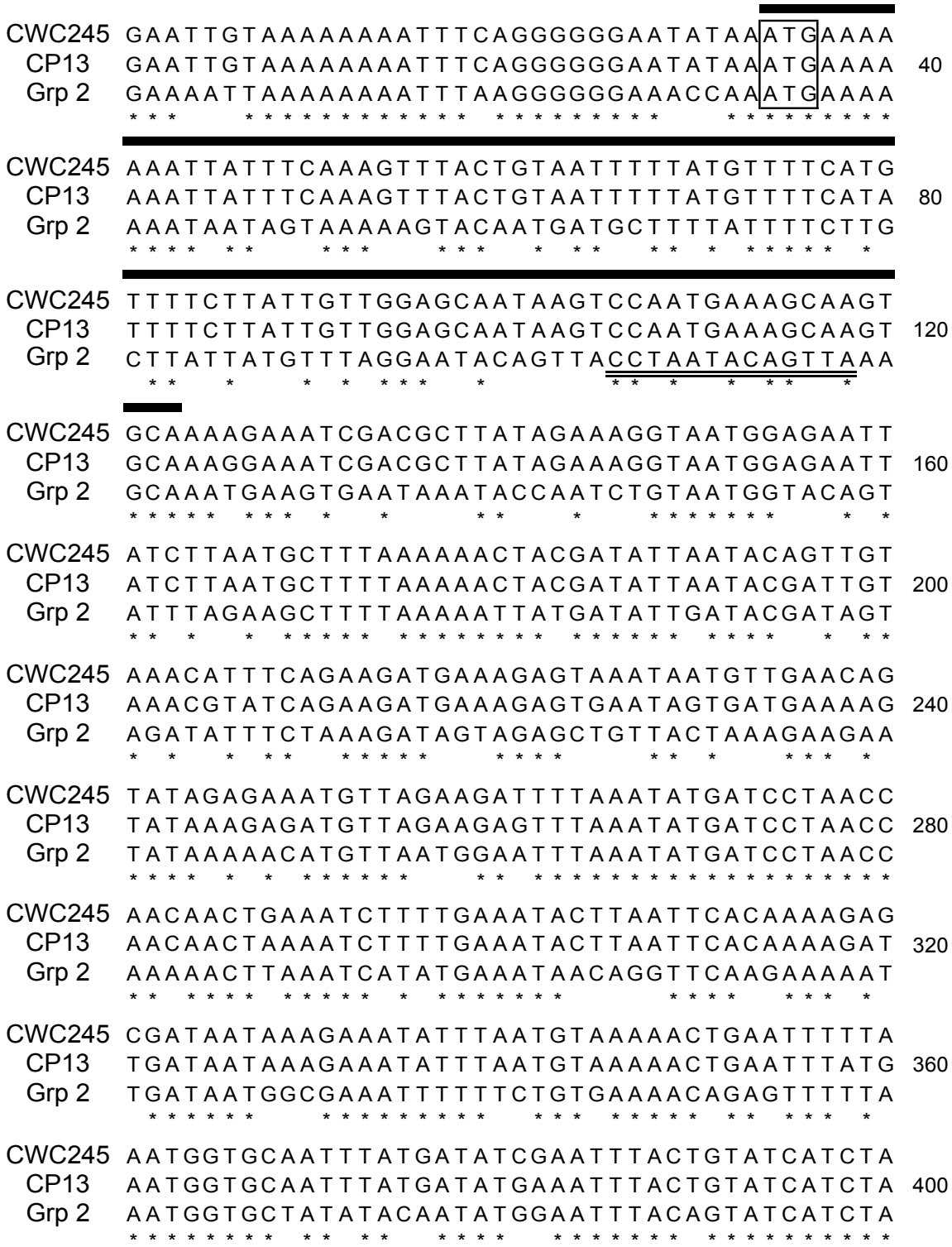


Figure 9. Comparison of the percentages of *C. perfringens* isolates that are positive for *cpa*, *cpa* and *cpb2*, *cpa* and *cpe*, and *cpa*, *cpb2*, and *cpe* for the three populations of *C. perfringens* isolates. Genotyping results are based on the multiplex PCR, and were verified by simplex PCR. The population of isolates from healthy human volunteers was considered the control group for statistical analysis, a $p<0.05$ was considered significantly different.


CWC245 GAATTGTAAAAAAAATTTCAGGGGGGAATATAAATGAAAA
CP13 GAATTGTAAAAAAAATTTCAGGGGGGAATATAAATGAAAA 40
Grp 2 GAAAATTAAAAAAAATTTAAGGGGGGAAACCAAATGAAAA
CWC245 AAATTATTTCAAAGTTTACTGTAATTTTTATGTTTTCATG
CP13 AAATTATTTCAAAGTTTACTGTAATTTTTATGTTTTCATA 80
Grp 2 AAATAATAGTAAAAAGTACAATGATGCTTTTATTTTCTTG
CWC245 TTTTCTTATTGTTGGAGCAATAAGTCCAATGAAAGCAAGT
CP13 TTTTCTTATTGTTGGAGCAATAAGTCCAATGAAAGCAAGT 120
Grp 2 CTTATTATGTTTAGGAATACAGTTACCTAATACAGTTAAA
CWC245 GCAAAAGAAATCGACGCTTATAGAAAGGTAATGGAGAATT
CP13 GCAAAGGAAATCGACGCTTATAGAAAGGTAATGGAGAATT 160
Grp 2 GCAAATGAAGTGAATAAATACCAATCTGTAATGGTACAGT
CWC245 ATCTTAATGCTTTAAAAAACTACGATATTAATACAGTTGT
CP13 ATCTTAATGCTTTTAAAAACTACGATATTAATACGATTGT 200
Grp 2 ATTTAGAAGCTTTTAAAAATTATGATATTGATACGATAGT
CWC245 AAACATTTCAGAAGATGAAAGAGTAAATAATGTTGAACAG
CP13 AAACGTATCAGAAGATGAAAGAGTGAATAGTGATGAAAAG 240
Grp 2 AGATATTTCTAAAGATAGTAGAGCTGTTACTAAAGAAGAA
CWC245 TATAGAGAAATGTTAGAAGATTTTAAATATGATCCTAACC
CP13 TATAAAGAGATGTTAGAAGAGTTTAAATATGATCCTAACC 280
Grp 2 TATAAAAACATGTTAATGGAATTTAAATATGATCCTAACC
CWC245 AACAACTGAAATCTTTTGAAATACTTAATTCACAAAAGAG
CP13 AACAACTAAAATCTTTTGAAATACTTAATTCACAAAAGAT 320
Grp 2 AAAAACTTAAATCATATGAAATAACAGGTTCAAGAAAAAT
CWC245 CGATAATAAAGAAATATTTAATGTAAAAACTGAATTTTTA
CP13 TGATAATAAAGAAATATTTAATGTAAAAACTGAATTTATG 360
Grp 2 TGATAATGGCGAAATTTTTTCTGTGAAAACAGAGTTTTTA
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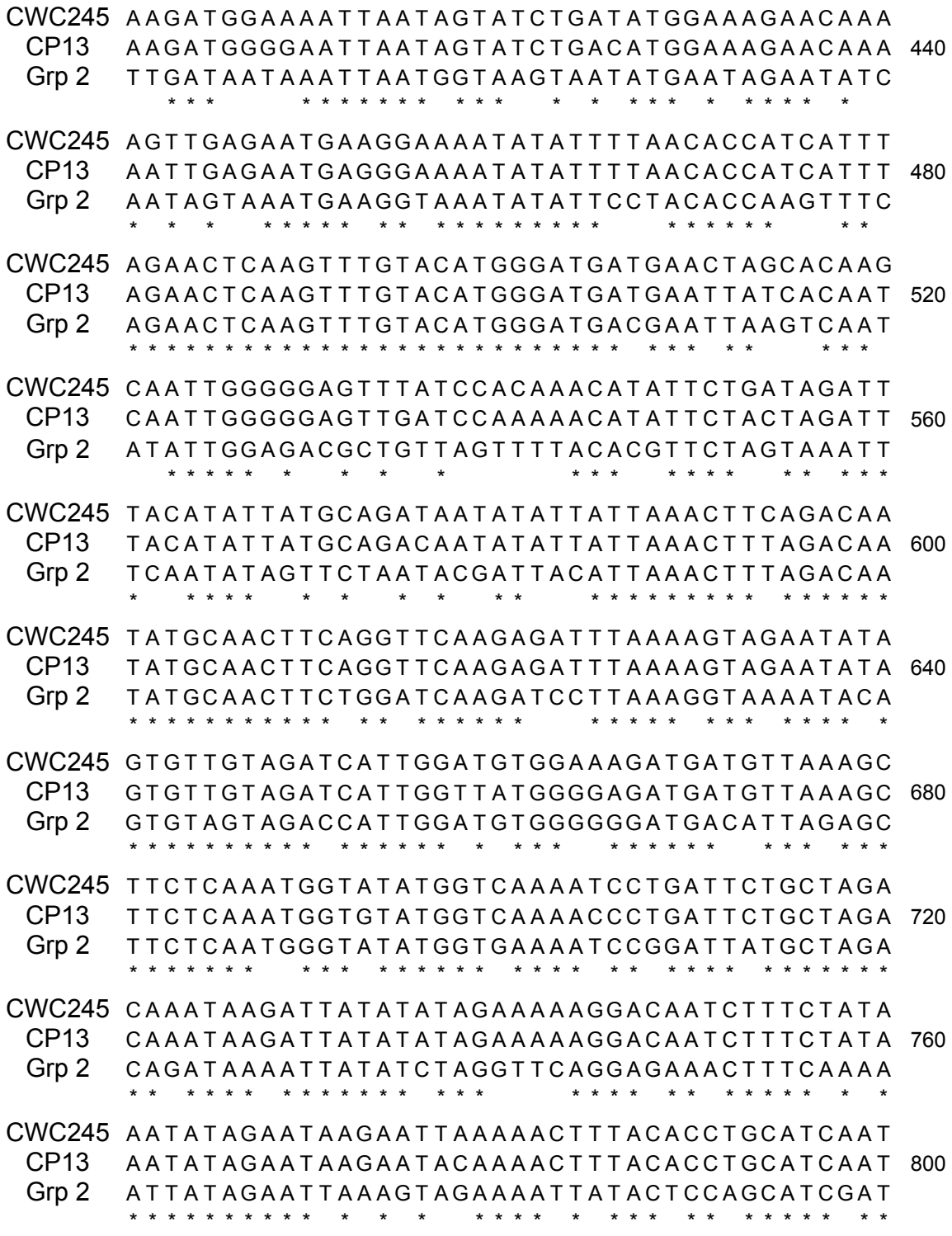
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CP13 AAGATGGGGAATTAATAGTATCTGACATGGAAAGAACAAA 440
Grp 2 TTGATAATAAATTAATGGTAAGTAATATGAATAGAATATC
CWC245 AGTTGAGAATGAAGGAAAATATATTTTAACACCATCATTT
CP13 AATTGAGAATGAGGGAAAATATATTTTAACACCATCATTT 480
Grp 2 AATAGTAAATGAAGGTAAATATATTCCTACACCAAGTTTC
CWC245 AGAACTCAAGTTTGTACATGGGATGATGAACTAGCACAAG
CP13 AGAACTCAAGTTTGTACATGGGATGATGAATTATCACAAT 520
Grp 2 AGAACTCAAGTTTGTACATGGGATGACGAATTAAGTCAAT
CWC245 CAATTGGGGGAGTTTATCCACAAACATATTCTGATAGATT
CP13 CAATTGGGGGAGTTGATCCAAAAACATATTCTACTAGATT 560
Grp 2 ATATTGGAGACGCTGTTAGTTTTACACGTTCTAGTAAATT
CWC245 TACATATTATGCAGATAATATATTATTAAACTTCAGACAA
CP13 TACATATTATGCAGACAATATATTATTAAACTTTAGACAA 600
Grp 2 TCAATATAGTTCTAATACGATTACATTAAACTTTAGACAA
CWC245 TATGCAACTTCAGGTTCAAGAGATTTAAAAGTAGAATATA
CP13 TATGCAACTTCAGGTTCAAGAGATTTAAAAGTAGAATATA 640
Grp 2 TATGCAACTTCTGGATCAAGATCCTTAAAGGTAAAATACA
CWC245 GTGTTGTAGATCATTGGATGTGGAAAGATGATGTTAAAGC
CP13 GTGTTGTAGATCATTGGTTATGGGGAGATGATGTTAAAGC 680
Grp 2 GTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGC
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CP13 TTCTCAAATGGTGTATGGTCAAAACCCTGATTCTGCTAGA 720
Grp 2 TTCTCAATGGGTATATGGTGAAAATCCGGATTATGCTAGA
CWC245 CAAATAAGATTATATATAGAAAAAGGACAATCTTTCTATA
CP13 CAAATAAGATTATATATAGAAAAAGGACAATCTTTCTATA 760
Grp 2 CAGATAAAATTATATCTAGGTTCAGGAGAAACTTTCAAAA
CWC245 AATATAGAATAAGAATTAAAAACTTTACACCTGCATCAAT
CP13 AATATAGAATAAGAATACAAAACTTTACACCTGCATCAAT 800
Grp 2 ATTATAGAATTAAAGTAGAAAATTATACTCCAGCATCGAT

Figure 10. Nucleotide alignment of three versions of *cpb2.* CWC245, a *C. perfringens* type C isolate, and CP13, a type A *C. perfringens* strain, are published sequences. The Grp2 *cpb2* sequence is a consensus sequence derived from 58 nucleotide sequences that were homologous to each other by 87% to 99%, that were determined in the course of this research study. The start and stop codons of the open reading frame are shown by a box around the bases. * Below the nucleotide sequences indicates a match between the base pairs. The black bar above represents the signal sequences that is removed when the protein is secreted. The double underline indicates the deletion seen in Group-2T, which results in a premature stop codon.

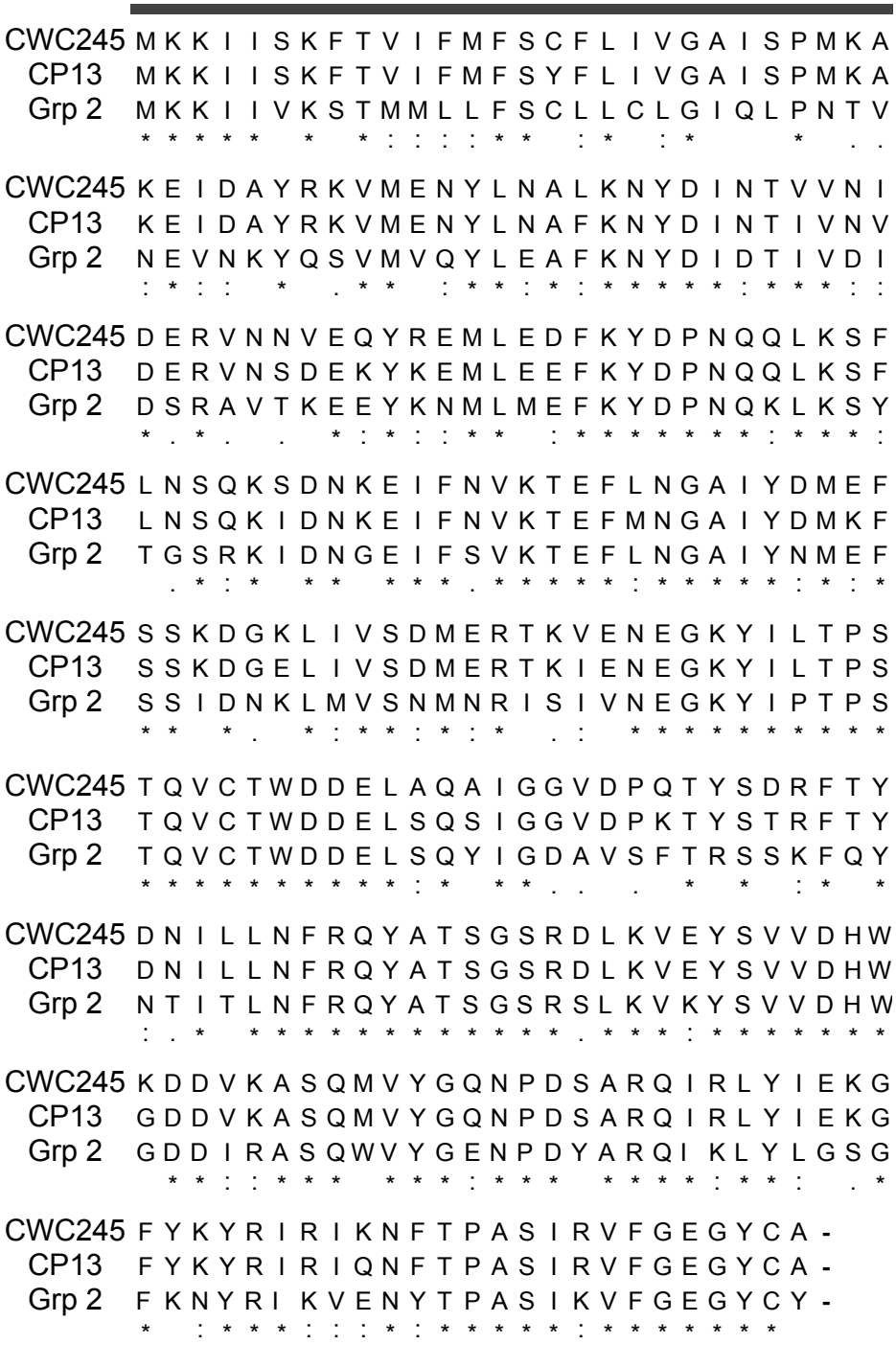

Figure 11. Alignment of the protein sequences of the three versions of the beta2 toxin, deduced from the gene sequences that were done as part of this investigation. The black bar represents the signal sequences that is removed when the protein is secreted. *Below the amino acid sequence indicates a match between amino acids. : Conserved amino acid substitution. . Semi-conserved amino acid substitution.

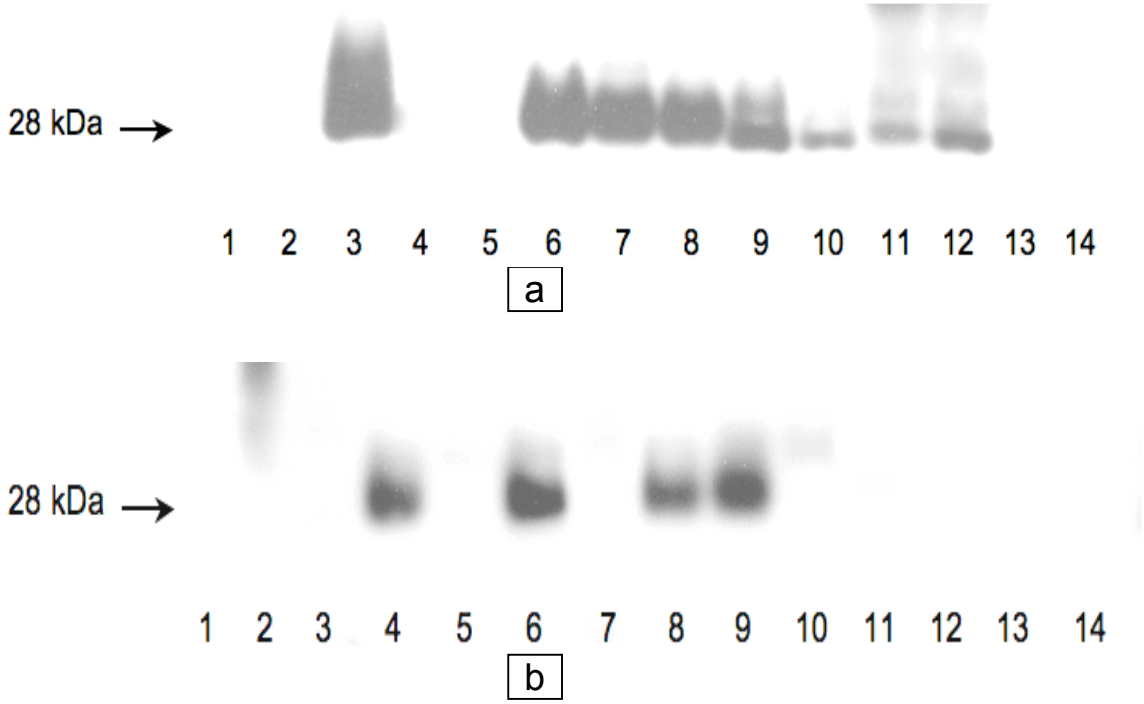

Figure 12. Western blotting analysis of beta2 protein toxin production by selected *cpb2* positive *C. perfringens* isolates. Cell culture supernatant protein from *C. perfringens* isolates was collected and subjected to SDS-polyacrylamide gel electrophoresis followed by western blotting using a poly-clonal anti-beta2 antibody.

Image a: *Clostridium perfringens* isolates from patients with gastrointestinal diseases. Lanes 1, 2, 4, 5, 13, and 14 are negative for production of the beta2 protein toxin. Lanes 3 and 6-12 are positive for production of the beta2 protein toxin. The arrow to the left indicates the migration size of the beta2 protein toxin immunoreactive band at 28 kDa.

Image b: *Clostridium perfringens* isolates from healthy human volunteers and from patients with non-gastrointestinal *C. perfringens* related diseases. Lanes 1-3, 5, and 10-14 are negative for production of the beta2 protein toxin. Lanes 4, 6, 8 and 9 are positive for production of the beta2 protein toxin. The arrow to the left indicates the migration size of the beta2 protein toxin immunoreactive band at 28 kDa.

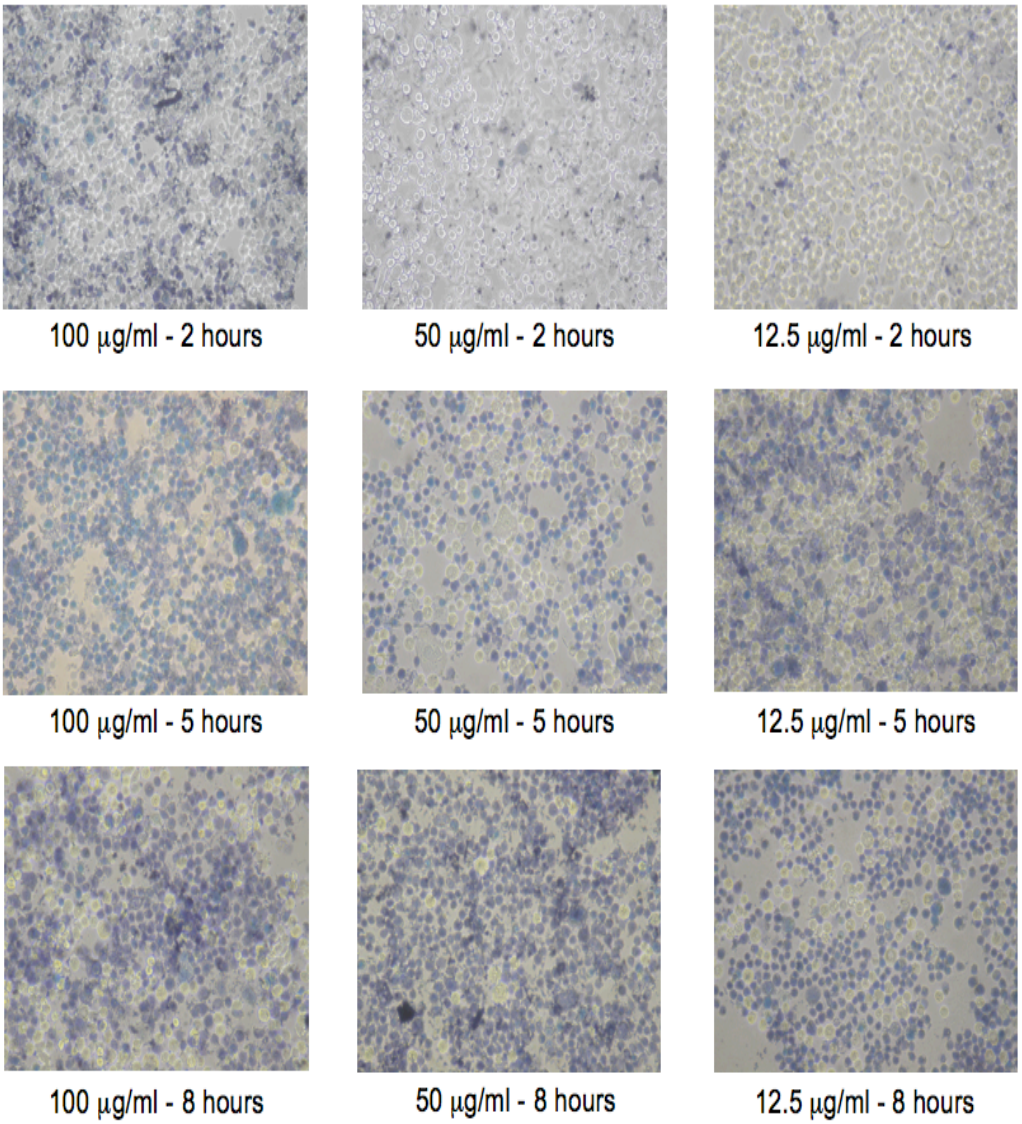

Figure 13. The effects of recombinant beta2 protein from clone pHRD103 on caco-2 cells, measured using the trypan blue exclusion assay. Caco-2 cells grown to 80% confluence were exposed to cell culture supernatant fluid from the *E. coli* clone with pHRD103, a recombinant group-1 *cpb2* plasmid, at three different concentrations: 100 μg/ml, 50 μg/ml, and 12.5 μg/ml. Trypan blue stain, which is taken up by cells that are dead or have damaged membranes, was added at three time points: 2 hours, 5 hours, and 8 hours to assess the effects of the three separate concentrations of the recombinant beta2 protein toxin over time. The percentage of cell death determined from these images and plotted in bar graph form is shown in figure 15.

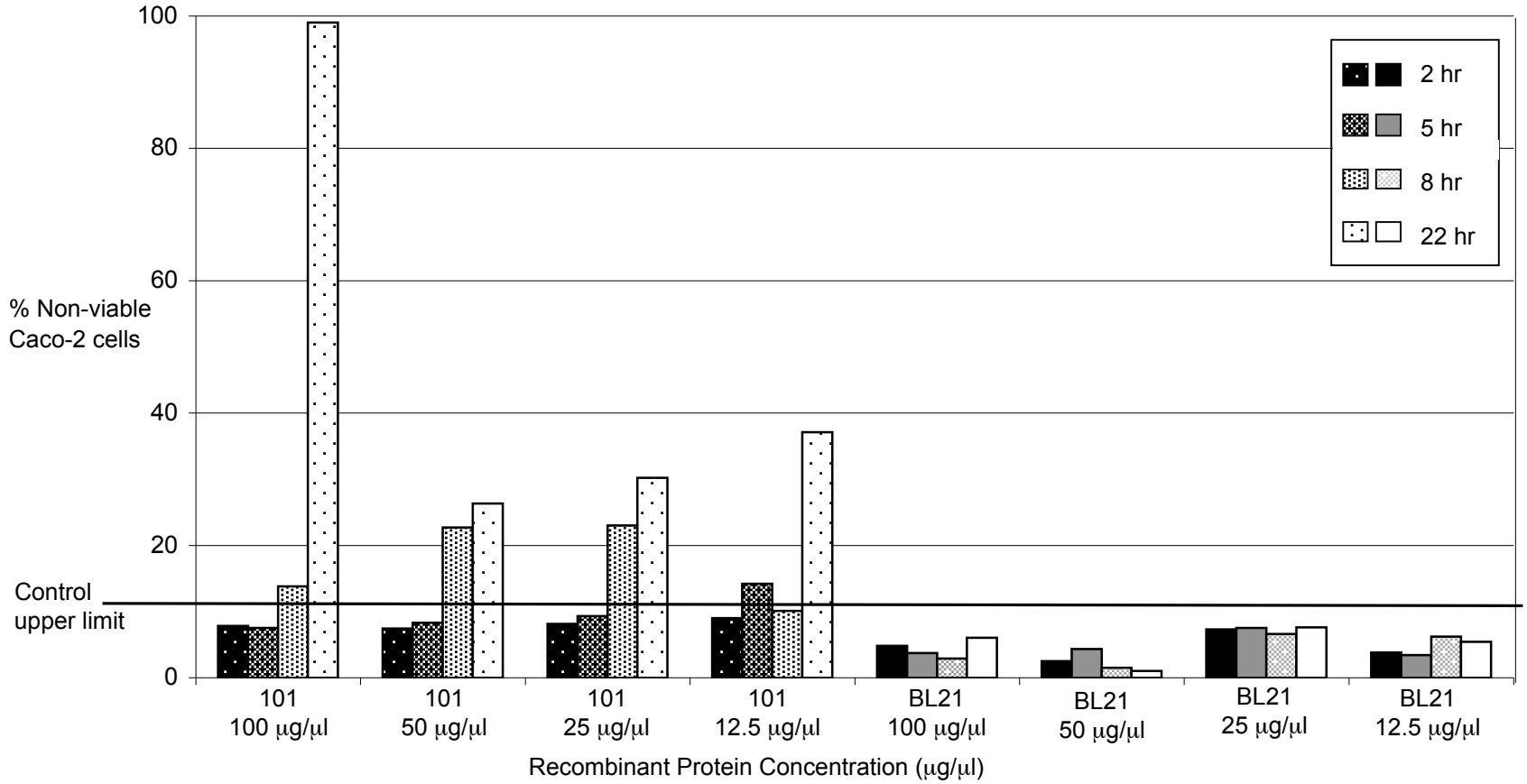

Figure 14. Trypan blue exclusion assay results for clone pHRD101 and the negative control clone BL21(DE3)pLysS. The highest percentage of caco-2 cell damage that was observed with exposure to the cell culture supernatant fluid from the BL21 control is indicated by the bold horizontal line that is labeled on the left side as the control upper limit. This control upper limit is also included on figures 15 - 18, the trypan blue exclusion assay bar graphs of the results of the remaining recombinant beta2 protein toxin samples.

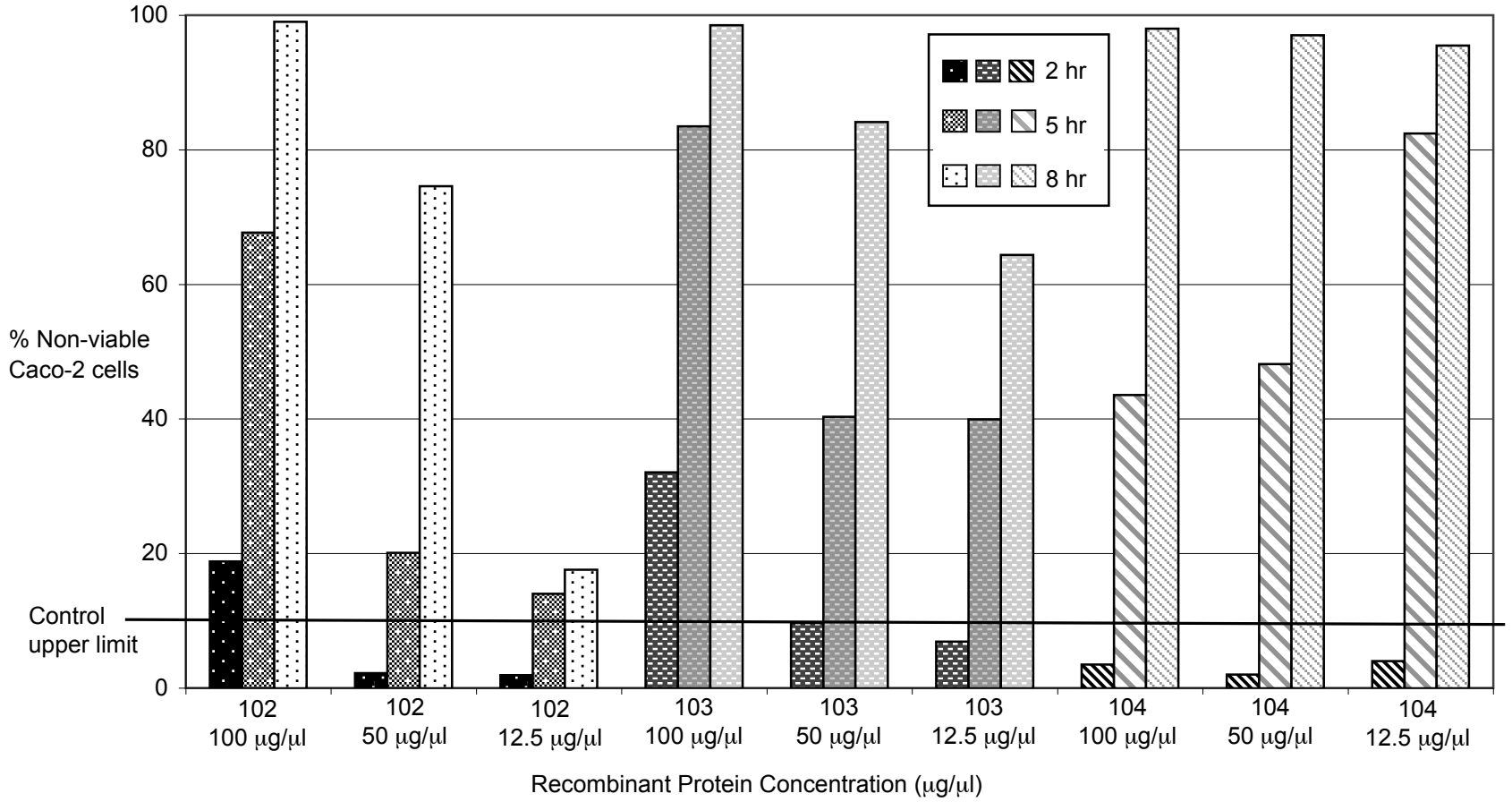

Figure 15. Trypan blue exclusion assay results for clones pHRD102, pHRD103, and pHRD104. The highest percentage of caco-2 cell damage that was observed with exposure to the cell culture supernatant fluid from the BL21 control is indicated by the bold horizontal line that is labeled on the left side as the control upper limit.

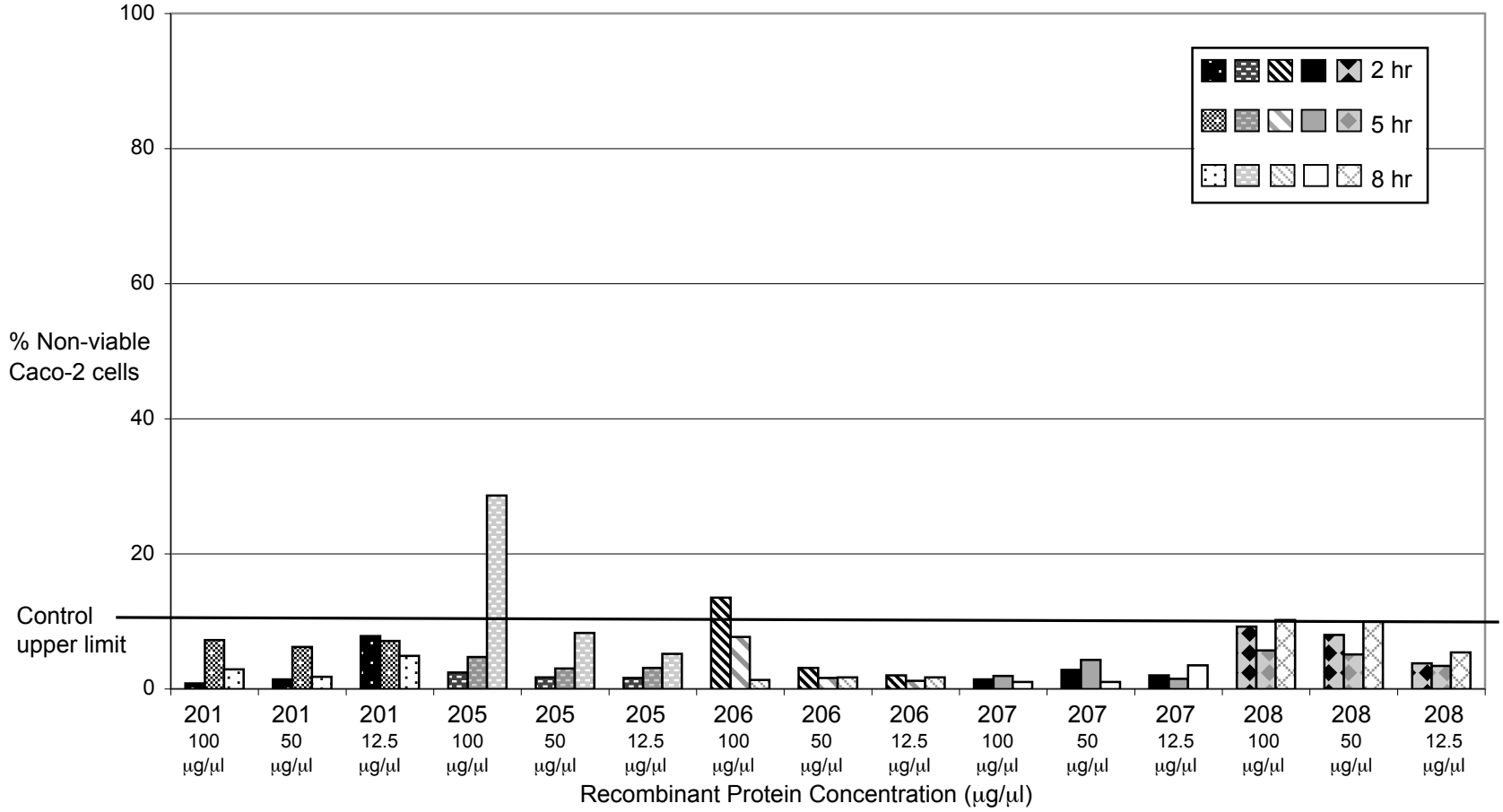

Figure 16. Trypan blue exclusion assay results for clones pHRD201, pHRD205, pHRD206, pHRD207, and pHRD208. The highest percentage of caco-2 cell damage that was observed with exposure to the cell culture supernatant fluid from the BL21 control is indicated by the bold horizontal line that is labeled on the left side as the control upper limit.

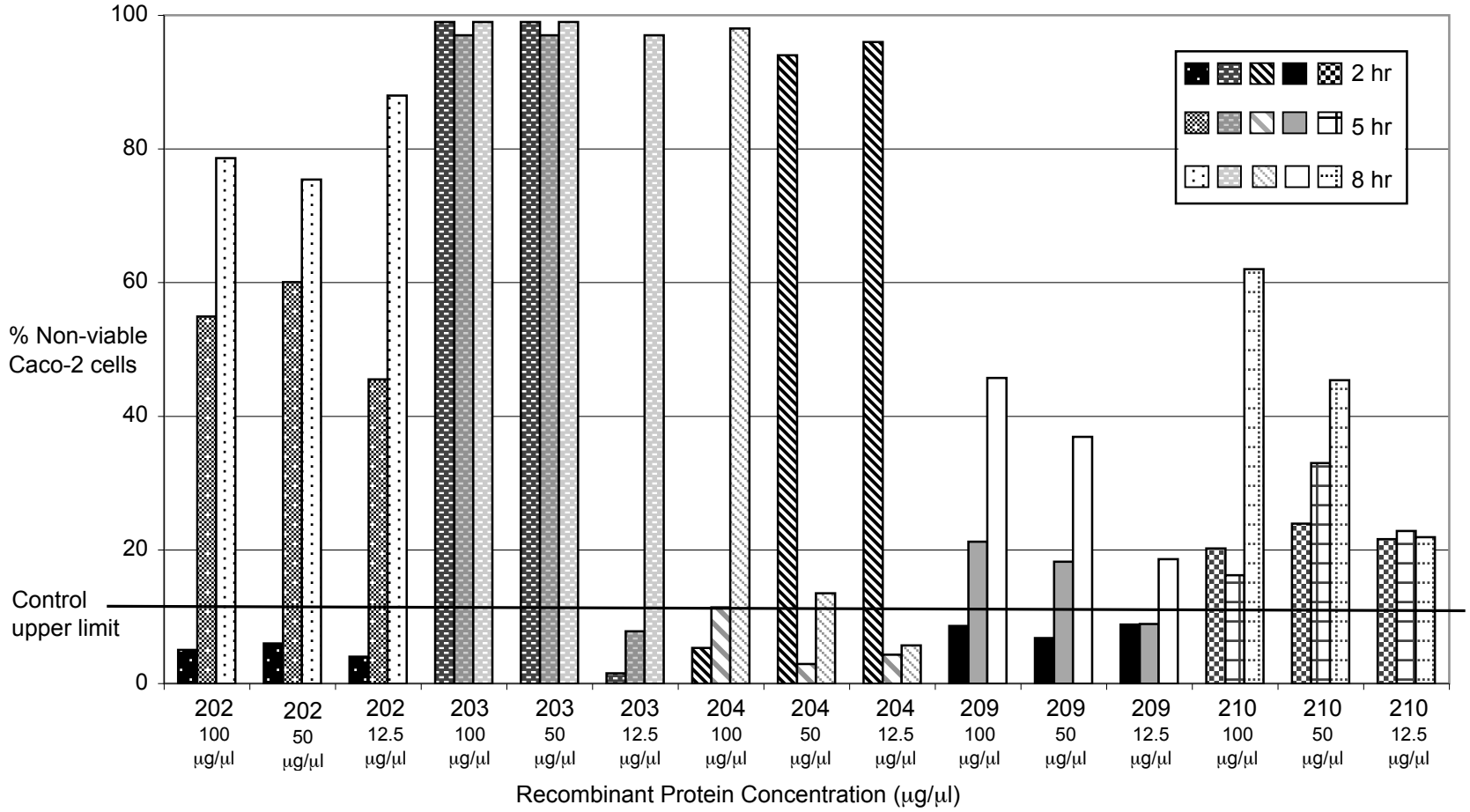

Figure 17. Trypan blue exclusion assay results for clones pHRD202, pHRD203, pHRD204, pHRD209, and pHRD210. The highest percentage of caco-2 cell damage that was observed with exposure to the cell culture supernatant fluid from the BL21 control is indicated by the bold horizontal line that is labeled on the left side as the control upper limit.

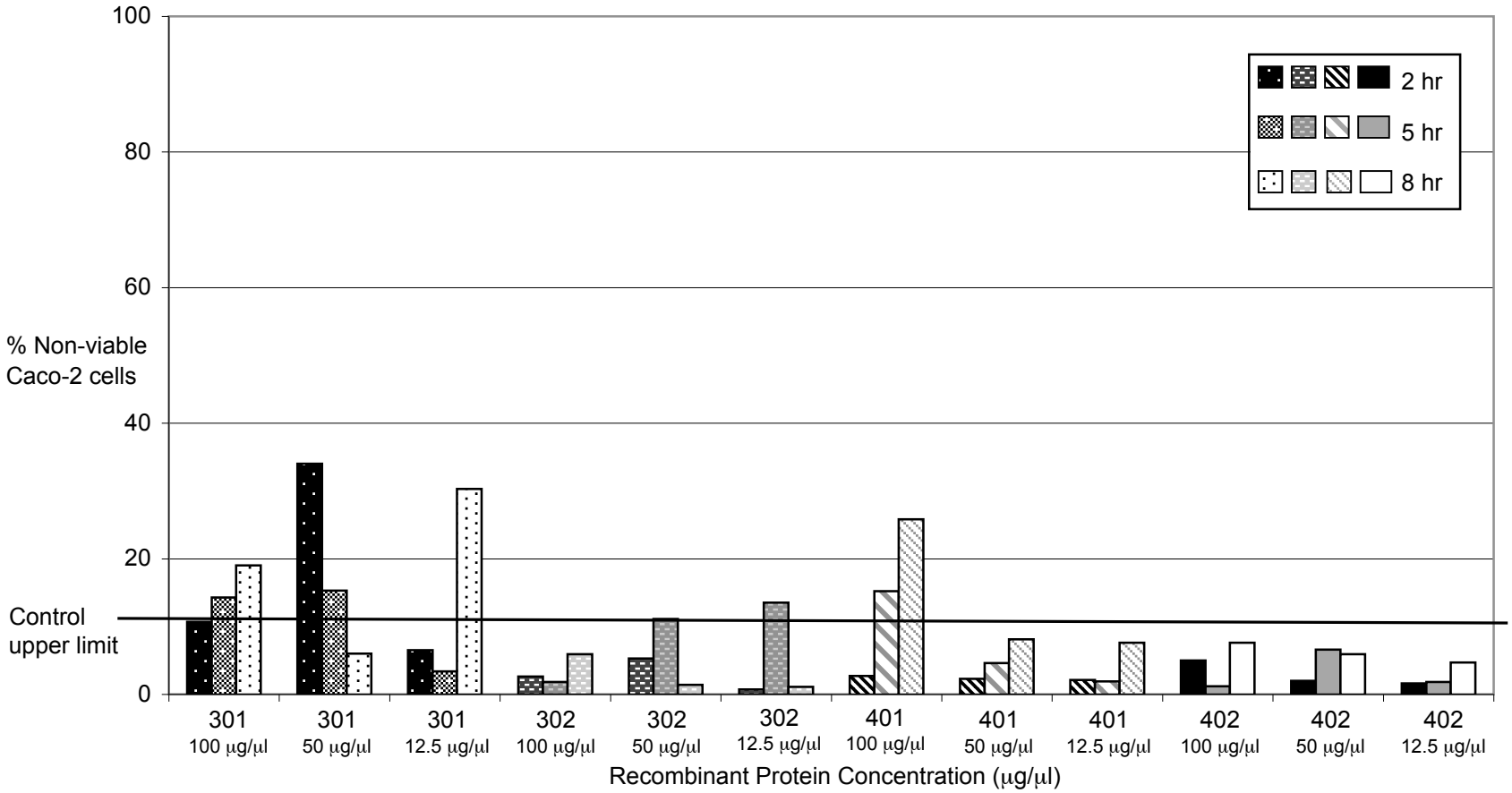

Figure 18. Trypan blue exclusion assay results for clones pHRD301, pHRD302, pHRD401, and pHRD402. The highest percentage of caco-2 cell damage that was observed with exposure to the cell culture supernatant fluid from the BL21 control is indicated by the bold horizontal line that is labeled on the left side as the control upper limit.

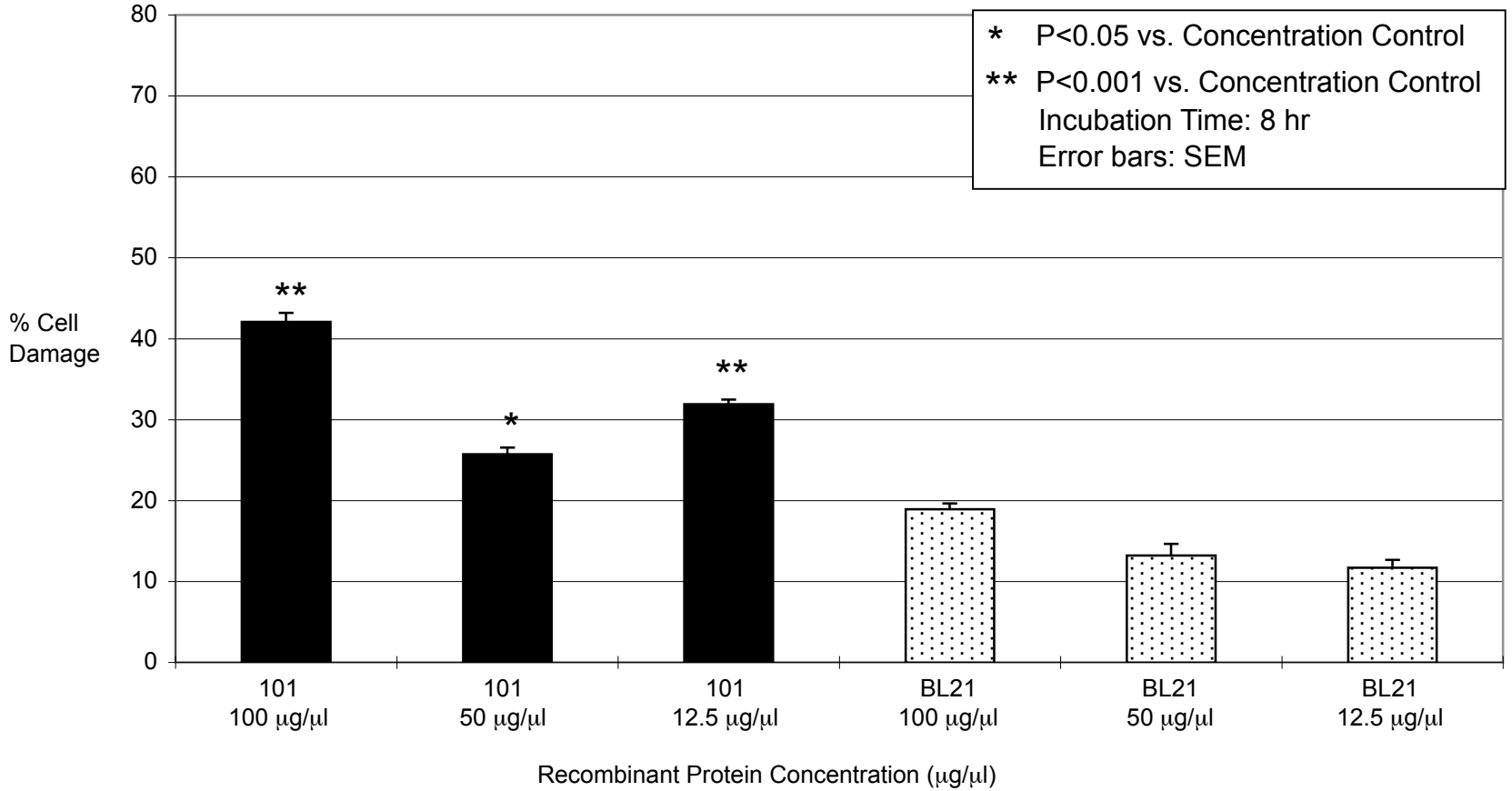

Figure 19. LDH release assay results comparing the effects of the recombinant beta2 protein from pHRD101 a group-1 *cpb2* clone, and the negative control BL21(DE3)pLysS (BL21). The percentage of cell damage that was observed at each of the three concentrations, 100 µg/ml, 50 µg/ml, and 12.5 µg/ml for the negative control BL21 is the concentration control that was used for comparison to the percentage of cell damage that was observed after exposure to the cell culture supernatant for each of the experimental *cpb2* clones, $p<0.05$ was considered significant.

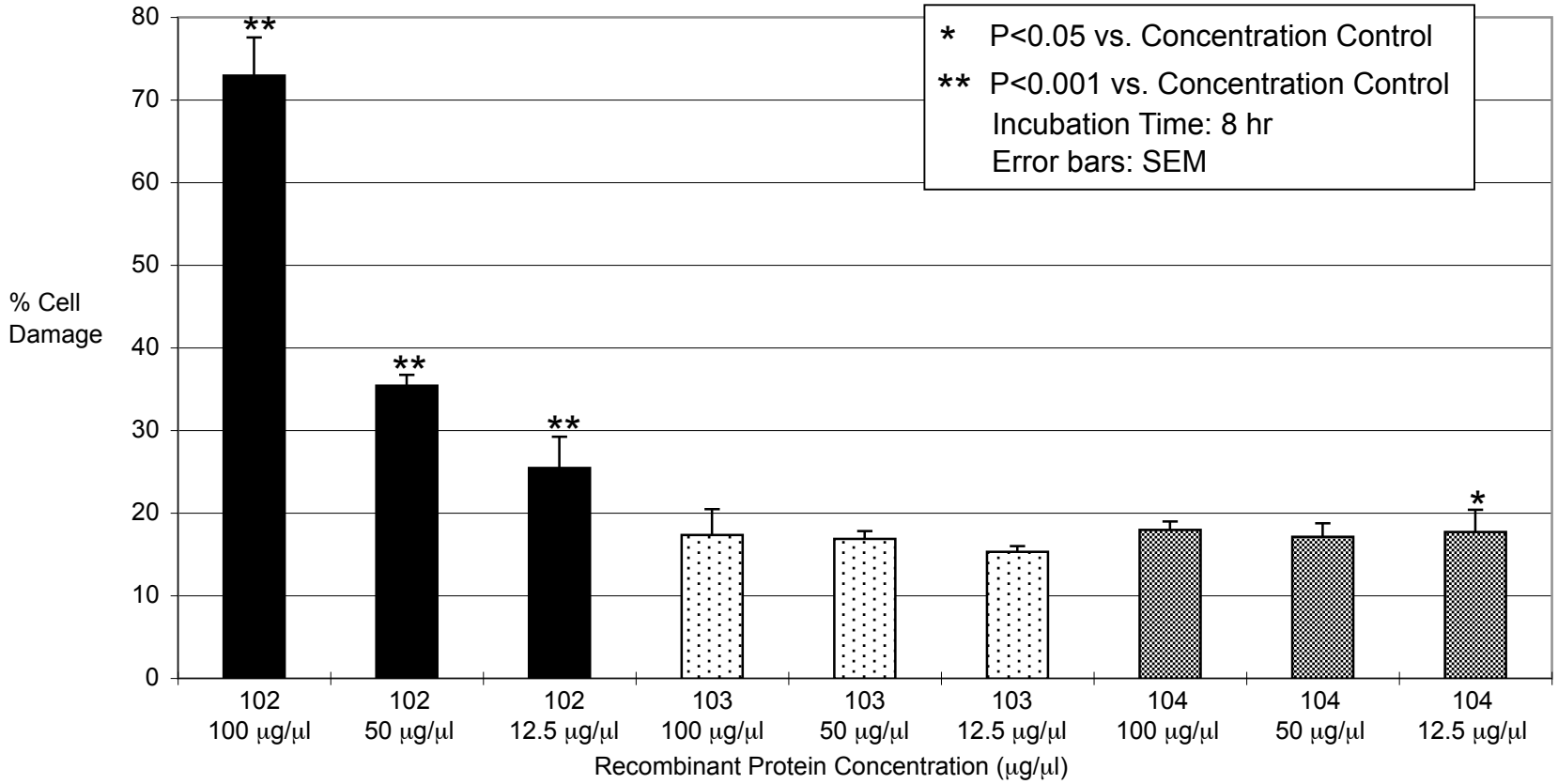

Figure 20. LDH release assay results comparing the effects of the recombinant beta2 protein from pHRD102, pHRD103, and pHRD104, group-1 *cpb2* clones after 8 hours of exposure to the cell culture supernatant with the recombinant beta2 protein toxin. The percentage of cell damage that was observed at each of the three concentrations, 100 µg/ml, 50 µg/ml, and 12.5 µg/ml for the negative control BL21 (shown in figure 19) is the concentration control that was used for comparison to the percentage of cell damage that was observed after exposure to the cell culture supernatant for each of the experimental *cpb2* clones, $p<0.05$ was considered significant.

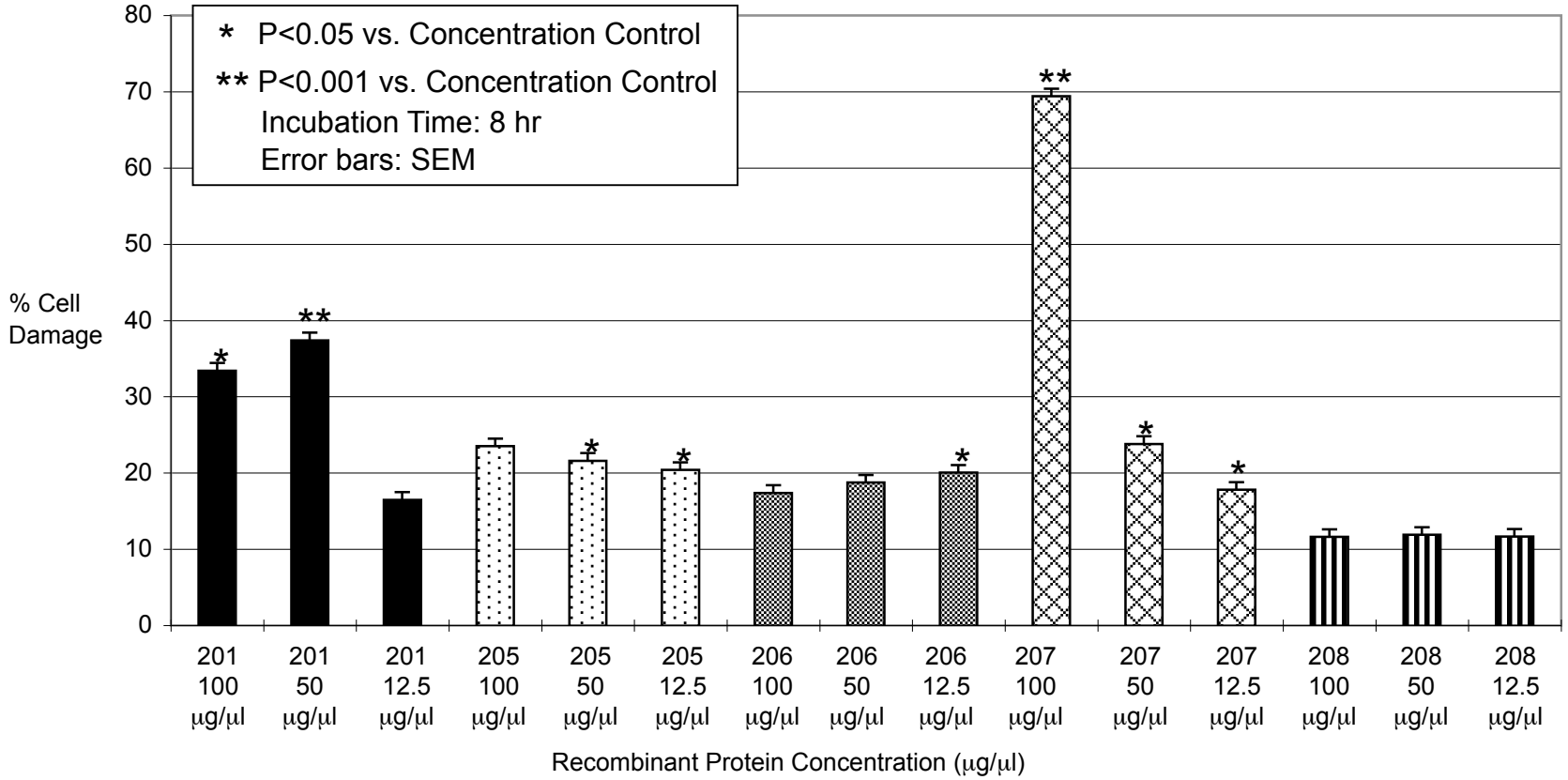

Figure 21. LDH release assay results comparing the effects of the recombinant beta2 protein from pHRD201, pHRD205, pHRD206, pHRD207, and pHRD208, group-2 *cpb2* clones after 8 hours of exposure to the cell culture supernatant with the recombinant beta2 protein toxin. The percentage of cell damage that was observed at each of the three concentrations, 100 µg/ml, 50 µg/ml, and 12.5 µg/ml for the negative control BL21 (shown in figure 19) is the concentration control that was used for comparison to the percentage of cell damage that was observed after exposure to the cell culture supernatant for each of the experimental *cpb2* clones, $p<0.05$ was considered significant.

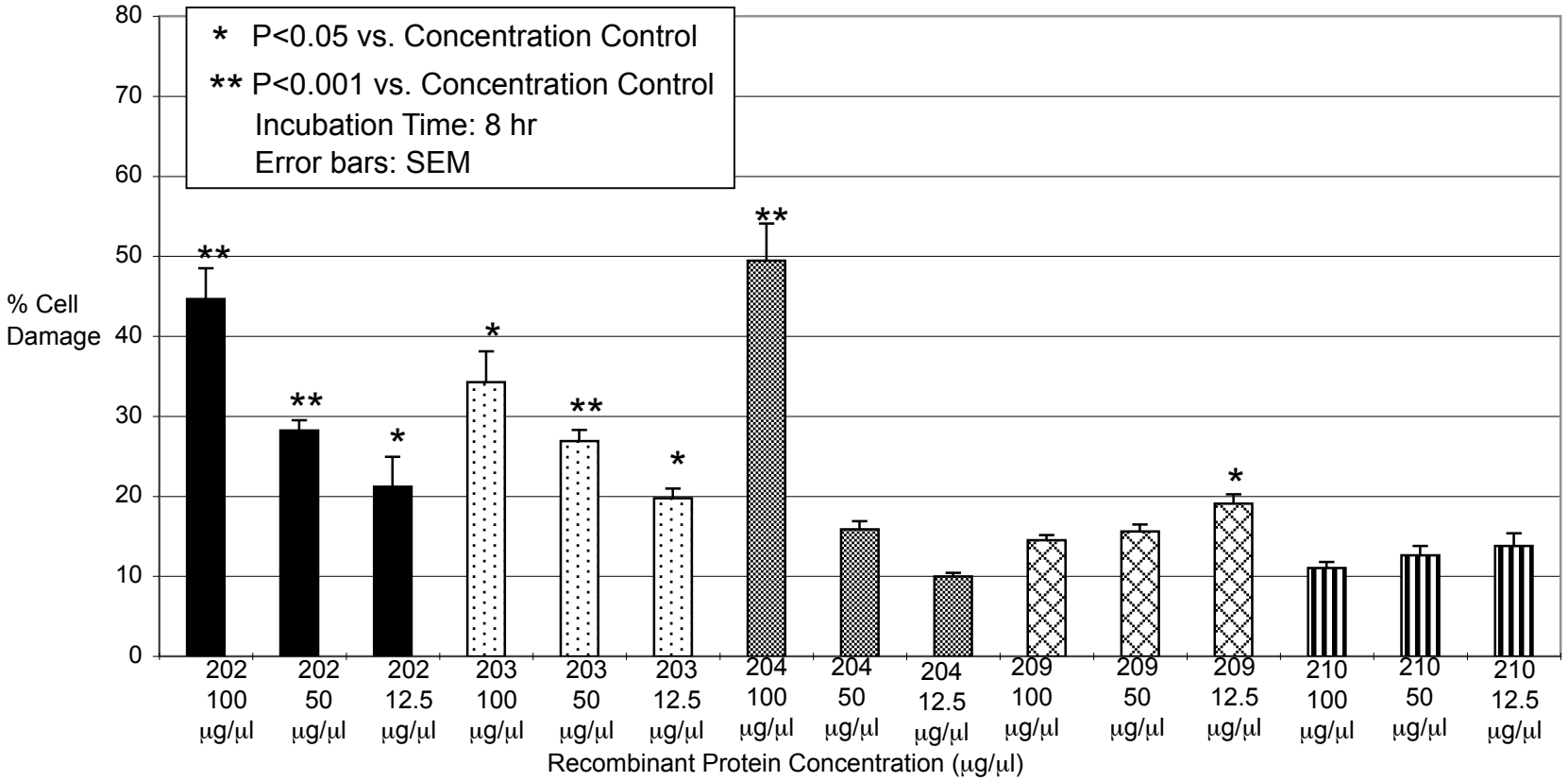

Figure 22. LDH release assay results comparing the effects of the recombinant beta2 protein from pHRD202, pHRD203, pHRD204, pHRD209, and pHRD210, group-2 *cpb2* clones after 8 hours of exposure to the cell culture supernatant with the recombinant beta2 protein toxin. The percentage of cell damage that was observed at each of the three concentrations, 100 µg/ml, 50 µg/ml, and 12.5 µg/ml for the negative control BL21 (shown in figure 19) is the concentration control that was used for comparison to the percentage of cell damage that was observed after exposure to the cell culture supernatant for each of the experimental *cpb2* clones, $p<0.05$ was considered significant.

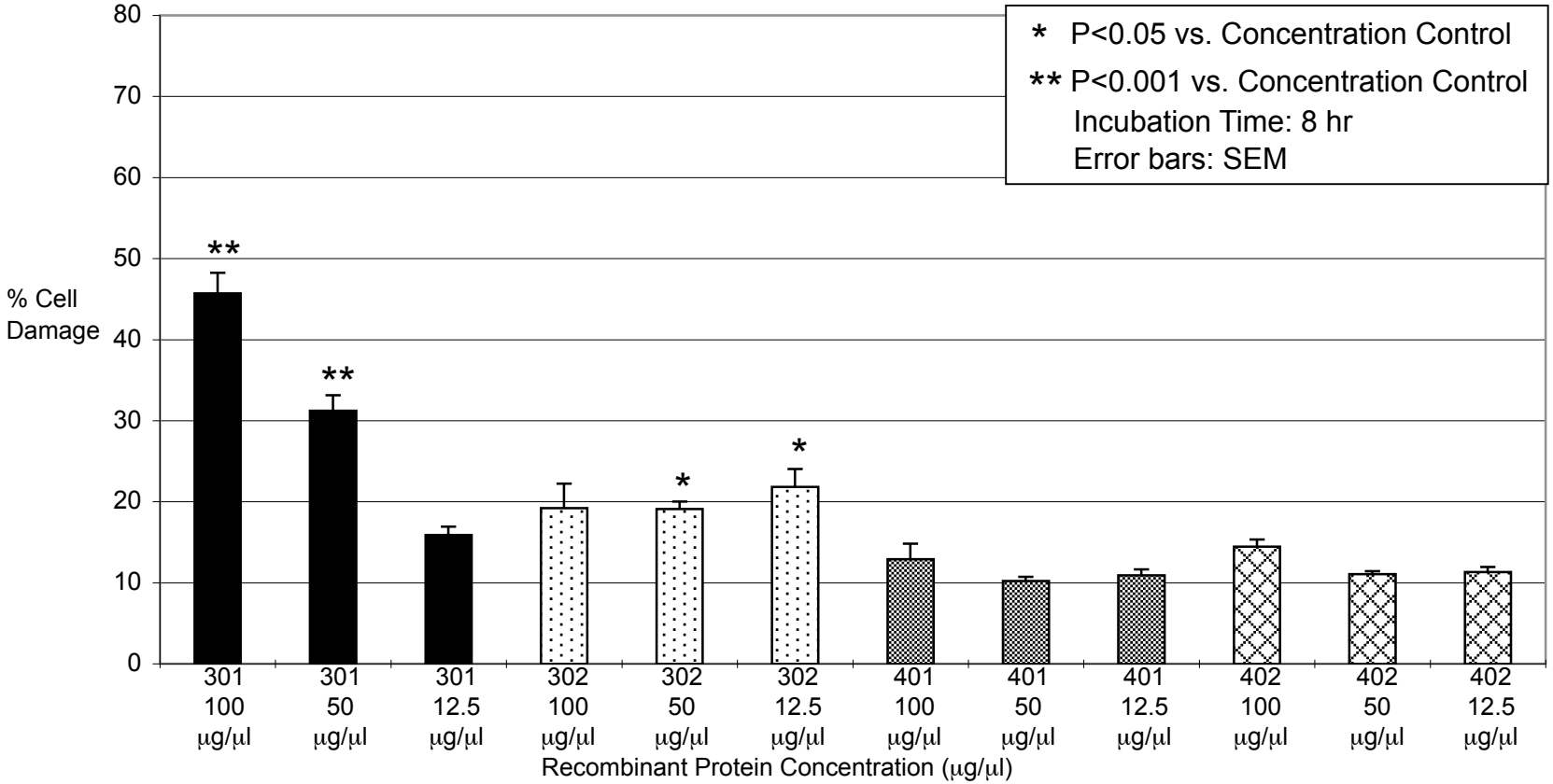

Figure 23. LDH release assay results comparing the effects of the recombinant beta2 protein from pHRD301, pHRD302, group-3 *cpb2* clones and pHRD401, and pHRD402, group-2 T *cpb2* clones after 8 hours of exposure to the cell culture supernatant with the recombinant beta2 toxin. The percentage of cell damage that was observed at each of the three concentrations, 100 µg/ml, 50 µg/ml, and 12.5 µg/ml for the negative control BL21 (shown in figure 19) is the concentration control that was used for comparison to the percentage of cell damage that was observed after exposure to the cell culture supernatant for each of the experimental *cpb2* clones, $p<0.05$ was considered significant.

APPENDIX

Table A. Specimen sources of the extra-intestinal *C. perfringens* isolates from humans.

Research Number	Specimen Source	Genotype[a]	*cpb2* group(s)[b]	Western Immunoblotting[c]
3017	Abdominal abscess	*cpa, cpb2*	2	N[d]
3033	Abdominal abscess	*cpa, cpb2*	2	N
3029	Abdominal fluid	*cpa*		
3039	Abdominal fluid	*cpa*		
3052	Abdominal fluid	*cpa, cpb2*	2	P
3018	Abdominal foreign body	*cpa*		
3014	Abdominal wound	*cpa*		
3041	Abdominal wound	*cpa*		
3044	Abdominal wound	*cpa, cpb2*	2	P
3045	Abdominal wound	*cpa*		
3059	Abdominal wound	*cpa*		
3049	Abdominal wound	*cpa*		
3043	Abdominal wound	*cpa*		
3001	Abscess	*cpa*		
3002	Abscess	*cpa, cpb2*	1 & 2T	N
3058	Abscess	*cpa*		
3005	Bile	*cpa*		
3009	Bile	*cpa*		
3010	Bile	*cpa*		
3013	Bile	*cpa*		
3015	Bile	*cpa*		
3024	Bile	*cpa*		
3025	Bile	*cpa*		
3026	Bile	*cpa*		
3027	Bile	*cpa*		
3030	Bile	*cpa*		
3038	Bile	*cpa, cpe*		
3012	Bile-autopsy	*cpa*		
3057	Bile-autopsy	*cpa*		
3008	Biliary aspirate	*cpa, cpb2*	1 & 2	N
3006	Blood	*cpa*		
3007	Blood	*cpa*		

3023	Blood	*cpa, cpb2*	2	P
3034	Blood	*cpa, cpe*		
3040	Blood	*cpa, cpb2*	2	P
3046	Blood	*cpa*		
3047	Blood	*cpa*		
3050	Blood	*cpa*		
3011	Blood-autopsy	*cpa*		
3032	Blood-autopsy	*cpa, cpb2*	2	P
3048	Blood-autopsy	*cpa*		
3053	Blood-autopsy	*cpa*		
3055	Buttock, wound	*cpa*		
3056	Duodenal fluid	*cpa*		
3031	Foot wound	*cpa*		
3035	Hematoma/ wound	*cpa*		
3036	Left leg wound	*cpa*		
3042	Perineal fluid	*cpa, cpb2*	2T	N
3028	Peritoneal	*cpa*		
3019	Peritoneal abscess	*cpa*		
3016	Peritoneal fluid	*cpa*		
3020	Peritoneal fluid	*cpa*		
3051	Peritoneal fluid	*cpa, cpb2*	2	N
3054	Peritoneal/abdominal wound	*cpa*		
3021	Skin wound	*cpa, cpe*		
3003	Spleen-autopsy	*cpa*		
3004	Uvula wound	*cpa*		
3037	Vaginal discharge	*cpa, cpb2*	2	N
3022	Wound, right 3rd toe	*cpa*		

[a] Genotype of each *C. perfringens* isolate was determined using the multiplex PCR (Garmory et al., 2000).

[b] Western immunoblotting assay for production of the beta2 protein toxin, using the polyclonal anti-beta2 antibody (Gibert et al., 1997).

[c] Indicates the group(s) of the *cpb2* for that isolate.

[d] N: negative result. P: positive result.

Table B. *C. perfringens* isolates from fecal specimens from Healthy Human volunteers.

Research Number	Genotype[a]	*cpb2* group(s)[b]	Western Immunoblotting[c]
1066	*cpa, cpb2*	1 & 2	P[d]
1067	*cpa, cpb2*	1 & 2	P
1068	*cpa, cpb2*	1 & 2	P
1069	*cpa, cpb2*	1 & 2	P
1009	*cpa, cpb2*	1	N
1010	*cpa, cpb2*	1	N
1014	*cpa, cpb2*	1	N
1017	*cpa, cpb2*	1	N
1008	*cpa, cpb2*	2	P
1093	*cpa, cpb2*	2	P
1028	*cpa, cpe, cpb2*	3	N
1084	*cpa, cpe*		
1001	*cpa*		
1002	*cpa*		
1003	*cpa*		
1004	*cpa*		
1005	*cpa*		
1006	*cpa*		
1007	*cpa*		
1011	*cpa*		
1012	*cpa*		
1013	*cpa*		
1015	*cpa*		
1016	*cpa*		
1018	*cpa*		
1019	*cpa*		
1020	*cpa*		
1021	*cpa*		
1022	*cpa*		
1023	*cpa*		
1024	*cpa*		
1025	*cpa*		
1026	*cpa*		
1027	*cpa*		
1029	*cpa*		

1030	*cpa*
1031	*cpa*
1032	*cpa*
1033	*cpa*
1034	*cpa*
1035	*cpa*
1036	*cpa*
1037	*cpa*
1038	*cpa*
1039	*cpa*
1040	*cpa*
1041	*cpa*
1042	*cpa*
1043	*cpa*
1044	*cpa*
1045	*cpa*
1046	*cpa*
1047	*cpa*
1048	*cpa*
1049	*cpa*
1050	*cpa*
1051	*cpa*
1052	*cpa*
1053	*cpa*
1054	*cpa*
1055	*cpa*
1056	*cpa*
1057	*cpa*
1058	*cpa*
1059	*cpa*
1060	*cpa*
1061	*cpa*
1062	*cpa*
1063	*cpa*
1064	*cpa*
1065	*cpa*
1070	*cpa*
1071	*cpa*
1072	*cpa*
1073	*cpa*

1074	*cpa*
1075	*cpa*
1076	*cpa*
1077	*cpa*
1078	*cpa*
1079	*cpa*
1080	*cpa*
1081	*cpa*
1082	*cpa*
1083	*cpa*
1085	*cpa*
1086	*cpa*
1087	*cpa*
1088	*cpa*
1089	*cpa*
1090	*cpa*
1091	*cpa*
1092	*cpa*
1094	*cpa*
1095	*cpa*
1096	*cpa*
1097	*cpa*
1098	*cpa*
1099	*cpa*
1100	*cpa*

[a] Genotype of each *C. perfringens* isolate was determined using the multiplex PCR (Garmory et al., 2000).

[b] Indicates the group(s) of the *cpb2* for that isolate.

[c] Western immunoblotting assay for production of the beta2 protein toxin, using the polyclonal anti-beta2 antibody (Gibert et al., 1997).

[d] N: negative result. P: positive result.

Table C. *C. perfringens* isolates from fecal specimens from patients with suspected antibiotic associated diarrhea (AAD).

Research Number	Genotype[a]	*cpb2* group(s)[b]	Western Immunoblotting[c]
2054	*cpa, cpb2*	1	N[d]
2010	*cpa, cpb2*	2	P
2048	*cpa, cpb2*	2	P
2049	*cpa, cpb2*	2	P
2051	*cpa, cpb2*	2	N
2061	*cpa, cpb2*	2	P
2062	*cpa, cpb2*	2	P
2063	*cpa, cpb2*	2	P
2064	*cpa, cpb2*	2	P
2065	*cpa, cpb2*	2	P
2067	*cpa, cpb2*	2	P
2068	*cpa, cpb2*	2	P
2069	*cpa, cpb2*	2	P
2070	*cpa, cpb2*	2	P
2071	*cpa, cpb2*	2	P
2073	*cpa, cpb2*	2	P
2082	*cpa, cpb2*	2	N
2093	*cpa, cpb2*	2	P
2107	*cpa, cpb2*	2	P
2110	*cpa, cpb2*	2	P
2115	*cpa, cpb2*	2	P
2125	*cpa, cpb2*	2	P
2128	*cpa, cpb2*	2	P
2132	*cpa, cpb2*	2	P
2133	*cpa, cpb2*	2	P
2134	*cpa, cpb2*	2	P
2137	*cpa, cpb2*	2	P
2139	*cpa, cpb2*	2	P
2140	*cpa, cpb2*	2	N
2052	*cpa, cpb2*	2T	N
2023	*cpa, cpb2*	1 & 2	P
2109	*cpa, cpb2*	1 & 2	P
2116	*cpa, cpb2*	1 & 2	P
2121	*cpa, cpb2*	1 & 2	P
2122	*cpa, cpb2*	1 & 2	P

2124	*cpa, cpb2*	1 & 2	P
2108	*cpa, cpb2*	2 & 3	N
2066	*cpa, cpe, cpb2*	2	P
2074	*cpa, cpe, cpb2*	2	P
2076	*cpa, cpe, cpb2*	2	N
2016	*cpa, cpe, cpb2*	1 & 2	N
2077	*cpa, cpe, cpb2*	1 & 2	N
2120	*cpa, cpe, cpb2*	1 & 2	N
2012	*cpa, cpe, cpb2*	1 & 2T	N
2096	*cpa, cpe, cpb2*	3	N
2006	*cpa, cpe*		
2007	*cpa, cpe*		
2008	*cpa, cpe*		
2009	*cpa, cpe*		
2015	*cpa, cpe*		
2017	*cpa, cpe*		
2018	*cpa, cpe*		
2047	*cpa, cpe*		
2078	*cpa, cpe*		
2079	*cpa, cpe*		
2086	*cpa, cpe*		
2087	*cpa, cpe*		
2097	*cpa, cpe*		
2098	*cpa, cpe*		
2099	*cpa, cpe*		
2100	*cpa, cpe*		
2102	*cpa, cpe*		
2104	*cpa, cpe*		
2118	*cpa, cpe*		
2001	*cpa*		
2002	*cpa*		
2003	*cpa*		
2004	*cpa*		
2005	*cpa*		
2011	*cpa*		
2013	*cpa*		
2014	*cpa*		
2019	*cpa*		
2020	*cpa*		
2021	*cpa*		

2022	*cpa*
2024	*cpa*
2025	*cpa*
2026	*cpa*
2027	*cpa*
2028	*cpa*
2029	*cpa*
2030	*cpa*
2031	*cpa*
2032	*cpa*
2033	*cpa*
2034	*cpa*
2035	*cpa*
2036	*cpa*
2037	*cpa*
2038	*cpa*
2039	*cpa*
2040	*cpa*
2042	*cpa*
2043	*cpa*
2044	*cpa*
2045	*cpa*
2046	*cpa*
2050	*cpa*
2053	*cpa*
2055	*cpa*
2056	*cpa*
2057	*cpa*
2058	*cpa*
2059	*cpa*
2060	*cpa*
2072	*cpa*
2075	*cpa*
2080	*cpa*
2081	*cpa*
2083	*cpa*
2084	*cpa*
2085	*cpa*
2088	*cpa*
2089	*cpa*

2090	*cpa*
2091	*cpa*
2092	*cpa*
2094	*cpa*
2095	*cpa*
2101	*cpa*
2103	*cpa*
2105	*cpa*
2106	*cpa*
2111	*cpa*
2112	*cpa*
2113	*cpa*
2114	*cpa*
2117	*cpa*
2119	*cpa*
2123	*cpa*
2126	*cpa*
2127	*cpa*
2129	*cpa*
2130	*cpa*
2131	*cpa*
2135	*cpa*
2136	*cpa*
2140	*cpa*
2141	*cpa*
2142	*cpa*
2143	*cpa*
2144	*cpa*

[a] Genotype of each *C. perfringens* isolate was determined using the multiplex PCR (Garmory et al., 2000).

[b] Indicates the group(s) of the *cpb2* for that isolate

[c] Western immunoblotting assay for production of the beta2 protein toxin, using the polyclonal anti-beta2 antibody (Gibert et al., 1997).

[d] N: negative result. P: positive result.

Table D. *C. perfringens* isolates from fecal specimens from patients with food-borne illness

Research Number	Genotype[a]	*cpb2* group(s)[b]	Western Immunoblotting[c]
2152	*cpa, cpe, cpb2*	3	P[d]
2153	*cpa, cpe, cpb2*	3	P
2145	*cpa, cpe*		
2155	*cpa, cpe*		
2157	*cpa, cpe*		
2158	*cpa, cpe*		
2160	*cpa, cpe*		
2161	*cpa, cpe*		
2146	*cpa*		
2147	*cpa*		
2148	*cpa*		
2149	*cpa*		
2150	*cpa*		
2151	*cpa*		
2154	*cpa*		
2156	*cpa*		
2159	*cpa*		

[a] Genotype of each *C. perfringens* isolate was determined using the multiplex PCR (Garmory et al., 2000).

[b] Indicates the group(s) of the *cpb2* for that isolate.

[c] Western immunoblotting assay for production of the beta2 protein toxin, using the polyclonal anti-beta2 antibody (Gibert et al., 1997).

[d] P: positive result.

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CURRICULUM VITAE

Heidi M. Roskens Dalzell

Education

M.D. Anticipated, May 2009
University of Minnesota Medical School

Ph.D. Experimental Pathology, September 2008
Indiana University
Department of Pathology and Laboratory Medicine

Concentration: Microbiology

Dissertation: A Study of the Beta2 Toxin Gene and the Beta2 Toxin in *Clostridium perfringens* Strains Isolated from Human Sources.

Advisor: Dr. Stephen D. Allen

M.S. Clinical Laboratory Sciences, August 2001
University of Minnesota
Department of Pathology and Laboratory Medicine

Concentration: Microbiology

Thesis: Development of an Enzyme Linked Immunosorbent Assay to Measure the Effects of Various Substances on the Attachment of *Giardia lamblia.*

Advisor: Dr. Stanley L. Erlandsen

B.S. Biology & Theater, December 1997
University of Wisconsin Stevens Point

Majors: Biology and Theatre Arts

Peer Reviewed Publication

Roskens H, and Erlandsen SL. 2002. Inhibition of In Vitro Attachment of *Giardia* Trophozoites by Mucin. *Journal of Parasitology* 88(5): 869 - 873.

Conference Presentations

Phenotypic/genotypic characterization of *cpb2* positive *Clostridium perfringens* isolates from humans with and without antibiotic-associated diarrhea.

Genetic variation of *cpb2* from *Clostridium perfringens* type A isolates from human sources.

Poster presentations. The 7th Biennial Meeting of the Anaerobe Society of the Americas. Annapolis, MD; July 18 - July 21, 2004.

Presence of the beta2 toxin gene in *Clostridium perfringens* isolates from humans with antibiotic-associated diarrhea and from healthy volunteers.

Oral presentation. The 3rd World Congress on anaerobic bacteria and infections. Glasgow, Scotland, UK; May 7 - May 9, 2003.

Mucin inhibits the in vitro attachment of *Giardia lamblia*, as measured by an ELISA assay.

Oral presentation. The 76th annual meeting of The American Society of Parasitologists. Albuquerque, NM; June 29 - July 3, 2001.

Conferences Attended

American Society of Microbiologists 104th General Meeting. New Orleans, LA; May 23 - May 27, 2004.

Clostridia '03 Pathogenesis. Woods Hole, MA; April 26 - April 30, 2003.

Anaerobe Olympiad 2002: The 6th Biennial Congress of the Anaerobe Society of the Americas. Park City, UT; June 29 - July 2, 2002.

Teaching Assistantship

Teaching Assistant for the Methods in Molecular Biology and Pathology Workshop. Indiana University - Purdue University Indianapolis.
June 9 - 30, 2003 and June 14 - July 2, 2004.

Honors and Awards

Medical Scholars Program award. A summer scholarship by the Infectious Diseases Society of America (IDSA) Education and Research Foundation, awarded to medical students in U.S. medical schools with mentorship by an IDSA member. Summer, 2008.

Class of 1943 (March) Scholarship. Awarded by the Minnesota Medical Foundation, University of Minnesota Medical School. 2005 - 2006.

First Place Award of Merit. For Student Presentation at: The 7th Biennial Meeting of the Anaerobe Society of the Americas. Annapolis, MD; July 18 - July 21, 2004.

Travel Fellowship. Awarded by the IUPUI Graduate Student Organization for travel to: The 3rd World Congress on Anaerobic Bacteria and Infections. Glasgow, Scotland, UK; May 7 - 9, 2003.

Educational Enhancement Grant. Awarded by the IUPUI Graduate Student Organization to support student research. November 2002.

University Fellowship. Awarded by IUPUI for the duration of the student's first year as a graduate student at the University. August 2001 - August 2002.

Travel Grant. Awarded by: The American Society of Parasitologists, to attend the 76th annual meeting. Albuquerque, NM; June 29 - July 3, 2001.

Dean's List/ Semester Honors

Dean's List: Spring 1995 and Fall 1998.

Semester Honors: Fall 1995, Spring 1996, and Fall 1996.

Semester High Honors: Fall 1997.

Semester Highest Honors: Spring 1998.