Published in final edited form as:
Cell Microbiol. 2009 September ; 11(9): 1306–1328. doi:10.1111/j.1462-5822.2009.01332.x.

Contact with enterocyte-like Caco-2 cells induces rapid upregulation of toxin production by *Clostridium perfringens* type C isolates

Jorge E. Vidal[1], **Kaori Ohtani**[2], **Tohru Shimizu**[2], and **Bruce A. McClane**[*,1,3]

[1]Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh PA, USA

[2]Department of Bacteriology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa Japan

[3]Australian Research Council Centre of Excellence in Structural and Functional Microbial Genetics, Department of Microbiology, Monash University, Victoria, Australia

Abstract

Clostridium perfringens type C isolates cause necrotizing enteritis in humans and domestic animals. *In vitro*, type C isolates often produce beta toxin (CPB), beta2 toxin (CPB2), alpha toxin (CPA), perfringolysin O (PFO), and TpeL during (or after) late log-phase growth. In contrast, the current study found that many type C isolates respond to close contact with enterocyte-like Caco-2 cells by producing all toxins, except TpeL, much more rapidly than occurs during *in vitro* growth. This *in vivo* effect involves rapid transcriptional upregulation of the *cpb*, *cpb*2, *pfo*A and *plc* toxin genes. Rapid Caco-2 cell-induced upregulation of CPB and PFO production involves the VirS/VirR two-component system, since upregulated *in vivo* transcription of the *pfo*A and *cpb* genes was blocked by inactivating the *vir*R gene and was reversible by complementation to restore VirR expression. However, the *lux*S quorum sensing system is not required for the rapid upregulation of type C toxin production induced by contact with Caco-2 cells. These results provide the first indication of host cell:pathogen cross-talk affecting toxin production kinetics by any pathogenic *Clostridium* spp., identify *in vivo* vs. *in vitro* differences in *C. perfringens* toxin expression, and implicate VirS/VirR as a possible contributor to some *C. perfringens* enteric diseases.

Keywords

Clostridum perfringens; host:pathogen cross-talk; toxin gene regulation; two-component regulatory system; quorum sensing

Introduction

The anaerobic, spore-forming bacterium *Clostridium perfringens* is one of the most important Gram-positive pathogens of humans and animals (McClane, 2006). *C. perfringens* diseases include numerous gastrointestinal syndromes and enterotoxemias, as well as gas gangrene and other histotoxic infections. The virulence of this bacterium is directly related to its production of potent toxins. Differential production of four lethal typing toxins [α, β, ε and/or ι] is used to classify *C. perfringens* isolates into 5 pathogenic types (A–E). Each *C.*

[*]Corresponding author: B. A. McClane 200 Lothrop St. E1240 BSTWR, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15261, USA bamcc@pitt.edu, · Tel.: +1-412-6489022, · Fax: +1-412-6241401.

perfringens type is associated with certain human or animal diseases (McClane, 2006; Smedley *et al.*, 2004).

By definition, *C. perfringens* type C isolates must produce both α toxin (CPA) and β toxin (CPB). Some type C isolates also make β2 toxin (CPB2), perfringolysin O (PFO) or enterotoxin (CPE) (Fisher *et al.*, 2006; Smedley *et al.*, 2004). Furthermore, many type C isolates also produce a newly discovered toxin named TpeL, which is a truncated homolog of *C. difficile* TcdA and other large clostridial toxins (Amimoto *et al.*, 2007).

C. perfringens type C isolates cause fatal diseases ranging from necrotizing enteritis to enterotoxemia in virtually all livestock species. Those type C-mediated animal diseases result in serious economic losses for the agricultural industry (McClane, 2004). In severe type C enterotoxemia, toxins are made in the intestines and then absorbed into the circulation, where they can cause rapid death of the infected animal (Songer and Uzal, 2005; Songer, 1996). Piglets are most commonly affected by type C isolates, with herd mortality rates between 50–100% despite a clinical course usually lasting <24 h (Songer, 1996; Niilo, 1988).

In humans, *C. perfringens* type C isolates cause enteritis necroticans, an often rapidly fatal disease that involves vomiting, diarrhea, severe abdominal pain, and the presence of blood in the stools (McClane, 2004). This illness is associated with low intestinal trypsin levels due to diet or disease, implicating trypsin as an important host defense mechanism against enteritis necroticans. On histologic examination, blunted villi and numerous bacteria are seen on the mucosal surface of the necrotic tissue (Walker *et al.*, 1980). Outbreaks of acute human enteritis necroticans caused by type C isolates were first recorded in post-war Germany, where the disease was known as Darmbrand. This illness, currently endemic throughout Southeast Asia, is most closely associated with New Guinea, where it is referred to as Pigbel. In the 1970´s, pigbel was the leading cause of death in children older than 1 year of age in the New Guinea highlands (Lawrence and Cooke, 1980). Although uncommon, human enteritis necroticans caused by type C isolates also occurs in developed countries (Matsuda *et al.*, 2007; Sobel *et al.*, 2005; Tonnellier *et al.*, 2001; Petrillo *et al.*, 2000). Importantly, diabetic patients infected with these bacteria often survive <48 h after the onset of symptoms (Tonnellier *et al.*, 2001; Severin *et al.*, 1984).

Several observations support CPB as the major cause of the clinical signs associated with type C disease (Sakurai and Duncan, 1977). First, immunohistochemistry studies detected CPB on the necrotic intestinal epithelium of patients suffering from type C infection (Matsuda *et al.*, 2007). In addition, we constructed and virulence-tested several toxin null mutants of type C isolate CN3685, which showed that beta toxin is necessary for this isolate to damage rabbit ileal loops (Sayeed *et al.*, 2008). We also showed that CPB is sufficient to cause enteric disease by experimentally reproducing necrotic enteritis in rabbit ileal, jejunal or duodenal loops (but not colonic loops) by injecting purified CPB, along with trypsin inhibitor to avoid CPB degradation by endogenous trypsin (Vidal *et al.*, 2008).

In other work, we showed that CPB, CPB2, PFO and PLC are produced during late log-phase by type C isolates growing in TGY medium (Fisher *et al.*, 2006). In that study, *in vitro* toxin production levels by type C isolates were found to vary using different bacterial culture media for growth, suggesting that environmental signals are important for regulating type C toxin production and, by extension, perhaps virulence.

Regulation of toxin production by *C. perfringens* vegetative cells has only been studied for gangrene-producing type A isolates growing *in vitro*. A two component regulatory system named VirS/VirR, comprised of (respectively) a membrane sensor and transcriptional regulator (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994), is encoded by the *vir*S and *vir*R genes

that form an operon transcribed as a single 2.1 kb mRNA molecule (Ba-Thein *et al.*, 1996). The VirS/VirR two component regulatory system helps to govern *in vitro* transcription of the chromosomal *plc*, *pfo*A, and *col*A toxin genes (encoding CPA, PFO and collagenase, respectively) and the plasmid-borne *cpb*2 gene encoding CPB2 toxin (Ohtani *et al.*, 2003; Ba-Thein *et al.*, 1996; Lyristis *et al.*, 1994; Shimizu *et al.*, 1994). In gangrene-producing type A strain 13, PLC and PFO toxin regulation also involves the *lux*S quorum-sensing mechanism (Ohtani *et al.*, 2002).

Whether contact with host cells during disease affects toxin gene transcription has not yet been studied for *C. perfringens* or any pathogenic *Clostridium spp*. This gap is significant since other pathogens regulate their virulence gene expression in response to stimuli from host cells. For example, upon host-cell contact, enteropathogenic *E. coli* upregulates transcription of genes involved in intimate adherence, pedestal formation (Leverton and Kaper, 2005), and EspC toxin secretion (Vidal and Navarro-Garcia, 2006). Contact with host cells induces *H. pylori* to produce surface appendages and activate invasion mechanisms (Rohde *et al.*, 2003). The presence of host cells stimulates *Salmonella* and *Shigella* to produce a functional type III secretion system and translocate invasion proteins into host cells (Demers *et al.*, 1998; Ginocchio *et al.*, 1994).

Therefore, the current study investigated toxin production by *C. perfringens* type C isolates in the presence of intestinal Caco-2 cells, as a model for human enterocytes. Type C isolates were found to sense Caco-2 cells and respond by quickly upregulating toxin production to exert cytotoxic consequences on host cells. This effect was shown to involve, in part, the VirS/VirR two component regulatory system but not *lux*S-controlled quorum sensing.

Results

Infection of Caco-2 cell cultures with *C. perfringens* type C strains induces a rapid accumulation of extracellular proteins

Since secretion of protein toxins is considered essential for the pathogenesis of type C intestinal disease (Sayeed *et al.*, 2008; Smedley *et al.*, 2004; Petit *et al.*, 1999), we first investigated by SDS-PAGE whether infection with type C isolates affects protein levels present in supernatants of Caco-2 cell cultures. After as little as 1 h post-infection with *C. perfringens* type C strains JGS1495 or CN3685 significant levels of proteins were detected in the supernatants of Caco-2 cultures (Figure S1A and S1C, respectively). The molecular size of those supernatant proteins ranged from 20 to <100 kDa. The levels of those supernatant proteins increased even further by 2 or 3 h post-infection. No secreted proteins were seen in the supernatant of *C. perfringens* type C strains grown similarly in either TGY or MEM without Caco-2 cells (Figures S1A and S1C), suggesting the protein secretion evident in Figures S1A and S1C had been stimulated by *C. perfringens* infection of Caco-2 cell cultures.

To evaluate whether these changes in supernatant protein patterns were specifically due to *C. perfringens* infection, SDS-PAGE was performed on the supernatants of non-infected Caco-2 cell cultures grown for 3 h in MEM without FBS (fetal bovine serum). A major band corresponding to a >60 kDa protein was detected in the supernatant of non-infected Caco-2 cells (Figures S1A and S1C, control lane). However these analyses also indicated that most proteins of <60 kDa present in the supernatants of infected Caco-2 cultures had resulted from *C. perfringens* type C infection. Since most toxins and proteolytic enzymes secreted by *C. perfringens* type C strains have a molecular mass of <60 kDa (i.e. CPB2, 28 kDa; CPB, 35 kDa; CPA, 47 kDa; and PFO, 54 kDa), the S1 figure results were consistent with the possibility of host cell-mediated stimulation of bacterial toxin secretion occurring early during infection.

To evaluate whether the enhanced protein secretion observed in Figures S1A and S1C was simply due to host cell stimulation of bacterial overgrowth, the colony forming units (CFU) of *C. perfringens* type C strains present under each culture condition were determined. After a 3 h infection of Caco-2 cells, the CFU of *C. perfringens* type C strain JGS1495 cultures were similar to, if not reduced from, the other two growing conditions, i.e., MEM alone (no Caco-2 cells) or TGY (Figure 1A). Similarly, after a 1.5 h infection of Caco-2 cell cultures, the CFU of *C. perfringens* type C strain CN3685 was similar to those obtained for the other two culture conditions, i.e., MEM (no Caco-2 cells) or TGY (Figure S1D). The CN3685 isolate was incubated for only 1.5 h because the presence of this strain was already inducing morphological damage in Caco-2 cells by 2 h post-infection (as described later). These results indicate that the increased protein secretion triggered by *C. perfringens* infection of Caco-2 cell cultures is not simply attributable to a stimulation of bacterial overgrowth.

The presence of Caco-2 cells and other mammalian cells causes rapid up-regulated secretion of *C. perfringens* beta toxin (CPB)

Since the S1 figure results indicated that some of the increased protein secretion stimulated by type C infection of Caco-2 cells included proteins matching the size of toxins, we next investigated whether CPB might be secreted rapidly into the supernatants of infected Caco-2 cell cultures. When those supernatants were analyzed by Western blotting using a monoclonal anti-CPB antibody, the presence of CPB was detected as early as 2 h post-infection in supernatants of Caco-2 cell cultures infected with *C. perfringen*type C strain JGS1495 (Figure S1B). In contrast, no CPB signal was detected in 2 h culture supernatants from MEM (no Caco-2 cells), TGY, or FTG (fluid thioglycolate medium) inoculated with JGS1495 (Figure S1B). CPB secretion into the supernatant of Caco-2 cells infected with JGS1495 increased further by 3 h (Figure 1B); in contrast, even after 3 h, no CPB signal was observed in the supernatant of the MEM, FTG or TGY cultures of JGS1495.

To evaluate the prevalence of this Caco-2 cell-induced upregulation of CPB secretion amongst type C isolates, Caco-2 cell cultures were separately infected with two other pathogenic *C. perfringens* type C strains that had been isolated from either a sheep with struck (CN3685) or a human pig-bel case (CN5383) (Fisher *et al.*, 2006; Sakurai and Duncan, 1977). Western blot analyses showed those two strains resemble JGS1495 in rapidly secreting CPB when incubated in the presence of Caco-2 cells (Figure 1C and Figure 2B), confirming that this upregulated CPB secretion phenomenon is common among *C. perfringens* type C isolates. ELISA analysis indicated that, compared to 3 h growth in TGY or MEM without Caco-2 cells, >3-fold higher levels of CPB were present in supernatants of Caco-2 cultures infected with CN3685 (Fig. 2A).

To investigate whether the rapid upregulation of CPB secretion can also be stimulated by the presence of other host cells besides Caco-2 cells, MDCK and Rat-1/R12 cell lines were similarly infected with *C. perfringens* type C isolates. This experiment revealed that rapid upregulation of CPB secretion can be triggered by the presence of dog-, rat- or human-derived cell cultures of kidney, fibroblast or intestinal origin (Figure 1D), suggesting that many mammalian cells produce factor(s) that stimulate *C. perfringens* type C strains to rapidly secrete CPB.

Secretion of PFO, CPA and CPB2 toxins by type C isolates is also rapidly up-regulated by the presence of Caco-2 cells

In addition to CPB, *C. perfringens* type C isolates commonly secrete CPB2, CPA and PFO into the medium during late log-phase growth in TGY broth (Fisher *et al.*, 2006; Smedley *et al.*, 2004; Petit *et al.*, 1999). *C. perfringens* alpha toxin (CPA) is a lethal toxin with

phospholipase C and sphingomyelinase activities, while PFO is a lethal, cholesterol-dependent cytolysin (Titball *et al.*, 1999; Tweten, 1988a); CPB2 action is unknown.

Therefore, studies were performed to evaluate whether the rapid host cell-induced CPB secretion noted in Fig. 1 also occurs for other toxins produced by type C isolates. Western blot analyses showed that CPA appears much earlier in the supernatant of Caco-2 cells infected with type C isolates compared to MEM or TGY cultures (Fig. 2D). An ELISA analysis revealed that, relative to equivalent MEM or TGY culture supernatants, CPA secretion is >4-fold higher in the supernatants of 3 h CN3685-infected Caco-2 cells (Fig. 2C).

Similarly, Western blot analyses demonstrated that, compared to MEM or TGY cultures, the presence of Caco-2 cells induces a more rapid secretion of PFO by type C isolates (Fig. 2F). The ability of *C. perfringens* supernatants to lyse horse erythrocytes and release hemoglobin (Hb) is specifically attributable to PFO activity (Lyristis *et al.*, 1994), as supported by the inability of CN3685 *pfo*A mutant (Sayeed *et al.*, 2008) to induce Hb release (data not shown). As shown in Fig. 2E, the horse erythrocyte lysis assay revealed that, compared to supernatants of 2 h MEM or TGY cultures of CN3685, there is a >4-fold increase in PFO levels present in supernatants of Caco-2 cell cultures infected for 2 h with *C. perfringens* type C strain CN3685.

Finally, CPB2 secretion was also specifically stimulated by the presence of Caco-2 cells. Western blotting detected CPB2 in supernatants of Caco-2 cell cultures within 2 h post-infection by strain JGS1495, whereas no CPB2 signal was detected in the supernatant of 2 h FTG, TGY or MEM (no Caco-2 cells) cultures inoculated with a similar amount of this type C isolate (Fig. 3A). Densitometric analysis of these gels identified a >5-fold increase in CPB2 levels in supernatants from 3 h cultures of CN3685-infected Caco-2 cells compared to equivalent TGY or MEM cultures (Fig. 3C)

The rapid host cell-mediated increase in *C. perfringens* type C toxin secretion follows early upregulated transcription of toxin genes

Collectively, the Figure 1–Figure 3 and S1 results suggested that a rapid global toxin up-regulation, likely part of a well-orchestrated virulence mechanism, is triggered when *C. perfringens* encounters its mammalian host and that this rapid host cell-induced toxin secretion is not attributable to stimulated bacterial growth. Therefore, RT-PCR analyses explored whether this effect might involve an early onset of toxin gene transcription.

Using 20, 50 or 100 ng levels of RNA extracted from Caco-2 cell cultures that had been infected for only 2 h with type C strain JGS1495, RT-PCR studies detected transcripts encoding CPB, CPB2, PFO and CPA (Figs. 4B, 4C, 4D and 4E respectively). In contrast, no mRNA encoding any of those toxins could be detected by RT-PCR using the same levels of RNA extracted from JGS1495 grown for 2 h in either TGY or MEM (no Caco-2 cells). The type C isolate CN3685 also quickly upregulated transcription of the *cpb* gene in the presence of Caco-2 cells (Fig. S2A).

The Fig. 4 results indicated that the rapid upregulated secretion of toxins involves stimulation of toxin gene transcription soon after type C isolates encounter Caco-2 cells. To explore whether this stimulation of toxin gene expression simply reflects a general host cell-mediated increase in transcription of all *C. perfringens* genes, primers were designed to amplify the alpha subunit of the DNA polymerase III gene (*pol*C), which is a *C. perfringens* type A housekeeping gene (Myers *et al.*, 2006). As shown in Figure 4A, the *pol*C gene could be PCR-amplified using template DNA extracted from either *C. perfringens* type C strain JGS1495 (Fig. 4A line 1) or CN3685 (result not shown). Moreover, RT-PCR detected

mRNA from the *pol*C gene in *C. perfringens* cultures growing for 2 h in either TGY, MEM or in the presence of Caco-2 cells (Fig. 4A).

Early transcription of the *tpe*L toxin gene is not stimulated by the presence of Caco-2 cells

TpeL, a recently described toxin that shares homology with TcdA and TcdB from *C. difficile*, is secreted by many *C. perfringens* type C isolates during the stationary phase of *in vitro* growth (Amimoto *et al.*, 2007). Therefore, experiments were performed to determine whether *tpe*L toxin gene transcription is also rapidly up-regulated when *C. perfringens* type C isolates encounter Caco-2 cells. PCR first confirmed that the *tpe*L gene is present in the type C isolates used in this study and, as previously reported (Amimoto *et al.*, 2007), that this gene in our type C isolates lacks the 3´fragment of the *tcd*A gene from *C. difficile* (Fig. 5A).

In contrast to mRNA for other type C toxin genes, *tpe*L mRNA (Fig. 5B) was not detected by RT-PCR following a 2 h infection of Caco-2 cell cultures by JGS1495 or after 2 h growth of this isolate in TGY or MEM alone. However, *tpe*L message was detected in a 24 h TY culture of JGS1495, confirming that our RT-PCR assay could detect *tpe*L transcript, if present (Fig. 5B).

Rapid host cell-mediated up-regulation of *C. perfringens* type C toxin secretion requires close contact between bacteria and host cells

The results presented above indicate that rapid host cell-stimulated secretion of most toxins made by *C. perfringens* type C isolates is triggered by an unknown host factor present during infection. Since some host proteins (mostly with molecular masses >60 kDa) are present in the supernatant of non-infected Caco-2 cells (Figs. S1A and S1C, control lanes), we next evaluated whether supernatants of non-infected Caco-2 cell cultures, referred to here as conditioned medium (CM), are sufficient to stimulate the secretion of *C. perfringens* type C toxins.

However, as shown in Figure 6A lane 3, CM (like MEM alone, Fig. 6A, lane 1) was unable to stimulate CPB secretion. Furthermore, when CM was added to the Caco-2 cell cultures before infection, CPB secretion remained similar to that of infected Caco-2 cells growing in fresh MEM (Figure 6A, compare line 2 and line 4). This negative result is less likely attributable to a soluble host cell factor being retained by the filter used for sterilizing supernatants since the same filter was freely permeable to a 70 kDa fluorescently-labeled dextran (data not shown). These results could suggest that a soluble factor secreted by Caco-2 cells is not responsible for stimulating rapid toxin secretion by *C. perfringens* type C isolates.

Since Fig. 6A was consistent with a Caco-2 cell surface component triggering the enhanced toxin secretion observed in infected Caco-2 cultures, this possibility was further tested by varying the numbers of Caco-2 cells infected with a constant number of bacteria. Those studies revealed that as the number of eukaryotic cells in a culture increased, so too did CPB secretion levels (Fig. 6B), consistent with these bacteria sensing the concentration of host cells and then triggering a rapid toxin secretion in response. The host factor responsible for inducing rapid CPB production was trypsin- and phospholipase C (PLC)-resistant, since rapid host cell-induced CPB secretion was unaffected if Caco-2 cells were pretreated with trypsin or CPA (which is a PLC) prior to infection by type C isolates (Figs. 7A and 7B). Interestingly, pretreatment of Caco-2 cells with Pronase enhanced the rapid secretion of CPB (Fig. 7C).

To further explore whether Caco-2 cell-associated surface components are sufficient for stimulating toxin secretion, *C. perfringens* type C strain JGS1495 was incubated for 3 h with

MEM containing either the insoluble membrane fraction of Caco-2 cells or Caco-2 cell lysates. Neither those isolated Caco-2 cell membranes nor Caco-2 cell lysates stimulated CPB secretion like intact, living Caco-2 cells (Fig. 6C), which could suggest that intact host cells, if not viable host cells, are needed to trigger rapid CPB secretion by type C isolates.

To evaluate whether viable host cells are needed to trigger rapid CPB secretion, Caco-2 cells were fixed using paraformaldehyde (PFA), which preserves the cell surface. CPB secretion was similar when *C. perfringens* type C strains JGS1495 or CN3685 were used to infect either living Caco-2 cells or the PFA prefixed-Caco-2 cells (Fig. 6D and Fig S2B, respectively). This experiment revealed that rapid toxin secretion by *C. perfringens* type C strains involves interactions with intact, but not necessarily viable, host cells. In addition, rapid CPB secretion in the presence of fixed cells was not seen when those PFA-fixed cells were incubated in PBS and that culture was then infected by CN3685, indicating a need for bacterial metabolism to upregulate Caco-2 cell-induced toxin production (Figure S2B).

To confirm definitively that close bacteria:host cell contact is a crucial step to trigger rapid toxin secretion, bacteria and Caco-2 cells were incubated in the same culture dish, but physically separated by a permeable 0.4 μm pore-size Transwell system membrane (see Materials and Methods). Consistent with our previous results, CPB secretion was stimulated when *C. perfringens* type C strain CN3685 or JGS1495 was allowed direct contact for 2 h or 3 h, respectively, with Caco-2 cells in the same chamber of the Transwell (Fig. 6E, line 3). However, rapid CPB secretion was abated when Caco-2 cells were similarly incubated with *C. perfringens* present in the same culture well but blocked for close physical contact by the Transwell membrane (Fig. 6E, line 4). This inhibition is less likely due to a soluble host cell factor being retained by the Transwell membrane since 70 kDa fluorescently-labeled dextran readily crossed this membrane (data not shown). These results support intimate bacteria:host cell contact as a key step for host cell-stimulated rapid toxin secretion by *C. perfringens* type C isolates.

Finally, the close physical contact needed to trigger rapid upregulation of toxin secretion by type C isolates does not involve tight bacterial adherence to Caco-2 cells, i.e., gentle washing displaced JGS1495 or CN3685 from the Caco-2 cell monolayer surface (not shown).

C. perfringens type C strains are rapidly cytotoxic for Caco-2 cells

We next investigated whether the rapid host cell-induced stimulation of toxin production and secretion by *C. perfringens* type C isolates has cytotoxic consequences for Caco-2 cells. To test this, Caco-2 cells were infected with *C. perfringens* type C strain CN3685 for 1 h. As a control, bacteria were similarly grown for 1 h in MEM (no Caco-2 cells). Both supernatants containing secreted proteins were collected, filter-sterilized and added to fresh Caco-2 cell cultures for 2 h. As shown in Figure 8B, the supernatant from bacteria that had been incubated for 1 h in MEM (without Caco-2 cells) did not damage Caco-2 cells within 5 h. However, by that same time, the supernatant collected from infected Caco-2 cell cultures had induced morphological damage, which was characterized by cell rounding, cellular contraction, and membrane blebbing (Figure 8C). After 6 h of incubation, this treated Caco-2 cell monolayer had detached from the glass substratum.

To better characterize this cytotoxicity phenotype, a Caco-2 cell monolayer was infected with either *C. perfringens* type C strain CN3685 or JGS1495 at a MOI=20 and cell morphology changes were then followed (with cytotoxicity scored as described in Material and Methods). Interestingly, as early as 2 h post infection, strain CN3685 induced swelling of Caco-2 cells; by 3 h post-infection, this effect turned into a striking cytotoxic phenotype characterized by rounding, detachment of cells from the glass, and formation of some

membrane blebs (Table 1 and Fig. 8E). A similar cytotoxic phenotype was induced by strains JGS1495 or CN5383, although those strains induced cytotoxicity more slowly than CN3685. Morphologic damage in JGS1495- or CN5383-infected Caco-2 cells cultures was observed by 3.5 h post-infection and rounding of the cell monolayer with membrane blebbing was seen by 5 h post infection (Table 1 and Fig. 8F). Together, these results indicate that *C. perfringens* type C strains produce toxins that are extremely cytotoxic for Caco-2 cells and that co-incubation of these bacteria with Caco-2 cells hastens the development of this cytotoxicity.

The *lux*S-controlled quorum sensing mechanism does not regulate rapid Caco-2 cell-induced CPB secretion

It was previously reported that a *lux*S-controlled quorum sensing mechanism partially regulates *in vitro* toxin production and toxin gene transcription for *C. perfringens* type A strain 13 (Ohtani *et al.*, 2002). To evaluate whether *lux*S-mediated quorum sensing is required for the rapid Caco-2 cell-induced upregulation of toxin gene transcription and toxin secretion observed for type C strains, the *lux*S gene was insertionally-inactivated in CN3685 by using our previously described Targetron® technology (Li and McClane, 2008; Sayeed *et al.*, 2008; Chen *et al.*, 2005). A Group II intron (~ 900 bp) was inserted, in the sense orientation, between nucleotides 295|296 of the CN3685 *lux*S ORF. The presence of this intron insertion into the *luxS*gene of the mutant (CPJV19) was shown by PCR using two *luxS*-specific primers that supported PCR amplification of an ~300 bp product from the wild-type *luxS*gene, but (due to the intron insertion) amplified a larger ~1.2 kb product from CPJV19 (Fig. 9A). A Southern blot confirmed the presence of only a single intron insertion in the CPJV19 genome (Fig. S3A) and RT-PCR analyses showed that *lux*S mRNA was not made by CPJV19 (Fig. 9B).

Culture supernatants from Caco-2 cells infected for 2 or 3 h with CN3685 (WT) or CPJV19 showed a similar CPB signal, as detected by Western blot (Fig. 9C). As expected, no signal was detected in the supernatant of MEM cultures of CN3685 or CPJV19 at those same time points (data not shown). RT-PCR analyses detected *cpb*, *plc*and *pfo*A transcripts after a 2 h infection period of Caco-2 cell cultures with the WT strain or CPJV19 (Fig. 9D). To further evaluate the role of LuxS-controlled quorum sensing for *in vitro* toxin production, a kinetic analysis of CPB secretion was conducted. In this experiment, CPB secretion by, and growth rates for, the *lux*S mutant remained similar to WT levels during a 7 h culture in TGY, indicating that CPB secretion was not affected by the absence of the *lux*S-controlled quorum sensing system (Fig. S3B and S3C).

The *vir*S/*vir*R two-component regulatory system regulates the rapidly increased production and secretion of CPB and PFO in the presence of Caco-2 cells

To address whether the VirS/VirR two-component regulatory system plays a role in Caco-2 cell-induced upregulation of toxin production and secretion by type C isolates, a CN3685 *vir*R mutant (CPJV47) was constructed using an *E. coli*-based suicide plasmid (pKOR) that contains an ~590 bp fragment of the *vir*R gene upstream of a tetracycline resistance gene (Shimizu *et al.*, 1994). As shown by PCR, a ~590 bp *vir*R fragment had replaced the ~710 bp wt *vir*R gene in the mutant strain CPJV47 (Fig. 10A). RT-PCR analyses showed that CPJV47 does not produce *vir*R mRNA (Fig. 10C) and Western blot analyses using an anti-VirR antibody confirmed that the VirR protein was not produced by CPJV47 (Fig. 10D).

When CPJV47 was incubated in the presence of Caco-2 cells, no CPB secretion was observed after 2 h (Fig. 10B). In addition, RT-PCR analyses detected no *cpb*or *pfo*A mRNA using template RNA isolated from CPJV47 following a 2 h infection of Caco-2 cells (Fig. 11). Quantitative RT-PCR confirmed that, compared to the wt levels, CPJV47 had a

significant decrease of *cpb* or *pfo*A mRNA levels. However, within 1 or 2 h post-infection, *plc* mRNA was produced by CPJV47 at the same levels as that of the wt strain, indicating that the rapid Caco-2 cell-induced increase in *plc* gene transcription can still occur in the absence of the *vir*S/*vir*R operon when this strain is grown in the presence of Caco-2 cells (Figs. 11C and 11D).

To confirm a role for the VirS/VirR system in Caco-2 cell induced rapid transcriptional upregulation of some toxin genes, a *vir*R antisense vector was constructed as described in Materials and Methods. The antisense *vir*R mRNA produced from this vector inhibits protein production by mediating the catalytic degradation of the target *vir*R mRNA or by binding to sites on *vir*R mRNA essential for translation (Laursen *et al.*, 2005). As shown in Figure 10B, CN3685 transformed with the *vir*R antisense vector (WT+pJVBM11) did not rapidly secrete CPB into the supernatant of infected Caco-2 cell cultures. This strain was also non-hemolytic when grown on blood agar plates [CN3685 produces PFO to induce beta-hemolysis (Sayeed *et al.*, 2008)] and RT-PCR analyses confirmed that the *pfo*A gene was not expressed after a 2 h infection of Caco-2 cell cultures. However, as also shown for CPJV47, *plc*mRNA was still produced within 2 h in the presence of Caco-2 cells (data not shown). Results presented above suggested that the *vir*S/*vir*R operon of CN3685 is required for regulating the early production of CPB and PFO, but not CPA, in the presence of enterocyte-like Caco-2 cells.

To prove that the loss of early CPB production by CPJV47 was specifically due to inactivation of its *vir*R gene, either the wild-type *vir*R gene alone or both the *vir*R and *vir*S genes together were amplified from type C strain CN3685 and ligated into the pJIR750 shuttle plasmid under the control of the *vir*R promoter, to generate pJVR4 and pJVRS3, respectively (Fig. 10E). Those plasmids were then individually electroporated into the *vir*R mutant strain CPJV47. In addition, the plasmid pTS405 (Okumura *et al.*, 2008) which contains a *Pst*I-digested fragment of Strain 13 chromosomal DNA that includes both the *vir*R and *vir*S genes, was also electroporated into CPJV47. The presence of the *vir*R gene in CPJV47(pJVR4), CPJV47(pJVRS3) and CPJV47(pTS405) was confirmed by PCR (Fig. 10A and not shown). Consistent with previous studies indicating that the *vir*R and *vir*S genes are co-transcribed in an operon (Shimizu *et al.*, 1994), RT-PCR analyses detected *vir*R mRNA in both CPJV47 derivatives [i.e. CPJV47(pJVRS3) and CPJV47(pTS405)] complemented with the *vir*S/*vir*R operon, but not in CPJV47(pJVR4), which only encodes the *vir*R gene (Fig. 10C). Western blot analyses using an anti-VirR antibody confirmed that the VirR protein had been produced by CPJV47(pJVRS3) (Fig. 10D).

This complementation restored CPB secretion by CPJV47(pJVRS3) and CPJV47(pTS405), but not by CPJV47(pJVR4) (Fig. 10B). RT-PCR detected transcription of *cpb* and *pfo*A mRNA using RNA isolated from CPJV47(pJVRS3) or CPJV47(pTS405) after a 2 h infection of Caco-2 cells (Fig. 11A and 11B). Moreover, qRT-PCR demonstrated the same levels of *pfo*A, *cpb* or *cpa* mRNA in the wt strain CN3685 and CPJV47(pJVRS3) after a 1 h infection of Caco-2 cells (Fig. 11D). Altogether, these results confirm that the *vir*S/*vir*R operon regulates the early transcription of *cpb* and *pfo*A by CN3685 in the presence of Caco-2 cells.

Finally, a Caco-2 cell cytotoxicity assay showed that the *vir*R mutant CPJV47 strain was less cytotoxic than the WT strain for Caco-2 cells. However, complementing back the *vir*S/*vir*R operon completely restored the cytotoxic phenotype of this strain (Table 1).

Discussion

To our knowledge, this is the first study of clostridial toxin gene regulation in the presence of host cells, an important topic since pathogenic clostridia often rely upon *in vivo* production of potent toxins to cause animal and human disease (McClane, 2006; Voth and Ballard, 2005; Songer, 1996). With specific respect to *C. perfringens* type C isolates, previous *in vitro* studies had indicated these bacteria produce toxins mainly during the late-log or stationary growth phases (Amimoto *et al.*, 2007; Fernandez-Miyakawa *et al.*, 2007; Fisher *et al.*, 2006; Sayeed *et al.*, 2005; Voth and Ballard, 2005), consistent with the absence of early toxin production noted in the current study for type C isolates growing in TGY medium. In contrast to those *in vitro* data, the current research revealed that the presence of intestinal-like Caco-2 cells causes *C. perfringens* type C isolates to rapidly upregulate their toxin gene expression. These kinetic differences between *in vivo* vs. *in vitro* toxin regulation suggest some need for caution when extrapolating *in vitro* toxin regulation results to the *in vivo* situation.

More importantly, the discovery of rapid host cell-induced toxin upregulation suggests new insights into clostridial virulence. Many human and animal clostridial diseases begin in the intestines (McClane *et al*., 2006), including human necrotic enteritis caused by *C. perfringens* type C isolates. This necrotic enteritis can be fatal within 48 h, as toxins produced quickly in the intestines are then absorbed into the circulation to affect internal organs (Matsuda *et al.*, 2007; Gui *et al.*, 2002). The same explosive disease progression can also occur in animals, e.g. type C isolates cause “struck” in lambs, a disease named because the animal dies so suddenly that it appears to have been struck by lightning (Songer, 1996). Our current results suggest that the rapid disease and death associated with type C infections may involve pathogenic *C. perfringens* type C isolates sensing the presence of host cells to rapidly upregulate their secretion of potent toxins such as CPB, CPB2, CPA and PFO. Consistent with rapid host-cell mediated toxin secretion upregulation contributing to the virulence of type C infection, the current study showed that supernatants from infected Caco-2 cells cultures cause cytotoxic consequences in Caco-2 cells more rapidly than do supernatants from MEM (no Caco-2 cells) cultures.

To our knowledge, the rapid Caco-2 cell-induced toxin secretion reported in this study represents the first identification of cross-talk host:pathogen communication affecting production of classical exotoxins, which are the hallmark of clostridial infections. This phenomenon appears to be widespread among pathogenic *C. perfringens* type C isolates, as several different type C strains isolated from diseased humans or several domestic animal species were shown to rapidly upregulate their toxin production and secretion in the presence of Caco-2 cells. Nor is this phenomenon restricted to type C isolates, as two *C. perfringens* type D isolates also rapidly upregulated their production and secretion of epsilon toxin in the presence of Caco-2 cells (Fig. S4).

Toxin upregulation in type C isolates is not only induced by contact with human-derived intestinal Caco-2 cells, since similar effects can be triggered by exposure to dog kidney MDCK cells or rat fibroblast 1R-12 cells. The ability of all three surveyed mammalian cell lines to enhance toxin secretion suggests that the factor(s) triggering this effect is widely distributed among host cells, which may be important since it could indicate that host cell-induced toxin upregulation can also occur in extraintestinal *C. perfringens* infections, possibly including gas gangrene (which is mediated by CPA and PFO (Lyristis *et al.*, 1994)).

To begin identifying the nature of the host cell factor(s) responsible for upregulated toxin secretion of CPB (and probably other toxins), we showed that toxin upregulation is

dependent upon close contact, although not tight adherence, between *C. perfringens* and host cells. The inability of sterile Caco-2 cell supernatants to stimulate CPB secretion, and the sharp reduction noted when type C isolates and Caco-2 cells were physically separated by a Transwell filter, could suggest that this signaling does not involve a soluble factor derived from host cells (further discussion later). Similarly, Caco-2 cell membrane preparations or Caco-2 cell lysates also failed to cause type C isolates to upregulate their CPB secretion. However, enhanced CPB secretion was observed when these *C. perfringens* isolates were incubated in the presence of PFA-fixed Caco-2 cells. These results could suggest (further discussion later) that, to trigger maximal upregulation of toxin secretion, *C. perfringens* type C isolates must recognize some factor(s) present in a proper conformation on the host cell surface. This possibility is consistent with the increased CPB production observed after Caco-2 cells were pretreated with pronase, which might unmask a signaling molecule on the Caco-2 cell surface.

With respect to bacterial mechanisms, our study found that this host cell-induced toxin secretion does not simply involve host cell stimulation of bacterial growth, as *C. perfringens* CFU values were similar in the presence or absence of Caco-2 cells. Instead, the quick appearance of secreted toxins in infected Caco-2 cell cultures correlated with a rapid onset of toxin gene transcription, since RT-PCR showed rapidly upregulated transcription of *cpb*, *cpb*2, *plc* and *pfo*A toxin genes in the presence of Caco-2 cells. However, the presence of Caco-2 cells does not induce transcription of all type C toxin genes, since transcription of the *tpe*L gene was not detectable during a 2 h infection period of Caco-2 cells, TGY or MEM alone. This result indicates that *tpe*L expression is regulated by a different regulatory mechanism from that of other type C toxin genes, which is consistent with previous studies showing that, unlike other type C toxins, TpeL is made *in vitro* only during stationary phase (Amimoto *et al.*, 2007).

Two component regulatory systems often mediate the responses of pathogenic bacteria to environmental alterations. In *C. perfringens* type A strains, a classic two component regulatory system named VirS/VirR was previously shown to control, at least in part, *in vitro* production of CPA, PFO, kappa-toxin (collagenase) and CPB2 (Ohtani *et al.*, 2003; Ba-Thein *et al.*, 1996; Lyristis *et al.*, 1994). VirS, a histidine kinase located in the bacterial membrane, undergoes autophosphorylation after activation by a still unidentified external stimulus. VirS then phosphorylates VirR, which in turn directly activates the transcription of *pfo*A by binding to a VirR box located −40 to −80 bp upstream of the transcriptional start site for *pfoA* (Cheung *et al.*, 2004). VirR also indirectly increases transcription of the *plc*, *col*A, *cpb*2 and other genes (Okumura *et al.*, 2008; Cheung *et al.*, 2004; Ohtani *et al.*, 2003; Lyristis *et al.*, 1994). However, VirR boxes are absent from the region upstream of those toxin genes; instead, *in vitro* transcription of those toxin genes is regulated by VirR binding to VirR boxes located upstream of the promoter controlling production of a regulatory RNA named VR-RNA (Okumura *et al.*, 2008; Banu *et al.*, 2000).

Our current results indicate that the early production of CPB and PFO (but not CPA) induced by the presence of Caco-2 cells is also dependent upon the VirS/VirR two-component regulatory system. For example, both CN3685 encoding a *vir*R antisense plasmid (WT+pJVBM11) and an isogenic CN3685 *vir*R mutant (CPJV47) did not secrete CPB or PFO after a 3 h infection of Caco-2 cell cultures (Figures 9B and not shown). Notably, CPJV47 was also less cytotoxic for Caco-2 cell cultures than the WT strain (Table 1). Rapid Caco-2 cell-induced CPB secretion, as well as Caco-2 cell cytotoxicity, was restored when CPJV47 was complemented with the *vir*S/*vir*R operon, indicating that a functional *vir*S/*vir*R operon is required for production of toxins and is also important for cytotoxicity *in vivo*. This discovery that a functional VirS/VirR system is necessary for the rapid Caco-2 cell-induced upregulation of *cpb* transcription (as well as *pfoA* transcription) is

interesting since VirR boxes are not readily identifiable immediately upstream of the *cpb* promoter. This may suggest that *cpb* transcription is under the control of a regulatory RNA, which could be VR-RNA or another recently-identified *C. perfringens* regulatory RNA (Okumura *et al.*, 2008). In contrast to the *cpb* results, rapid Caco-2 cell-induced upregulation of *plc* transcription occurred even in a *vir*R null mutant of a type C isolate. Unfortunately, the involvement of VirS/VirR in regulating the *in vivo* transcription of *cpb2* by type C isolates could not be assessed because our *cpb2*-positive type C isolates were not sufficiently transformable to construct a VirR mutant.

Despite several qualitative similarities between VirS/VirR-mediated toxin gene regulation during *in vivo* versus *in vitro* growth (e.g., *pfoA* transcription is completely dependent on a functional VirS/VirR system both *in vivo* and *in vitro*, while *plc* transcription is not), an obvious difference between *in vitro* vs. *in vivo* toxin gene regulation by VirS/VirR concerns transcriptional kinetics. Type C isolates slowly produce PFO, PLC, CPB2 and CPB in TGY medium (Fisher *et al.*, 2006), but the presence of Caco-2 cells cause more rapid toxin production by wild-type type C isolate compared to an isogenic *vir*R mutant (this study). This suggests that a major role for VirS/VirR during type C disease may be to mediate rapid host cell-induced upregulation of some toxins, including CPB. This *in vivo* VirS/VirR toxin regulation should have significant pathogenic consequences since CPB is required for type C isolate virulence (Sayeed *et al.*, 2008). If future animal studies confirm the importance of VirS/VirR for type C virulence, this would provide the first linkage of VirS/VirR to some *C. perfringens* enteric diseases. VirS/VirR has already been shown to be important for *C. perfringens*-induced gas gangrene in the mouse model (Lyristis *et al.*, 1994); as mentioned earlier, this requirement for VirS/VirR in *C. perfringens*-induced gas gangrene could involve, at least in part, mediating the upregulation of toxin production after type A isolates contact muscle or other host cells.

It is not yet clear why *C. perfringens* transcribe toxin genes more quickly in the presence of host cells. However, these differences could involve, in part, the signaling that activates the VirS sensor. VirS/VirR signaling molecules remain poorly understood, but this two-component system might become more rapidly activated in the *in vivo* environment because either, i) VirS responds to a different signal under *in vivo* vs. *in vitro* conditions (e.g. perhaps a Caco-2 cell surface molecule provides the *in vivo* signal?) or ii) the same signal is produced by *C. perfringens* under both *in vivo* and *in vitro* conditions, but the presence of Caco-2 cells directly or indirectly triggers greater or faster production of this signal.

Quorum sensing molecules often provide the signals that modulate two component regulatory systems to upregulate virulence factor expression during bacterial disease (Novick, 2003) (Clarke *et al.*, 2006). In *C. perfringens* type A strain 13, the *lux*S gene product (which is involved in production of the AI-2 quorum sensing autoinducer) has been previously implicated in regulating the *in vitro* transcription of the *plc* and *pfoA* genes (Ohtani *et al.*, 2002). However, the current study still observed rapid transcriptional upregulation of the *cpb*, *pfoA* and *plc* genes when a *lux*S mutant of CN3685 was used to infect Caco-2 cell cultures, indicating the AI-2 autoinducer is not required for rapid *in vivo* toxin gene transcription by this type C isolate.

Finally, further studies are clearly needed to better identify the mechanism by which contact with Caco-2 cells induces *C. perfringens* to rapidly upregulate production of several toxins. It is particularly interesting that this effect can involve VirS, which is located in the plasma membrane and thus buried under a peptidoglycan cell wall. Among several possibilities to explain how VirR could be activated upon close contact of *C. perfringens* with host cells, VirR activation might involve interactions between surface factors on the host and bacterial cells, with the *C. perfringens* surface factor then signaling VirS. This hypothesis would

appear to conflict with the inability of isolated Caco-2 cell membranes to mediate upregulated toxin production; however that negative result might simply reflect the loss or inactivation of the necessary Caco-2 cell surface factor during membrane isolation. An alternative explanation is that a soluble host-derived factor mediates VirR activation. Although appearing inconsistent with the lack of toxin upregulation observed, i) using conditioned supernatants from infected cultures or ii) when bacteria and host cells are separated by Transwell membranes, this possibility should not yet be eliminated. For example, supernatants of infected cultures, or Transwell-separated bacteria and host cells, may not show toxin upregulation because a host cell-derived soluble factor might only induce signaling when present at locally high concentrations, as occurs when Caco-2 cells and *C. perfringens* are in close contact. If a soluble host cell-derived factor does mediate the signaling that triggers upregulation of toxin production, this factor could be present on the host surface and then released be by one of the many potent exoenzymes (e.g., proteases, sialidases, phospholipases) produced by *C. perfringens*. The increased toxin production observed after pronase pretreatment of host cells is consistent with signalling involving either direct host cell-bacterial cell surface factor interaction or a soluble host factor, i.e., pronase-induced removal of some host surface protein(s) might expose the signaling model for direct interactions with a bacterial surface factor or for cleavage by a *C. perfringens* exoenzyme. Further study of the signaling process behind upregulated toxin production is currently underway to better address these uncertainties.

Experimental procedures

Bacterial strains and culture conditions

C. difficile isolate CD00030 (*tcd*A$^+$/*tcd*B$^+$) was kindly provided by Dr. Scott Curry. The toxin genotypes of *C. perfringens* isolates used in this study are described in Table 2. *C. perfringens* toxin genotypes had been already determined using a previously described mutiplex PCR assay (Fisher *et al.*, 2006); in addition, PCR amplification of *tpe*L or *tcd*A genes was performed using primers listed in Table 3. Frozen (−20°C) stock cultures of each isolate were prepared in cooked meat medium (Difco Laboratories). Those stock cultures were routinely used to inoculate 10 ml tubes of FTG (Difco Laboratories), which were then incubated overnight at 37°C. To obtain isolated colonies, each FTG culture was inoculated onto an TCS agar plate, (SFP agar [Difco Laboratories] containing 0.1% D-cycloserine [Sigma-Aldrich]) which was then incubated overnight at 37°C under anaerobic conditions. For experiments, a single bacterial colony from a TCS agar plate was inoculated into ~10 ml of FTG, which was grown for 12 h at 37°C. This culture was then centrifuged at 8000 × *g* for 20 min at 4°C. That bacterial pellet was washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4) before delicate resuspension in 10 ml of ice-cold PBS containing 0.1% of cysteine (Difco Laboratories). Those washed bacteria were then immediately used to infect cell cultures as described below.

Cell culture

Human-derived enterocyte-like Caco-2 cells were routinely cultured in Eagle's Minimum Essential Medium (MEM) (Sigma) supplemented with heat-inactivated 10% fetal calf serum (FCS) (Mediatech Incorporated), 1% non-essential amino acids (Sigma), 1% glutamine (Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml). Madin-Darby canine kidney (MDCK) cells were cultured in a 50:50 mixture of Dulbeco´s Modified Eagle´s Media (DMEM) (Sigma) and Ham's F12 (Sigma) supplemented with heat-inactivated 3% FCS, 1% non-essential amino acids, 1% glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Rat1-R12 fibroblasts were maintained in DMEM with 10% tet-off-certified fetal bovine serum, penicillin (100 U/ml), geneticin (100 μg/ml) and streptomycin (100 μg/ml). Each cell

line was normally harvested with 0.25% trypsin (Gibco), resuspended in the cell culture medium, and incubated at 37°C in 5 % CO_2 humidified atmosphere.

C. perfringens infection of Caco-2 cell cultures

For all experimental infections, Caco-2 cells were grown for 4–5 days until reaching confluency in either 100 mm tissue culture dishes (containing 1.2×10^7 cells/dish) or 24-well microplates (containing 7×10^5 cells/well). Prior to bacterial infection, the confluent Caco-2 cell cultures were washed three times with pre-warmed PBS (pH 7.4) and then incubated with 10 ml of MEM containing no additives, i.e. no serum or antibiotics (this was considered as a mock infection). Caco-2 cells were then infected with *C. perfringens* isolates at a multiplicity of infection of 20 (MOI=20) and incubated for indicated times at 37°C. Infection of MDCK cell cultures or Rat1-R12 fibroblast cultures with *C. perfringens* was performed similarly.

To compare the secretion of proteins and toxins by *C. perfringens* during *in vitro* growth versus growth in the presence of Caco-2 cells, the same number of bacteria were inoculated into tissue culture wells containing either TGY [3% tryptic soy broth (Difco), 2% glucose (Sigma), 1% yeast extract (Difco), 0.1% cysteine (Difco)], FTG or MEM and no Caco-2 cells. Those cultures were then incubated under the same conditions used for *C. perfringens*-infected Caco-2 cell cultures.

Determination of *C. perfringens* colony-forming units (CFU)

To determine the total number of bacteria present in each culture, Caco-2 cell cultures growing in a 24-well cell culture microplate (containing 7×10^5 cells/well) were infected with 1.5×10^7 CFU of *C. perfringens* strains (MOI=20) for 3 h at 37°C. The same number of bacteria were also inoculated into TGY or MEM added to a 24-well tissue culture plate lacking any Caco-2 cells. The supernatant containing bacteria (10 ml) was aspirated and those wells were extensively washed by pipetting up and down five times to detach all bacteria attached to the Caco-2 cell surface or to the bottom of the tissue culture microplate wells. Supernatants containing the harvested bacteria were then serially diluted in BHI broth (10 ml final volume) (brain heart infusion, Difco Laboratories) and 1 ml was plated onto BHI agar plates. After a 24 h incubation under anaerobic conditions at 37°C, CFU were determined. In addition, detachment of all bacteria from the Caco-2 cells was verified by scrapping these cells in cold PBS with a pipette tip and determining, as described above, CFU of the cell homogenate. Finally, to confirm that no bacteria were intracellular, Caco-2 cells were scraped into cold PBS containing 0.1% Triton X-100 (Sigma), vigorously vortexed for 30 s, and CFU were then recorded. No CFU differences were detected using any of these methods.

Analysis of protein and toxin secretion

To compare the secretion kinetics of proteins and toxins by *C. perfringens* isolates growing in the presence or absence of Caco-2 cells, confluent Caco-2 cell cultures grown in 100 mm tissue culture dishes (containing 1.2×10^7 cells/dish) were infected, for the indicated times, with 2.5×10^8 CFU of *C. perfringens* strains (MOI=20). For comparison, 100 mm tissue culture dishes containing an equivalent volume of TGY or MEM lacking Caco-2 cells were similarly infected. The supernatant of each culture, which contained *C. perfringens* secreted proteins and toxins, was aspirated and centrifuged 8000 × *g* at 4°C for 30 min. That supernatant was then filter sterilized using a 0.22 μm filter (Millipore) and the sterile filtrate was processed for the following analysis.

i) SDS-PAGE analysis of secreted proteins—The sterile supernatants prepared above were each concentrated 10-fold using an Amicon Ultra centrifugal filter device with a 10-

kDa cutoff (Millipore, Bedford, Mass.). Equal volumes (25 µl) of the concentrated supernatants were then subjected to SDS-PAGE on a 12% polyacrylamide gel and stained with Coomassie blue to visualize total protein patterns in each supernatant sample (Laemmli, 1970). To evaluate whether the secreted protein patterns of Caco-2 cell cultures reflected *C. perfringens* infection, the supernatants of non-infected Caco-2 cells growing for 3 h in MEM without additives were similarly concentrated and evaluated by SDS-PAGE.

ii) Western blots of secreted toxins—To compare the secretion of CPB, CPB2 or ETX toxins in the presence or absence of Caco-2 cells, unconcentrated sterile supernatants, prepared as described above, were subjected to SDS-PAGE o n a 12% polyacrylamide gel and then transferred onto a nitrocellulose membrane. Those membranes were blocked with PBS-Tween 20 (0.05% v/v) and non fat dry milk (5% wt/v) for 1 h and then probed with either a rabbit polyclonal anti-CPB2 antibody (Fisher *et al.*, 2005), a mouse monoclonal anti-CPB antibody, kindly provided by Dr. Paul Hauer, or with an monoclonal anti-ETX antibody (Sayeed *et al.*, 2005). Bound antibody was then detected after incubation with a horseradish peroxidase-conjugated secondary anti-species specific antibody and addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce). Where noted, purified CPB, CPB2 or ETX were added as a Western blot control; those purified toxins were obtained as previously described (Vidal *et al.*, 2008; Sayeed *et al.*, 2007; Fisher *et al.*, 2005).

To quantify CPB2 secretion, different amounts of purified CPB2 or sterile supernatant from Caco-2 cells, MEM or TGY infected with JGS1495 (MOI=20) were run in a 12% SDS-PAGE and Western blotted. After scanning, the intensity of each band, in arbitrary units (pixels), was obtained using SigmaGel (Jandel Scientific) software and plotted graphically.

To analyze by Western blot the secretion of CPA and PFO toxins in the presence or absence of Caco-2 cells, the sterile culture supernatants were dialyzed overnight at 4°C versus 10 mM Tris, pH 7.5. The dialyzed supernatants were then lyophilized (using a Labconco freeze-dry system) and resuspended in ice-cold PBS (pH 7.4) to obtain a 200-fold concentrated supernatant. Identical amounts of lyophilized material were electrophoresed on a 10% acrylamide gel containing SDS. After transfer of separated proteins onto a nitrocellulose membrane, the membranes were blocked in PBS-Tween 20 (0.05% v/v) with non-fat dry milk (5% wt/v) for 1 h and probed with either a mouse monoclonal anti-CPA antibody (a kind gift of Dr. P. Hauer) or a rabbit polyclonal anti-PFO antibody (a kind gift of Dr. R. Tweten). Western blotting was then performed as described above.

iii) Hemoglobin (Hb) release assay to quantify secreted PFO activity—Horse red blood cell (RBC, Becton-Dickinson Laboratories) were washed three times with sterile phosphate-buffered saline (PBS) and resuspended in PBS to give a final 1% RBC suspension. Sterile Caco-2 cell supernatants, prepared as described above, were mixed with the erythrocyte suspension (1:1) and incubated at 37°C for 30 min. As controls, MEM, TGY or 0.1% saponin (positive control) were also incubated with the RBC suspension. PFO was purified from the supernatant of CN3685, essentially as described earlier (Tweten, 1988b) and different amounts were incubated with RBC as described. After incubation, the RBC suspension was centrifuged at 500 × *g* for 5 min at 4°C to pellet non-lysed erythrocytes and/or cell debris. Hb released into the supernatant (Abs_{570nm}) was then measured using a microplate reader (Dinex technologies).

iv) ELISA to quantify secreted CPA and CPB levels—*C. perfringens* alpha toxin (Sigma Aldrich), purified CPB or sterile supernatants, were serially diluted with 1 M carbonate buffer (0.5 M Na_2CO_3 and 0.5 M $NaHCO_3$; pH 9.5) in a 96-well ELISA microplate (Corning, Inc.). After overnight incubation at 4°C, plates were washed 3 times with PBS-Tween (PBST, 0.05% Tween 20) and blocked with bovine serum albumin (BSA,

1%) for 1 h at 37°C. The plates were then washed 10 more times and incubated with a mouse monoclonal anti-CPA antibody (1:5000) or monoclonal anti-CPB (1:5000) for 1 h at 37°C. After that incubation, the plates were washed ten times with PBST and incubated with a HRP-conjugated anti-mouse antibody (1:5000) for 1 h at 37°C. After 10 more washings with PBST, the bound HRP-conjugated antibody was detected using tetramethylbenzidine (TMB) substrate solution (Thermo scientific). After 15 min, the color reaction was stopped with sulphuric acid (0.18 M) and the sample absorbance at 450 nm (A_{450} nm) was determined using an ELISA reader (Dinex Technologies).

RT-PCR and qRT-PCR analyses

Supernatants containing bacteria were removed from infected Caco-2 cell cultures and pelleted at 8000 × *g* for 20 min at 4°C. MEM, FTG, TGY or TY [3% tryptic soy broth (Difco), 1% yeast extract (Difco), 0.1% cysteine (Difco)] cultures were similarly centrifuged. Total bacterial RNA was extracted from the centrifuged pellets using a RiboPure bacteria kit (Ambion), as described by the manufacturer. All RNA samples were treated with DNase I at 37°C for 30 min. RT-PCR reactions were then performed on those DNase-treated RNA samples using the AccesQuick RT-PCR system (Promega). Briefly, 20, 50 or 100 ng of each RNA sample were reverse-transcribed to cDNA at 45°C for 1 h and then used as template for PCR (denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min) with the toxin gene-specific primers listed in Table 3. RT-PCR reactions yielded the same results using any of the three tested RNA concentrations. Control RT-PCR reactions were similarly performed, except for the omission of reverse transcriptase. As an additional control, another reaction amplifying each toxin gene was performed by adding, into the RT-PCR reaction cocktail, DNA that had been extracted from *C. perfringens* type C isolates using the MasterPure Gram Positive DNA Purification kit (Epicentre Biotechnologies).

Quantitative RT-PCR (qRT-PCR) was performed using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) and the iCycler thermal cycler with a 96-well reaction module (Bio-Rad). qRT-PCR reactions were performed in triplicate with 20 ng of total RNA, 500 nM concentration of each primer (Table 3) and the following conditions; 1 cycle at 50°C for 30 min, 1 cycle at 95°C for 10 min and 40 cycles of 95°C for 15, 55°C for 1 min. Melting curves were generated by a cycle of 95°C for 1 min, 55°C for 1 min and 80 cycles of 55°C with 0.5°C increments. The relative quantitation of mRNA expression was normalized to the constitutive expression of the housekeeping *pol*C gene and calculated by the comparative C_T ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001).

Studies of Caco-2 cell mediated up-regulation of *C. perfringens* toxin expression

i) CPB secretion in conditioned medium (CM)—A confluent monolayer of Caco-2 cells was washed three times and then incubated for 3 h at 37°C in MEM without additives (serum or antibiotics). This medium containing secreted or released Caco-2 cells proteins (termed conditioned medium [CM]) was collected, centrifuged 7000 × *g* to remove cellular material, and filtered-sterilized.

C. perfringens type C strain JGS1495 or CN3685 was then inoculated (1.5×10^7 CFU) into 1 ml of CM alone, 1 ml of MEM alone (without additives), Caco-2 cell cultures treated with 1 ml of CM, or Caco-2 cells in fresh (non-conditioned) MEM in a 24-well tissue culture plate. After 3 h of incubation at 37°C, supernatants were centrifuged 8000 × *g* at 4°C for 30 min, filter-sterilized, and analyzed by Western blot for β-toxin production using a mouse monoclonal anti-CPB antibody, as described earlier.

ii) Effects of Caco-2 cell number on CPB secretion levels—Caco-2 cell cultures were grown for 3–4 days in 24-well microplates to the following confluency: 100% (containing 7×10^5 cells/well), 80% (containing 5.5×10^5 cells/well), 50% (containing 4×10^5 cells/well), 30% (containing 2.5×10^5 cells/well) or MEM alone (mock infection). Each well was then infected with 1.5×10^7 CFU of *C. perfringens* type C strain JGS1495 and then incubated for 3 h at 37°C. After this incubation, the culture supernatants were harvested and centrifuged 8000 × *g* at 4°C for 30 min. The supernatants were then filter-sterilized and analyzed by Western blot for CPB levels using a mouse monoclonal anti-CPB antibody, as described earlier.

iii) CPB secretion levels after incubation with Caco-2 cell lysates or Caco-2 cell membranes—Washed Caco-2 cells (1.2×10^7 cells) were scraped from a 100 mm tissue culture dish using a rubber policemen and homogenized in 500 µl of ice-cold PBS (pH 7.4). Cell homogenates were then lysed by sonication. Some of the lysates were centrifuged at 21 000 × *g* for 30 min at 4°C to obtain a Caco-2 membrane fraction.

C. perfringens type C strain JGS1495 (2.5×10^8 CFU) was inoculated into a 100 mm tissue culture dish containing 12 ml of MEM without additives, MEM without additives but with Caco-2 cell lysates, MEM without additives but with the Caco-2 cell membrane fraction, or MEM plus confluent living Caco-2 cells (MOI=20). After 3 h of infection at 37°C, supernatants were collected, centrifuged at 8000 × *g* at 4°C for 30 min, and analyzed by Western blot for CPB levels using a mouse monoclonal anti-CPB antibody, as described earlier.

iv) CPB secretion levels in the presence of fixed Caco-2 cells—Caco-2 cells were grown to confluency in 24-well microplates and washed twice with pre-warmed PBS (pH 7.4). The washed cultures were then fixed for 20 min with 2% paraformaldehyde (PFA), extensively washed with pre-warmed PBS (pH 7.4) and incubated with MEM without additives or with PBS. Those PFA-fixed cells were infected with *C. perfringens* (MOI=20), and incubated for the indicated time at 37°C. Supernatants were then collected, centrifuged 8000 × *g* at 4°C for 30 min and filter-sterilized. The sterile supernatants were analyzed for CPB levels by Western blotting using a mouse monoclonal anti-CPB antibody, as described earlier.

v) CPB secretion levels in the presence of trypsin, EDTA, PLC or pronase treated Caco-2 cells—Caco-2 cells growing in a 24-well microplate were treated with either; a) 0.25% trypsin or a 0.2% EDTA solution for 15 min; b) phospholipase C in the form of CPA (1×10^{-3} U/ml) or (1×10^{-5} U/ml) for 1 h or c) pronase (Roche) (100 µg/ml) for 20 min. All cell treatments were done at 37°C. Treated cells were washed three times with sterile PBS and resuspended in MEM (trypsin-, EDTA- or pronase-treated cells) or added with MEM (PLC-treated cells). The cell density was obtained by standard procedures using a cell counting chamber. Cell suspensions (7×10^5 cells/well) were infected with CN3685 or JGS1495 (MOI=20) and incubated for the indicated time at 37°C. The supernatant was obtained and analyzed by western blot for CPB levels as described earlier.

vi) CPB secretion levels in Transwell cultures of Caco-2 cells—Caco-2 cells were seeded into the membrane chamber of a Transwell-Col filter (0.4 µm pore size, Corning) installed in a 12-well cell culture plate. The cultures were then grown to confluency over four days. Before infection, the Caco-2 transwell cultures were carefully washed three times with pre-warmed PBS (pH 7.4) and incubated at 37°C in 2 ml of MEM (without additives) for 30 min. As a control, an uninoculated Transwell filter was sometimes installed in wells where Caco-2 cells (1.5×10^6 cells/well) were growing in the bottom of the culture well.

For infection, *C. perfringens* type C isolates were inoculated (3×10^7 CFU) directly into the Transwell filter chamber (that did or did not contain Caco-2 cells) and incubated for 3 h at 37°C. The supernatant from the Transwell filter chamber was collected, centrifuged at 8000 × *g* for 30 min, and filter-sterilized. The sterile supernatant was then analyzed by Western blot for CPB levels using a mouse monoclonal anti-CPB antibody, as described earlier.

Cytotoxicity of *C. perfringens* type C strains

To evaluate the cytotoxic consequences of Caco-2 cell-induced toxin up-regulation, we first inoculated Caco-2 cell cultures or MEM alone with *C. perfringens* type C strain CN3685 for 1 h. The culture supernatants were then removed and filtered-sterilized using a 0.22 μm filter (Millipore). Each sterilized supernatant was then added to fresh confluent Caco-2 cells and incubated for 2 h at 37°C. After this treatment, the Caco-2 cell cultures were washed three times with pre-warmed PBS (pH 7.4), fixed with 70% methanol and stained with Giemsa stain.

To directly observe cytotoxicity during infection, Caco-2 cells were infected with *C. perfringens* type C strains (MOI=20) and the development of morphological changes was then followed every 30 min using a Zeiss Axiovert 25 inverted microscope. Cytotoxicity induced by infection was scored as follows, (+) indicates that >80% of Caco-2 cells had swelled, (++) <50% of Caco-2 cells had swelled and <50% of cells had rounded and (+++) indicates that >80% of Caco-2 cells were rounded and had detached from the glass. Pictures of infected-Caco-2 cells were then taken using a Canon Powershot G5 fitted to the Zeiss Axiovert 25 microscope. We were unable to fix and stain infected Caco-2 cells since the development of morphological damage (i.e. swelling on >80% of cells) coincided with Caco-2 cell detachment from the glass after PBS washing.

Construction of a CN3685 *lux*S null mutant using TargeTron® technology

To inactivate the *lux*S gene, the wild-type *lux*S gene sequence was first entered into the Intron prediction program (www.sigma-genosys.com/targetron/). This program predicted a sense insertion site, with a low e-score (0.170), between the nucleotides 295|296 of the *lux*S gene. The primers, luxS-IBS, luxS-EBS1d and luxS-EBS2 (Table 3), were used to generate a 350 bp intron targeting sequence to this *lux*S gene. The amplified 350-bp fragment was then digested with *Hin*dIII and *Bsr*GI and ligated into pJIR750ai, (a TargeTron®-carrying vector we created previously to construct a *plc* null mutant (Chen *et al.*, 2005) in type A isolate ATCC3624), which had been similarly digested with the same two restriction enzymes. The resultant plasmid (pJVLux), carrying a *lux*S-targeted intron, was electroporated into wild-type *C. perfringens* type C isolate CN3685, as described previously (Chen *et al.*, 2005). Transformants were plated onto BHI agar plates containing 15 μg/ml of chloramphenicol. Colonies were then PCR screened using *lux*S-specific primers that supported PCR amplification of an ~300 bp product from the wild-type *luxS* gene, but amplified a larger ~1.2 kb product from those mutants. The mutant PCJV19 was then shown by PCR to have the intron inserted in the *lux*S gene and by Southern blotting, to have a single intron insertion (Figure S3A). All PCR-screened reactions were done using Taq 2X Master Mix (New England Biolabs) and primers final concentration of 1 μM in a Techne (Burkhardtsdorf, Germany) thermocycler.

Inactivation of the *vir*R gene in type C CN3685 isolate

To inactivate the *vir*R gene, the *E. coli*-based *C. perfringens* suicide plasmid (pKOR) (Shimizu *et al.*, 1994), which contains a ~590 bp fragment of the *vir*R gene cloned upstream the tetracycline resistant gene, was electroporated into CN3685. Transformants were plated onto BHI agar plates containing 2.5 μg/ml of tetracycline. Only those bacteria with the ~590 bp fragment of the *vir*R gene integrated in their genome were capable of growth in the

presence of tetracycline. Tetracycline-resistant colonies were PCR screened using primers that supported the amplification of a ~590 bp product or primers that amplify the entire ~710 bp *vir*R wt gene. A mutant, CPJV47, that had lost the ~710 bp PCR product (Figure 9A), was then grown in blood agar plates to test for PFO-induced beta hemolysis.

Complementation of *vir*R mutant

To complement the *vir*R mutant (CPJV47), the entire *vir*S/*vir*R operon from CN3685, (including the *vir*R promoter), was PCR amplified using the primers virRP-LKpnI and virS-RXbaI. The product was then ligated into the *E. coli-C. perfringens* shuttle vector pJIR750 to generate pJVRS3 (Figure 9D). To construct pJVR4, the *vir*R promoter and *vir*R gene from CN3685 were PCR amplified using the primers virRP-LKpnI and virR-RXbaI and cloned into pJIR750 (Figure 9D). Plasmid pTS405, which encodes the *vir*S/*vir*R operon, was constructed by digesting Strain 13 DNA with *Pst*I and cloned in *E. coli-C. perfringens* shuttle vector pJIR418. Those plasmids were electroporated into the *vir*R mutant strain CPJV47, as described (Chen *et al.*, 2005).

Construction of a *vir*R antisense vector

To knockdown the *vir*R gene with an antisense mRNA, a PCR product containing the *vir*R promoter was cloned between the *Kpn*I-*Xba*I sites of pJIR750. The *vir*R gene was then amplified using the primers virRanti-L (*Hin*dIII) and virRanti-R (*Xba*I) and directionally cloned, in the antisense orientation, between the *Xba*I-*Hin*dIII sites of pJIR50, that lie upstream of the *vir*R promoter (Figure 9D). The resulting plasmid, pJVBM11, was electroporated into the WT CN3685.

Southern blot hybridization

C. perfringens genomic DNA from wild type CN3685 or CPJV19 was obtained, as described earlier. This DNA was digested with *Eco*RI and run on a 0.8% agarose gel. After transfer to a nylon membrane (Roche), the blot was probed with a digoxigenin-labeled probe specific for the intron sequence. This probe was prepared using the primer pair luxS-IBS and luxS-EBS1d (Table 3) and labeled with a DIG labeling kit obtained from Roche Applied Science. CSPD substrate (Roche Applied Science) was used for detection of DIG-labeled hybridized probes, according to the manufacturer's instruction.

Extraction of *C. perfringens* cytoplasmic proteins

An overnight FTG culture (1 ml) was inoculated in 20 ml of TGY and incubated for 4 h at 37°C and then centrifuged at 10 000 × *g* at 4°C for 30 min. The resulting pellet was washed twice with cold PBS and resuspended in 5 ml of PBS. Bacteria were lysed by passing trough a French Press (psi 1600) twice and the cytoplasmic fraction was obtained after centrifugation at 10 000 × g for 30 min. Cytoplasmic proteins (20 μg) were electrophoresed in a 12% SDS-PAGE, transferred to nitrocellulose membranes and probed by Western blot using rabbit polyclonal anti-VirR antibody (McGowan *et al.*, 2002; Cheung and Rood, 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was generously supported by grant R01 AI056177-04 (BAM) from National Institute of Allergy and Infectious Diseases. JEV also received generous support from the Mexican National Council of Science and Technology (CONACyT). The authors thank Dr. P. Hauer for supplying monoclonal antibodies against CPB, CPA

and ETX, Dr. R. Tweten for the anti-PFO antibody and Dr. Julian I. Rood for supplying the anti-VirR antibody. Also thanks to Dr. Jackie Chueng for her technical advice and Dr. J. Carroll and Christy Nolder for their assistance and helpful advice with qRT-PCR experiments.

References

Amimoto K, Noro T, Oishi E, Shimizu M. A novel toxin homologous to large clostridial cytotoxins found in culture supernatant of *Clostridium perfringens* type C. Microbiology. 2007; 153:1198–1206. [PubMed: 17379729]

Ba-Thein W, Lyristis M, Ohtani K, Nisbet IT, Hayashi H, Rood JI, Shimizu T. The *vir*R/*vir*S locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. J Bacteriol. 1996; 178:2514–2520. [PubMed: 8626316]

Bannam TL, Rood JI. *Clostridium perfringens-Escherichia* coli shuttle vectors that carry single antibiotic resistance determinants. Plasmid. 1993; 29:233–235. [PubMed: 8356117]

Banu S, Ohtani K, Yaguchi H, Swe T, Cole ST, Hayashi H, Shimizu T. Identification of novel VirR/VirS-regulated genes in *Clostridium perfringens*. Mol Microbiol. 2000; 35:854–864. [PubMed: 10692162]

Chen Y, McClane BA, Fisher DJ, Rood JI, Gupta P. Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. Appl Environ Microbiol. 2005; 71:7542–7547. [PubMed: 16269799]

Cheung JK, Rood JI. The VirR response regulator from *Clostridium perfringens* binds independently to two imperfect direct repeats located upstream of the *pfo*A promoter. J Bacteriol. 2000; 182:57–66. [PubMed: 10613863]

Cheung JK, Dupuy B, Deveson DS, Rood JI. The spatial organization of the VirR boxes is critical for VirR-mediated expression of the perfringolysin O gene, *pfo*A, from *Clostridium perfringens*. J Bacteriol. 2004; 186:3321–3330. [PubMed: 15150217]

Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V. The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci U S A. 2006; 103:10420–10425. [PubMed: 16803956]

Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW, Harrison LH. *tcd*C genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. J Clin Microbiol. 2007; 45:215–221. [PubMed: 17035492]

Demers B, Sansonetti PJ, Parsot C. Induction of type III secretion in *Shigella flexneri* is associated with differential control of transcription of genes encoding secreted proteins. Embo J. 1998; 17:2894–2903. [PubMed: 9582283]

Fernandez-Miyakawa ME, Fisher DJ, Poon R, Sayeed S, Adams V, Rood JI, et al. Both epsilon-toxin and beta-toxin are important for the lethal properties of *Clostridium perfringens* type B isolates in the mouse intravenous injection model. Infect Immun. 2007; 75:1443–1452. [PubMed: 17210666]

Fisher DJ, Miyamoto K, Harrison B, Akimoto S, Sarker MR, McClane BA. Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol Microbiol. 2005; 56:747–762. [PubMed: 15819629]

Fisher DJ, Fernandez-Miyakawa ME, Sayeed S, Poon R, Adams V, Rood JI, et al. Dissecting the Contributions of *Clostridium perfringens* Type C Toxins to Lethality in the Mouse Intravenous Injection Model. Infect Immun. 2006; 74:5200–5210. [PubMed: 16926413]

Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiol Infect. 2000; 124:61–67. [PubMed: 10722131]

Ginocchio CC, Olmsted SB, Wells CL, Galan JE. Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. Cell. 1994; 76:717–724. [PubMed: 8124710]

Gui L, Subramony C, Fratkin J, Hughson MD. Fatal enteritis necroticans (pigbel) in a diabetic adult. Mod Pathol. 2002; 15:66–70. [PubMed: 11796843]

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680–685. [PubMed: 5432063]

Laursen BS, Sorensen HP, Mortensen KK, Sperling-Petersen HU. Initiation of protein synthesis in bacteria. Microbiol Mol Biol Rev. 2005; 69:101–123. [PubMed: 15755955]

Lawrence G, Cooke R. Experimental pigbel: the production and pathology of necrotizing enteritis due to *Clostridium welchii* type C in the guinea-pig. Br J Exp Pathol. 1980; 61:261–271. [PubMed: 6252934]

Leverton LQ, Kaper JB. Temporal expression of enteropathogenic *Escherichia coli* virulence genes in an in vitro model of infection. Infect Immun. 2005; 73:1034–1043. [PubMed: 15664947]

Li J, McClane BA. A novel small acid soluble protein variant is important for spore resistance of most *Clostridium perfringens* food poisoning isolates. PLoS Pathog. 2008; 4:e1000056. [PubMed: 18451983]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Lyristis M, Bryant AE, Sloan J, Awad MM, Nisbet IT, Stevens DL, Rood JI. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. Mol Microbiol. 1994; 12:761–777. [PubMed: 8052128]

Matsuda T, Okada Y, Inagi E, Tanabe Y, Shimizu Y, Nagashima K, et al. Enteritis necroticans 'pigbel' in a Japanese diabetic adult. Pathol Int. 2007; 57:622–626. [PubMed: 17685936]

McClane, BA.; Lyerly, DL.; Wilkins, TD. Enterotoxic clostridia: *Clostridium perfringens* type A and *Clostridium difficile*. In: Fischetti, VA.; R.P.N.; Feretti, JJ.; Portnoy, DA.; Rood, JI., editors. Gram positive pathogens. Washington DC: ASM Press; 2006. p. 703-714.

McClane, BA.; Uzal, FA.; Fernandez-Miyakawa, M.; Lyerly, D.; Wilkins, TD. The Enterotoxigenic Clostridia. In: Dworkin, M.; S.F.; Rosenburg, E.; Schleifer, KF.; Stackebrandt, E., editors. The Prokaryotes. New York, NY: Springer-Verlag; 2004. p. 698-752.

McGowan S, Lucet IS, Cheung JK, Awad MM, Whisstock JC, Rood JI. The FxRxHrS motif: a conserved region essential for DNA binding of the VirR response regulator from *Clostridium perfringens*. J Mol Biol. 2002; 322:997–1011. [PubMed: 12367524]

Myers GS, Rasko DA, Cheung JK, Ravel J, Seshadri R, DeBoy RT, et al. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Res. 2006; 16:1031–1040. [PubMed: 16825665]

Niilo L. *Clostridium perfringens* Type C Enterotoxemia. Can Vet J. 1988; 29:658–664. [PubMed: 17423103]

Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol. 2003; 48:1429–1449. [PubMed: 12791129]

Ohtani K, Hayashi H, Shimizu T. The *lux*S gene is involved in cell-cell signaling for toxin production in *Clostridium perfringens*. Mol Microbiol. 2002; 44:171–179. [PubMed: 11967077]

Ohtani K, Kawsar HI, Okumura K, Hayashi H, Shimizu T. The VirR/VirS regulatory cascade affects transcription of plasmid-encoded putative virulence genes in *Clostridium perfringens* strain 13. FEMS Microbiol Lett. 2003; 222:137–141. [PubMed: 12757957]

Okumura K, Ohtani K, Hayashi H, Shimizu T. Characterization of genes regulated directly by the VirR/VirS system in *Clostridium perfringens*. J Bacteriol. 2008; 190:7719–7727. [PubMed: 18790863]

Petit L, Gibert M, Popoff MR. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol. 1999; 7:104–110. [PubMed: 10203838]

Petrillo TM, Beck-Sague CM, Songer JG, Abramowsky C, Fortenberry JD, Meacham L, et al. Enteritis necroticans (pigbel) in a diabetic child. N Engl J Med. 2000; 342:1250–1253. [PubMed: 10781621]

Rohde M, Puls J, Buhrdorf R, Fischer W, Haas R. A novel sheathed surface organelle of the *Helicobacter pylori cag* type IV secretion system. Mol Microbiol. 2003; 49:219–234. [PubMed: 12823823]

Sakurai J, Duncan CL. Purification of beta-toxin from *Clostridium perfringens* type C. Infect Immun. 1977; 18:741–745. [PubMed: 201565]

Sayeed S, Li J, McClane BA. Virulence plasmid diversity in *Clostridium perfringens* type D isolates. Infect Immun. 2007; 75:2391–2398. [PubMed: 17339362]

Sayeed S, Fernandez-Miyakawa ME, Fisher DJ, Adams V, Poon R, Rood JI, et al. Epsilon-toxin is required for most *Clostridium perfringens* type D vegetative culture supernatants to cause lethality

in the mouse intravenous injection model. Infect Immun. 2005; 73:7413–7421. [PubMed: 16239541]

Sayeed S, Uzal FA, Fisher DJ, Saputo J, Vidal JE, Chen Y, et al. Beta toxin is essential for the intestinal virulence of *Clostridium perfringens* type C disease isolate CN3685 in a rabbit ileal loop model. Mol Microbiol. 2008; 67:15–30. [PubMed: 18078439]

Severin WP, de la Fuente AA, Stringer MF. *Clostridium perfringens* type C causing necrotising enteritis. J Clin Pathol. 1984; 37:942–944. [PubMed: 6088590]

Shimizu T, Ba-Thein W, Tamaki M, Hayashi H. The *vir*R gene, a member of a class of two-component response regulators, regulates the production of perfringolysin O, collagenase, and hemagglutinin in *Clostridium perfringens*. J Bacteriol. 1994; 176:1616–1623. [PubMed: 8132455]

Smedley JG 3rd, Fisher DJ, Sayeed S, Chakrabarti G, McClane BA. The enteric toxins of *Clostridium perfringens*. Rev Physiol Biochem Pharmacol. 2004; 152:183–204. [PubMed: 15517462]

Sobel J, Mixter CG, Kolhe P, Gupta A, Guarner J, Zaki S, et al. Necrotizing enterocolitis associated with *Clostridium perfringens* type A in previously healthy north american adults. J Am Coll Surg. 2005; 201:48–56. [PubMed: 15978443]

Songer JG. Clostridial enteric diseases of domestic animals. Clin Microbiol Rev. 1996; 9:216–234. [PubMed: 8964036]

Songer JG, Uzal FA. Clostridial enteric infections in pigs. J Vet Diagn Invest. 2005; 17:528–536. [PubMed: 16475510]

Titball RW, Naylor CE, Basak AK. The *Clostridium perfringens* alpha-toxin. Anaerobe. 1999; 5:51–64. [PubMed: 16887662]

Tonnellier M, Maury E, Guglielminotti J, Offenstadt G. A fatal sandwich. Lancet Infect Dis. 2001; 1:202. [PubMed: 11871497]

Tweten RK. Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin O and pneumolysin. Infect Immun. 1988a; 56:3235–3240. [PubMed: 2903128]

Tweten RK. Cloning and expression in *Escherichia coli* of the perfringolysin O (theta-toxin) gene from *Clostridium perfringens* and characterization of the gene product. Infect Immun. 1988b; 56:3228–3234. [PubMed: 2903127]

Vidal JE, Navarro-Garcia F. Efficient translocation of EspC into epithelial cells depends on enteropathogenic *Escherichia coli* and host cell contact. Infect Immun. 2006; 74:2293–2303. [PubMed: 16552060]

Vidal JE, McClane BA, Saputo J, Parker J, Uzal FA. Effects of *Clostridium perfringens* beta-toxin on the rabbit small intestine and colon. Infect Immun. 2008; 76:4396–4404. [PubMed: 18625730]

Voth DE, Ballard JD. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev. 2005; 18:247–263. [PubMed: 15831824]

Walker PD, Murrell TG, Nagy LK. Scanning electronmicroscopy of the jejunum in enteritis necroticans. J Med Microbiol. 1980; 13:445–450. [PubMed: 6251221]

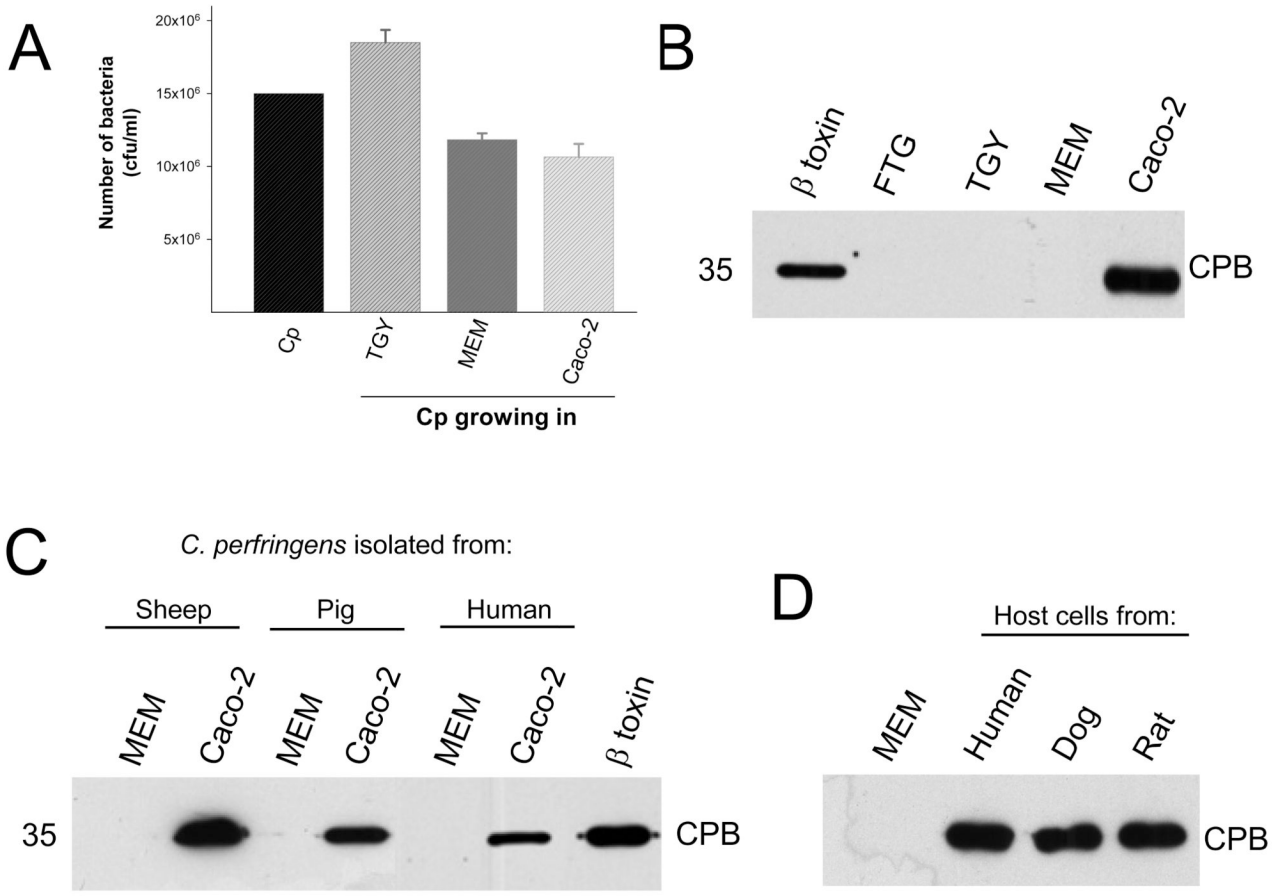


Figure 1. CPB secretion is upregulated during infection of mammalian Caco-2 cells

(A) Cell culture dishes containing Caco-2 cell cultures, TGY or MEM were each inoculated with 1.5×10^7 CFU of *C. perfringens* (Cp) type C isolates JGS1495 and incubated for 3 h at 37°C (MOI=20). The culture supernatants containing bacteria (10 ml) were aspirated, serially diluted in BHI broth (10 ml final volume), and then plated (1 ml) onto BHI agar plates. The number of bacteria (cfu/ml) growing in each culture condition were recorded after a 24 h incubation under anaerobic conditions at 37°C. (B) Cell culture dishes containing FTG, TGY, MEM (no cells) or Caco-2 cells were inoculated with *C. perfringens* strain JGS1495 and then incubated for 3 h at 37°C. (C) Cell culture dishes containing MEM (no cells) or Caco-2 cells were infected with *C. perfringens* type C strains isolated from sheep (CN3685), pig (JGS1495) or human (CN5383) for 3 h at 37°C. Culture supernatant was then removed and filter-sterilized. Equal amounts of each supernatant were subjected to SDS-PAGE on a 12% acrylamide gel and proteins were then transferred to nitrocellulose membrane. Purified 35 kDa CPB (0.5 μg) was also included (β toxin lane). Membranes were blocked for 1 h before probing with a mouse monoclonal anti-CPB antibody. Bound antibody was detected with a horseradish peroxidase-conjugated secondary anti-species specific antibody, followed by incubation of blots with a chemiluminescent substrate. D) Caco-2 cells (Human), MDCK cells (Dog) or rat 1R-12 fibroblasts were infected with *C. perfringens* type C strain JGS1495 for 3 h at 37°C. Culture supernatants were then analyzed as described above. CPB molecular weight is shown at left of each figure in kDa. Figures shown are representative of at least 4 independent experiments.

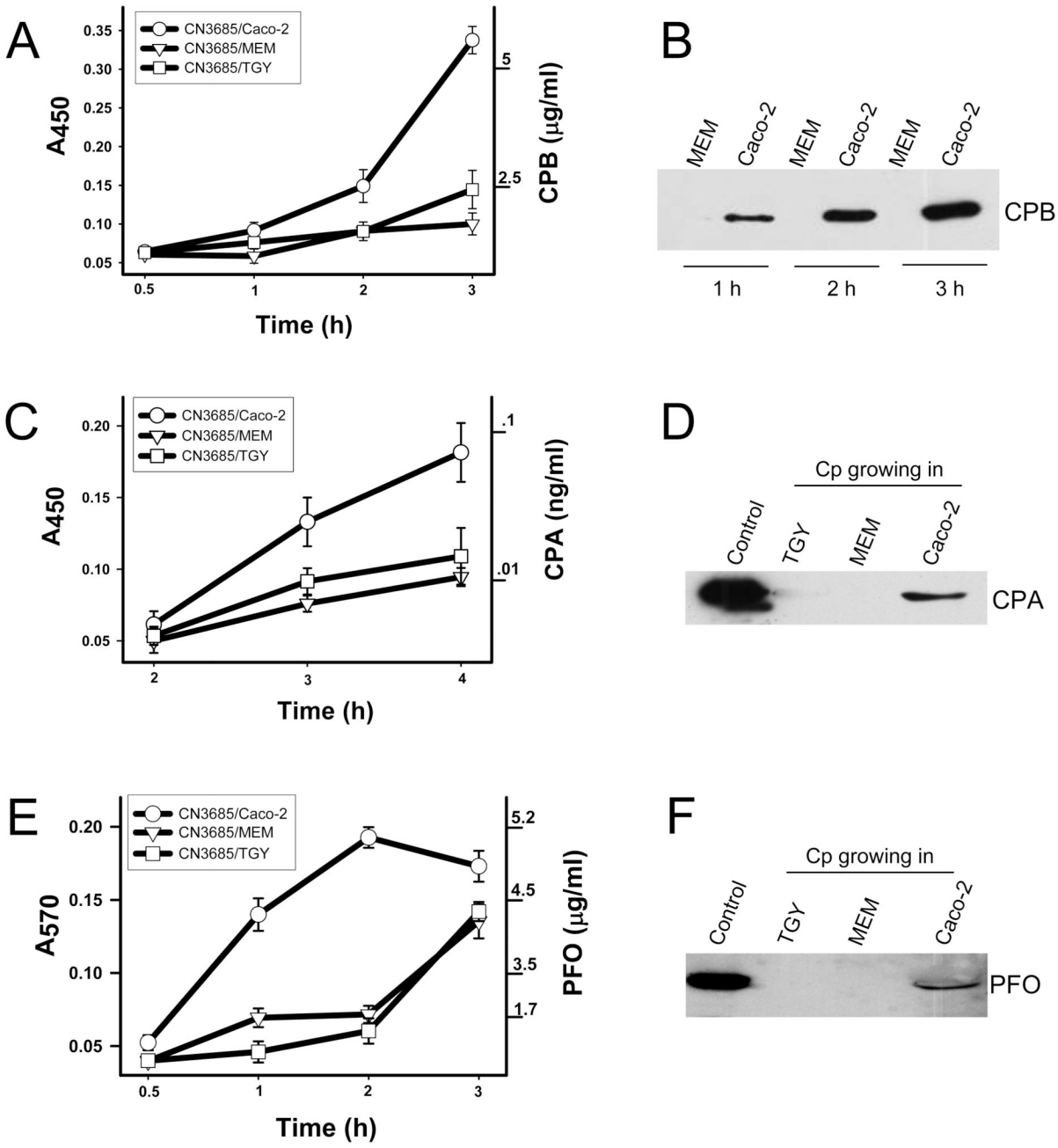


Figure 2. Levels of CPB, CPA and PFO toxins secreted in the presence of Caco-2 cells

ELISA analyses (A and C). *Clostridium perfringens* type C strain CN3685 was inoculated into Caco-2 cells (CN3685/Caco-2), MEM (CN3685/MEM) or TGY (CN3685/TGY) (MOI=20) and incubated for the indicated time at 37°C. Supernatants were removed and sterilized by filtration. Sterile supernatants and purified CPB (A) or purified CPA (C) were coated in separate wells of an ELISA microplate overnight at 4°C. The wells were incubated with a mouse monoclonal anti-CPB (A) or anti-CPA antibody (C) followed by a HRP-conjugated anti-mouse antibody. The bound antibody was detected with a TMB substrate solution and the color reaction stopped with sulphuric acid (0.18 M). Absorbance at 570 (A_{450}) was determined using an ELISA reader. Western blot analyses (right panels). (B)

CPB secretion. Caco-2 cells or MEM cultures were infected with CN3685 for 1, 2 or 3 h and incubated at 37°C. Equal amounts of sterile supernatants were analyzed by Western blot using a mouse monoclonal anti-CPB antibody. Secretion of 47 kDa CPA (D) and 54 kDa PFO (F) by Caco-2 cells grown in 100-mm culture dishes prior to infection for 3 h at 37°C with *C. perfringens* type C isolate JGS1495 (MOI=20). For comparison, culture dishes containing TGY or MEM (no cells) were similarly inoculated and then incubated under the same conditions. For D and F, supernatants were removed, filter sterilized and concentrated 200-fold (see material and methods). Control sample is an infected 8 h TGY culture that was similarly concentrated. The same amounts of each concentrated supernatant were subjected to SDS-PAGE on a 12% acrylamide gel and then analyzed by Western blotting using a mouse monoclonal anti-CPA (D) antibody or a rabbit-raised polyclonal anti-PFO antibody (F) as described. (E) Hemoglobin (Hb) release assay for PFO activity. Supernatants obtained as above or purified PFO were incubated (1:1) with a 1% suspension of horse red blood cells for 30 min at 37°C. PFO-induced Hb release was detected by obtaining the absorbance at 570 (A_{570}).

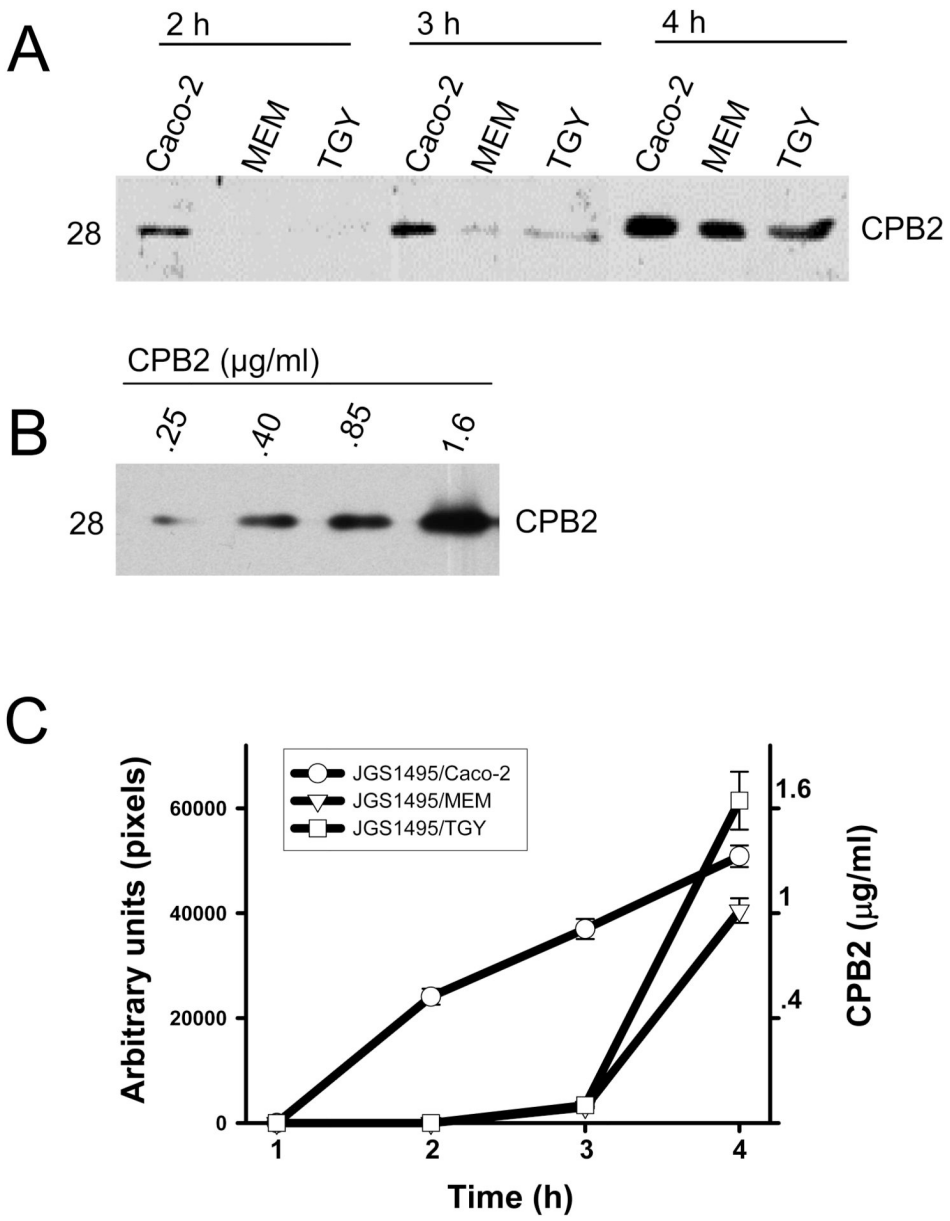


Figure 3. Levels of CPB2 secreted in the presence of enterocyte-like Caco-2 cells

Clostridium perfringens type C strain JGS1495 was inoculated into Caco-2 cell cultures (JGS1495/Caco-2), MEM (JGS1495/MEM) or TGY (JGS1495/TGY) (MOI=20) and incubated for the indicated time at 37°C. Supernatants were removed and sterilized. (A) Equal amounts (25 µl) of each supernatant or different amount of purified CPB2 (B) were subjected to SDS-PAGE on a 12% acrylamide gel and proteins were then transferred to nitrocellulose membrane. Membranes were probed by Western blot using a polyclonal anti-CPB2 antibody. CPB2 molecular weight is shown at left of each figure in kDa. C) Quantification of CPB2 secretion. Experiments described above were repeated three times. Western blots were then scanned and their pixel intensity [(A) *x*-axis or (B) *y*-axis right] was quantified and graphically integrated against a standard curve of purified CPB2 to determine CPB2 levels.

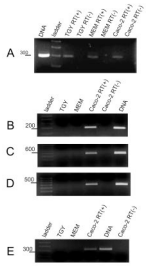


Figure 4. The transcription of *cpb*, *cbp*2, *plc* and *pfo*A toxin genes is quickly upregulated during Caco-2 infection

Type C strain JGS1495 was inoculated into TGY, MEM or Caco-2 cell cultures (MOI=20) and then incubated for 2 h at 37°C. Bacteria were then collected and pelleted by centrifugation. Total RNA was extracted and treated with DNase I. R T-PCR reactions were performed with 20–100 ng of RNA (results shown are for 100 ng) and using a specific pair of primers to amplify (A) the house keeping *pol*C gene, (B) *cpb*, (C) *cpb*2, (D) *pfo*A, or (E) *plc* toxin genes. Where indicated, reverse transcriptase (RT) was (+) or was not (−) added into reaction tubes as a control to confirm RT-PCR signals were from RNA rather than DNA. DNA from JGS1495 strain was also included, as a control reaction. Molecular markers shown are a 100 bp increment ladder with selected marker size, in bp, shown at left of each gel. For all panels, products were electrophoresed on a 2% agarose gel and then stained with ethidium bromide for visualization. Shown are representative figures of three independent experiments.

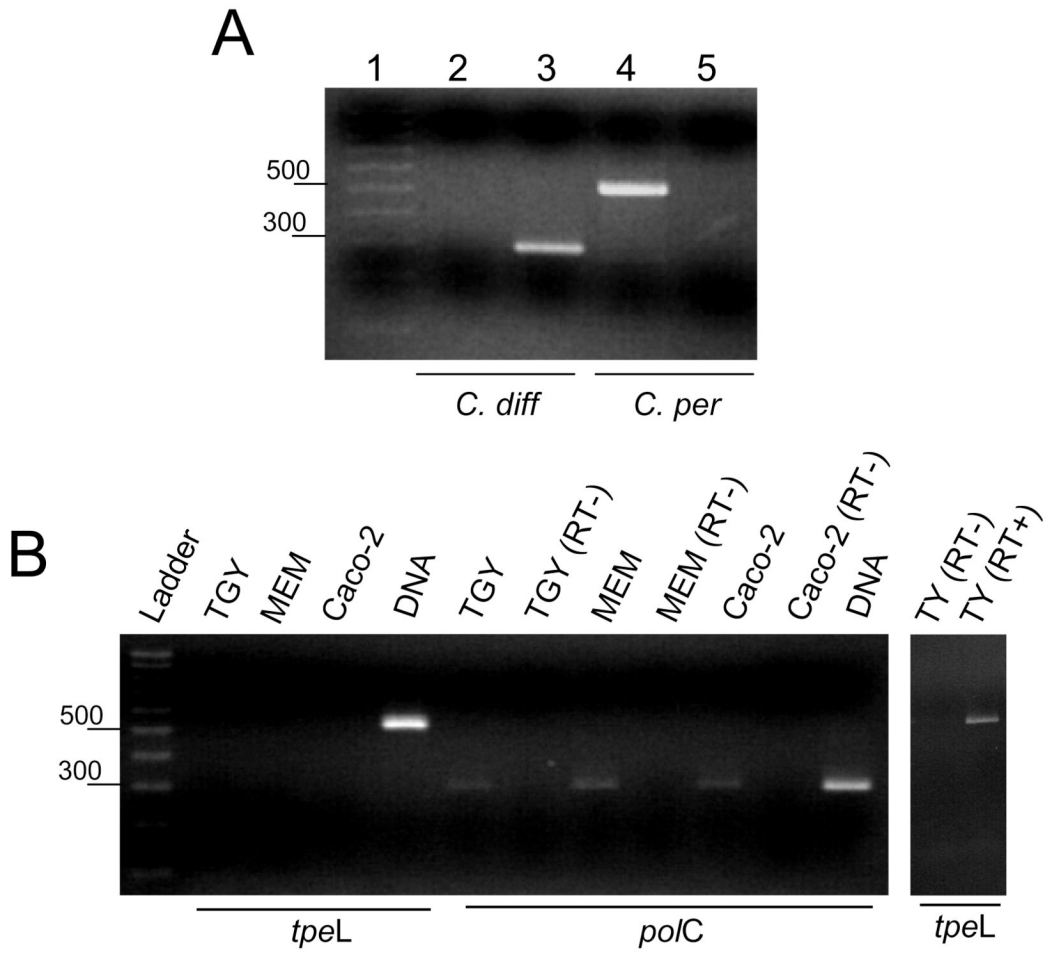
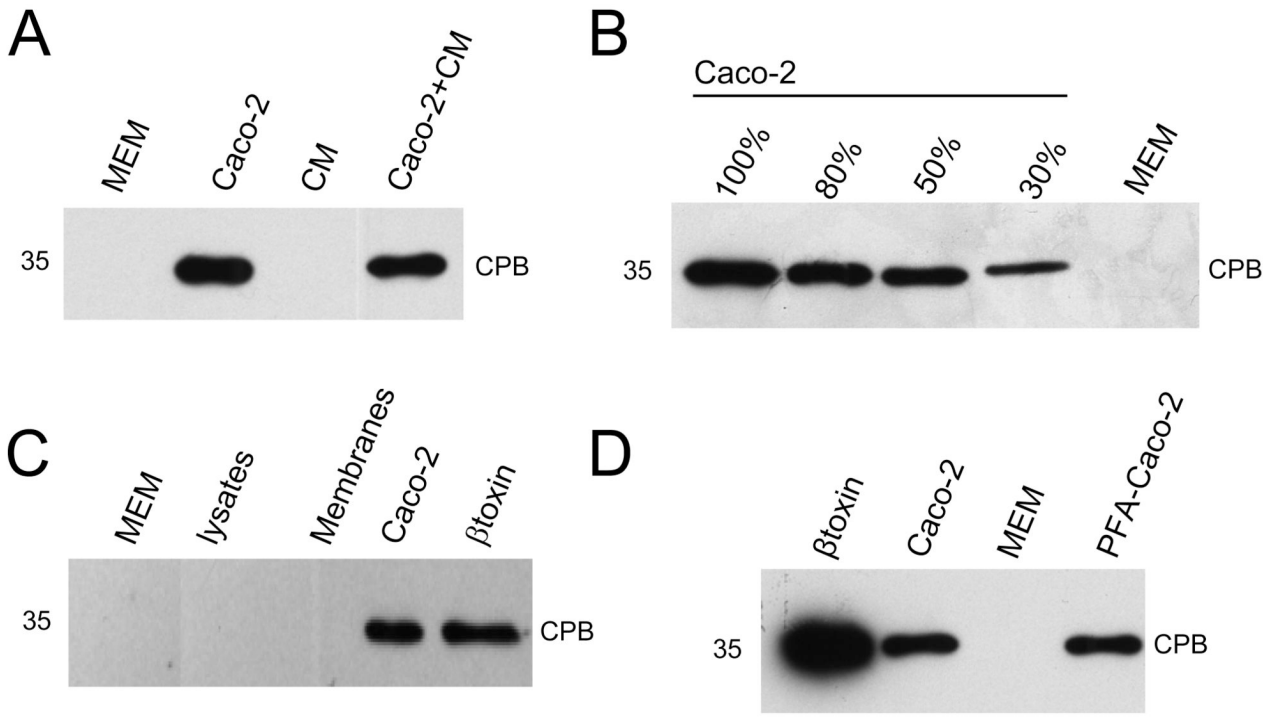


Figure 5. Transcription of the *C. perfringens tpe*L toxin gene is not upregulated early during infection of Caco-2 cell cultures

(A) PCR was performed using primers to specifically amplify either the *C. perfringens tpe*L gene (lanes 2 and 4) or a 3´ fragment of the *C. difficile tcd*A gene (lanes 3 and 5) that is not present in the *tpe*L gene (Amimoto *et al.*, 2007). As template, DNA extracted from *C. difficile* strain 00030 (*C. diff*) or *C. perfringens* strain JGS1495 (*C. per*) was used. Lane 1 contains 100 bp ladder, selected marker size is shown at left of each gel. (B) RT-PCR for *tpe*L or *pol*C transcripts. Type C strain JGS1495 was inoculated into a TGY, MEM or a Caco-2 cell culture and then incubated for 2 h at 37°C, or inoculated in TY and incubated for 24 h. Bacteria were collected from growing conditions and pelleted by centrifugation. Total RNA was extracted from those pellets and treated with DNase I. RT-PCR reactions were then performed with 20–100 ng of RNA (results shown are for 100 ng) and using a specific pair of primers to amplify either *tpe*L or the house keeping *pol*C gene. Where indicated, reverse transcriptase (RT) was (+) or was not (−) added into the reaction tubes. As a control, DNA from JGS1495 was added into a reaction tube. Molecular markers were increments of a 100 bp ladder, size of selected markers, in bp, is shown at the left of the gel.



E

	Control		Transwell	
Caco-2	-	Bottom	Top	Top
C. perfringens	Bottom	Bottom	Top	Bottom

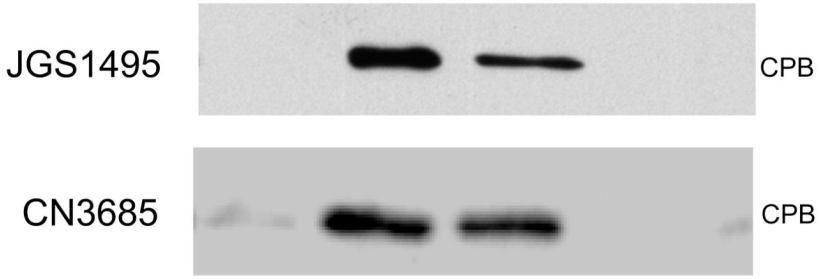


Figure 6. Upregulation of CPB secretion requires close contact between *C. perfringens* and host cells

(A) Conditioned medium (CM) does not stimulate CPB secretion. *C. perfringens* type C strain JGS1495 was inoculated into a well of a 24-well tissue culture microplate containing; 1 ml of CM alone, 1 ml of MEM alone, Caco-2 cell cultures treated with 1 ml of CM, or Caco-2 cells in 1 ml of fresh (non-conditioned) MEM. After 3 h of incubation at 37°C, supernatants were analyzed by Western blot for CPB production using a mouse monoclonal anti-CPB antibody (expected migration of a 35 kDa protein indicated at left of each blot in panels A–D). (B) CPB secretion increases in proportion to the number of host cells in a culture. *C. perfringens* type C strain JGS1495 (1.5×10^7 CFU) was inoculated into Caco-2

cell cultures which were at the following confluency: 30, 50, 80 or 100% and then incubated for 3 h at 37°C. A mock infection without cells (MEM) was also included. Equal amounts of each sterilized supernatant were electrophoresed, transferred to nitrocellulose membranes and analyzed as described above. (C) Living cells, but not Caco-2 cell membranes or cell lysates, stimulated CPB secretion. JGS1495 was inoculated (2.5×10^8 CFU) into 100 mm tissue culture dishes containing 12 ml of MEM without additives, MEM without additives but with Caco-2 cell lysates, MEM without additives but with the Caco-2 cell membrane fraction or MEM plus confluent living Caco-2 cells. After 3 h of infection at 37°C, supernatants were analyzed by Western blot for CPB levels as described. (D) Fixed Caco-2 cells stimulate CPB secretion. JGS1495 was inoculated into MEM (no cells), Caco-2 cell cultures or plates with paraformaldehyde (PFA)-fixed Caco-2 cells and incubated for 3 h at 37°C. Supernatants were filter-sterilized and then analyzed by Western blot for CPB levels. Purified CPB (0.5 μg) was included (β toxin). (E) *C. perfringens* type C isolates JGS1495 or CN3685 was inoculated (3×10^7 CFU) into i) wells of a 12-well culture microplate containing MEM (no cells [-]) or a Caco-2 cell culture or ii) the Transwell filter chamber (Top) containing a confluent culture of Caco-2 cells or iii) the well (Bottom) in which a Transwell filter chamber containing Caco-2 cells was installed (i.e. bacteria and cells are present in the same well but physically separated). The infected cultures were incubated for 3 h at 37°C and the supernatants were collected and filter-sterilized. The sterile supernatants were analyzed by Western blot as above. Shown are representative results from at least four independent experiments.

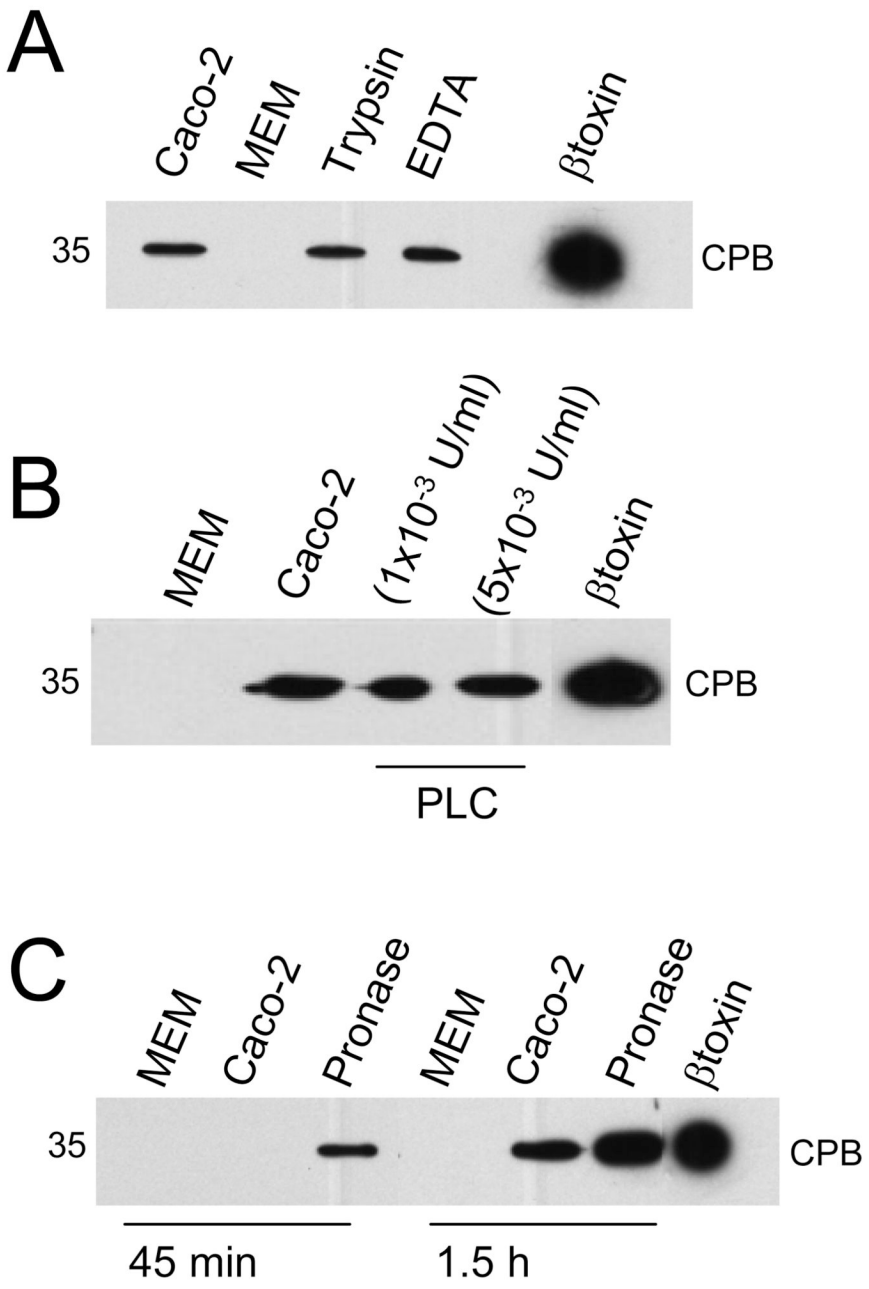


Fig. 7. Treatment of Caco-2 cell cultures with pronase, but not trypsin or PLC, enhances CPB secretion

(A) Caco-2 cells were treated with trypsin or EDTA for 15 min at 37°C. Detached cells were washed and resuspended in MEM to a final cell density of 70×10^5 cells/ml. Caco-2 cell cultures, MEM, trypsin-detached or EDTA-detached Caco-2 cells were then infected, in a 24-well microplate, with CN3685 for 1.5 h at 37°C. The supernatants were analyzed for CPB levels by Western blot. (B) Caco-2 cells were treated with 1×10^{-3} or 5×10^{-3} U/ml of phospholipase C (PLC) in the form of CPA for 1 h at 37°C. Cells were then washed and added with MEM. Untreated Caco-2 cell cultures, MEM or PLC-treated Caco-2 cells were infected with CN3685 for 1.5 h at 37°C. The supernatants were analyzed for CPB levels by

Western blot. (C) Caco-2 cells were treated with pronase (100 μg/ml) for 20 min at 37°C. Detached cells were thoroughly washed and resuspended in MEM to a final cell density of 7×10^5 cells/ml. MEM, Caco-2 cell cultures or pronase-treated Caco-2 cells were then infected, in a 24-well microplate, with CN3685 for 45 min or 1.5 h at 37°C. The supernatants were analyzed for CPB levels by Western blot. Expected migration of a 35 kDa protein indicated at left of each blot.

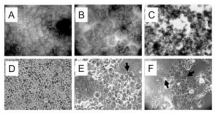


Figure 8. *C. perfringens* type C strains are cytotoxic for Caco-2 cells

(A–C) Cytotoxic consequences of host cell-induced stimulation of toxin secretion. *C. perfringens* type C strain CN3685 was inoculated into tissue culture wells containing MEM (no cells) or Caco-2 cells for 1 h. The culture supernatant was removed and filter-sterilized. The sterilized supernatant from inoculated MEM alone (no Caco-2 cells) (B) or infected Caco-2 cell cultures (C) was individually added to fresh confluent Caco-2 cells and incubated for 5 h at 37°C. Then, treated or untreated Caco-2 cells (A) were washed three times with pre-warmed PBS, fixed with 70% methanol and stained with Giemsa stain. Slides were analyzed at a magnification of 40X with standard bright-field light microscopy. (D–F) Cytotoxicity induced by *C. perfringens* type C infection. Phase-contrast microscopy of confluent Caco-2 cell cultures left uninfected (D) or infected with CN3685 (E) or JGS1495 (F) (MOI=20) for 5 h at 37°C. Arrows show membrane blebs.

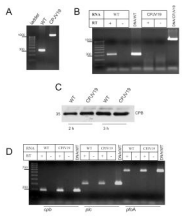


Figure 9. *lux*S-controlled quorum sensing mechanism is not required for Caco-2 cell induced upregulation of toxin production by CN3685

A) PCR showing an intron insertion in the *lux*S gene. Primers LuxS-L and LuxS-R amplified a 300 bp product of the *lux*S gene using DNA isolated from CN3685 (WT) or a 1.2 kb *lux*S-intron product using Δ*lux*S DNA (CPJV19). B) *lux*S mRNA is not produced by CPJV19. RNA (100 ng) isolated from an overnight TGY culture of CN3685 (WT) or CPJV19 was used as template for RT-PCR reactions using the LuxS-L and LuxS-R primers. Where indicated, retrotranscriptase (RT) was (+) or was not (−) added into the reaction tubes. As additional controls, reactions containing DNA from the WT or CPJV19 strain were included. Shown at left is a 100 bp ladder; selected marker size, in bp, are noted at left of the gel. C) CPB secretion is not affected in CPJV19. CN3685 (WT) or CPJV19 was infected in Caco-2 cell cultures for 2 h or 3 h (MOI=20). Sterile culture supernatants were obtained and analyzed by Western blot using a mouse monoclonal anti-CPB; expected migration of the 35 kDa CPB protein is noted on the left of the blot. D) Transcription of *cpb*, *plc* and *pfo*A toxin genes by CPJV19. Caco-2 cells were infected with CN3685 (WT) or CPJV19 for 2 h. Bacterial RNA (100 ng) was then isolated and used in RT-PCR reactions with primers to amplify the *cpb*, *plc* or *pfo*A genes. Where indicated, retrotranscriptase (RT) was (+) or was not (−) added into the reaction tubes. Reactions containing DNA from the WT was included. A 100 bp ladder is shown at left and selected marker size, in bp, are noted at left of the gel. Figures shown are representatives of at least three independent experiments.

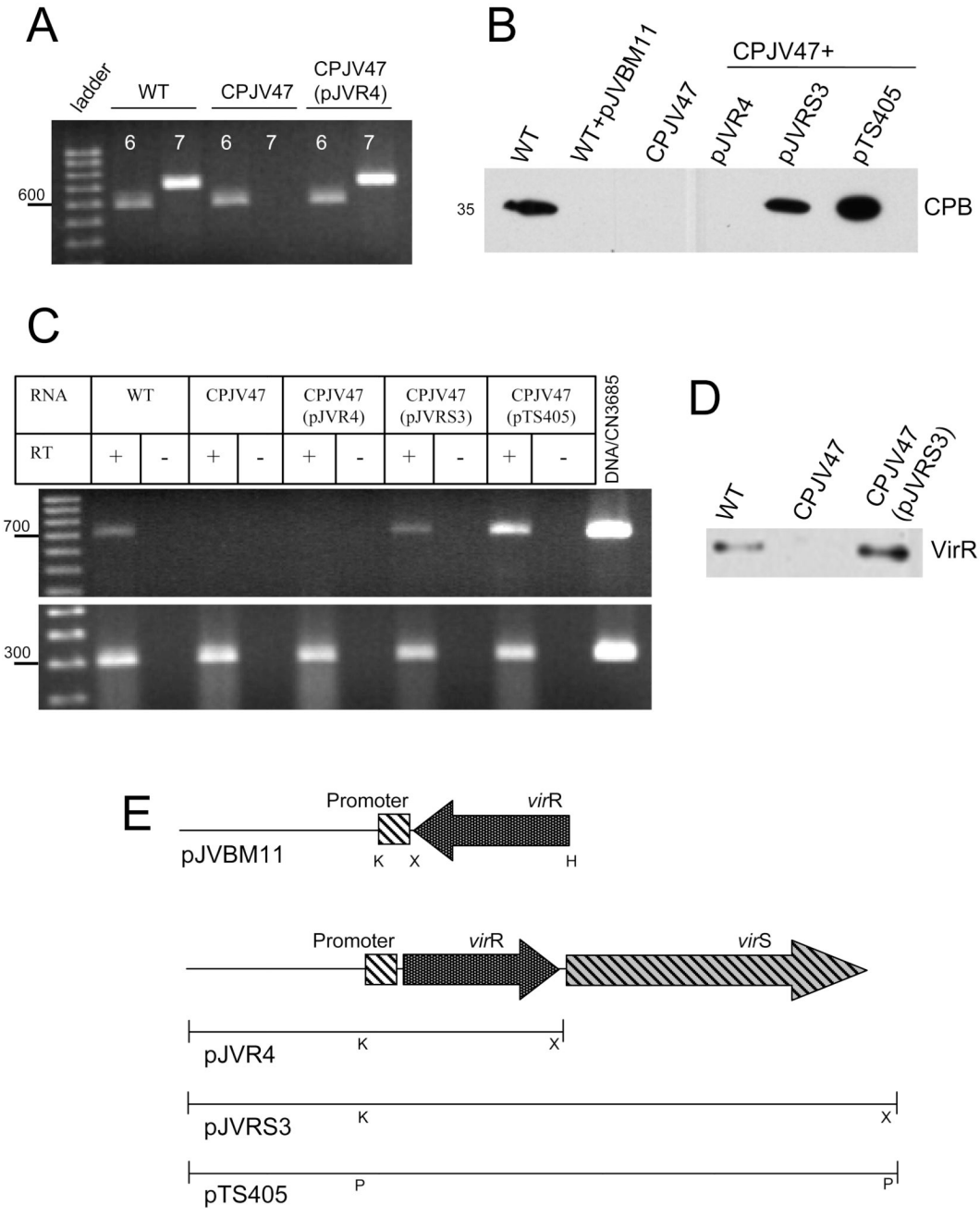


Figure 10. The *vir*S/*vir*R two-component regulatory system regulates the Caco-2 cell-induced increase in CPB secretion

A) Inactivation of the *vir*R gene. PCR was performed with primers that amplify (6) a ~590 bp product or (7) ~710 bp *vir*R wt gene using DNA from CN3685 (WT), CPJV47 or CPJV47(pJVR4). A 100 bp ladder is shown at left and migration of the 600 bp marker is noted. B) CPB secretion. Caco-2 cell cultures were infected with the indicated strain for 2 h at 37°C. Sterile culture supernatants were obtained and analyzed by Western blot using a mouse monoclonal anti-CPB (migration of the 35 kDa CPB protein is noted at left and right of the blot). C) Caco-2 cells were infected with CN3685 (WT), CPJV47, CPJV47(pJVR4), CPJV47(pJVRS3) or CPJV47(pTS405) for 1 h. RNA (50 ng) isolated from bacteria was

used as template in RT-PCR reactions with primers that amplify (top panel) the *vir*R gene or (bottom panel) the housekeeping *pol*C gene. Where indicated, retrotranscriptase (RT) was (+) or was not (−) added into the reaction tubes. Reactions containing DNA from the WT strain was included. A 100 bp ladder is shown at left and the location of 700 bp (top panel) and 300 bp (bottom panel) markers is noted. Figures shown are representatives of at least three independent experiments. D) Production of the VirR protein. Cytoplasmic proteins (20 μg) from CN3685 (WT), CPJV47 or CPJV47(pJVR3) were electrophoresed and transferred to nitrocellulose membranes. The membranes were probed by Western blot using an anti-VirR antibody. E) Schematic diagrams showing the *vir*R antisense vector (pJVBM11) or the plasmids pJVR4, pJVRS3 and pTS405. Note that pJVBM11 has the *vir*R gene in the antisense orientation with respect to the *vir*R promoter. pJVR3 only encodes the CN3685 *vir*R gene, pJVRS3 encodes the *vir*S/*vir*R operon from CN3685, and pTS405 encodes the *vir*S/*vir*R operon from Strain 13. K, *Kpn*I; X, *Xba*I; H, *Hin*dIII, P, *Pst*I.

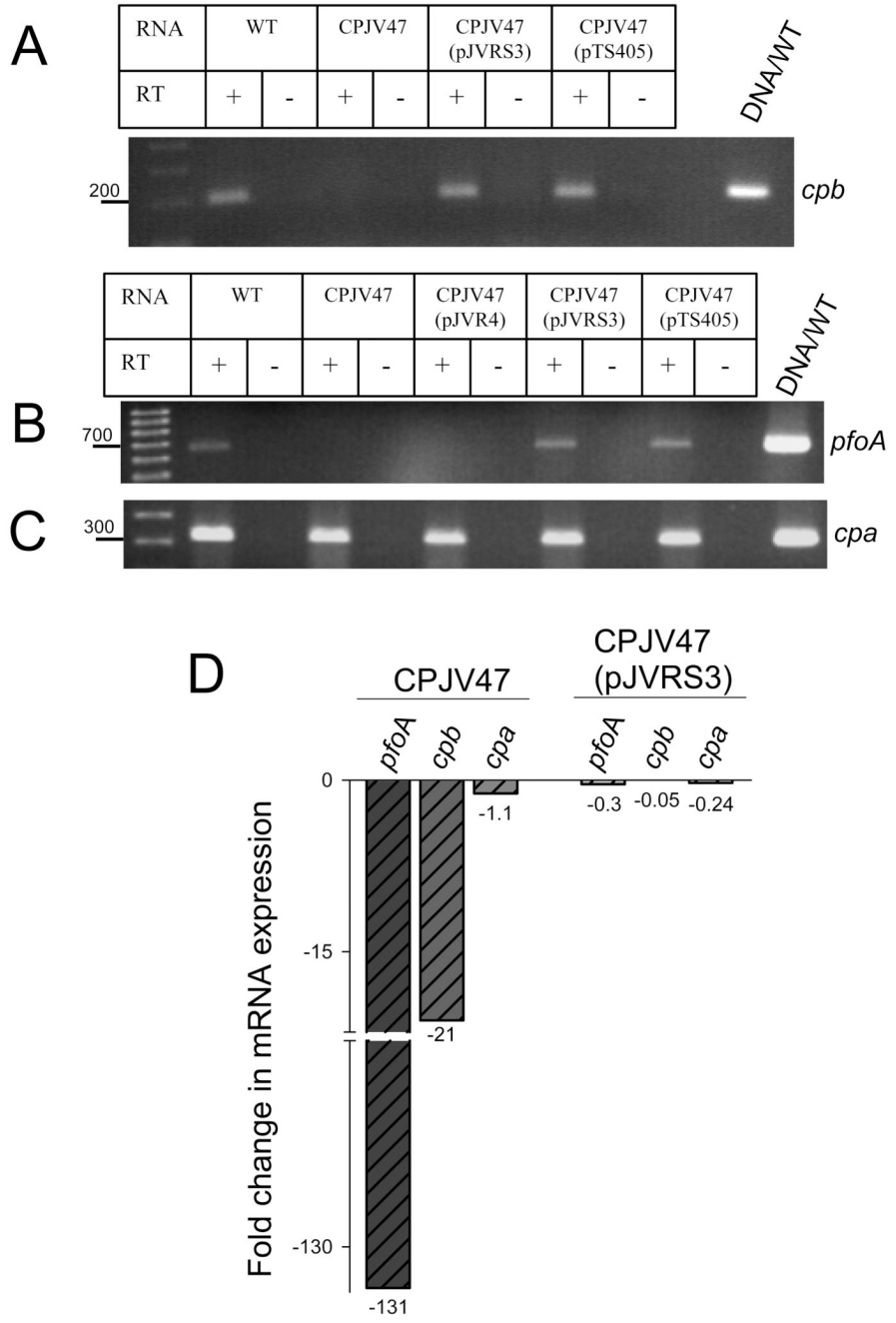


Figure 11. The *vir*S/*vir*R two-component regulatory system controls the Caco-2 induced increase in transcription of *cpb* and *pfo*A

Caco-2 cells were infected with CN3685 (WT), CPJV47, CPJV47(pJVR4), CPJV47(pJVRS3) or CPJV47(pTS405) for 1 h at 37°C. Bacteria-containing supernatants were obtained to isolate the RNA as described in Material and Methods. Panels A–C, RT-PCR reactions were then conducted with 50 ng of RNA and primers that amplify the genes, (A) *cpb*, (B) *pfo*A or (C) *plc*/*cpa*. Where indicated, retrotranscriptase (RT) was (+) or was not (−) added into the reaction tubes. Reactions containing DNA from the WT was included. A 100 bp ladder is shown at left, with location of 200 bp (A), 700 bp (B) or 300 bp (C) markers is noted. Shown are representative figures of at least three independent experiments.

Panel (D), Quantitative RT-PCR was then performed with 20 ng of the indicated RNA from infected Caco-2 cell cultures and primers that amplified the *pfo*A, *cpb* or *plc*/*cpa* gen. Average C_T values were normalized to the housekeeping *pol*C gene and the fold differences were calculated using the comparative C_T method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen, 2001). Values below each bar indicate the calculated fold change relative to the wt strain CN3685. Shown is a representative graphic of three independent experiments.

Table 1

Morphological changes induced on Caco-2 cells by *C. perfringens* strains.

Strain	Time (h)	Cytotoxicity*
CN3685	1.5	−
	2	+
	2.5	++
	3	+++
CPJV47	1.5	−
	2	−
	2.5	+
	3	+
CPJV47(pJVRS3)	1.5	−
	2	+
	2.5	++
	3	+++
JGS1495	1.5	−
	2	−
	2.5	−
	3	−
	3.5	+
	4	++
	5	+++
CN5383	1.5	−
	2	−
	2.5	−
	3	−
	3.5	+
	4	++
	5	+++

* (+) >80% of Caco-2 cells were swollen, (++) <50% of Caco-2 cells were swollen and <50% of cells were rounded and (+++) >80% of Caco-2 cells were rounded and detached from the glass.

Table 2

C. perfringens type C isolates, mutants and plasmids used in this study.

Strain	Description	Origen or reference
JGS1495	*cpb*$^+$, *cpb*2$^+$, *plc*$^+$, *pfo*$^+$ and *tpe*L$^+$	Piglet with necrotic enteritis (Fisher *et al.*,2006).
CN3685	*cpb*$^+$, *plc*$^+$, *pfo*$^+$ and *tpe*L$^+$	Sheep with struck, (Fisher*et al.*, 2006).
CN5383	*cpb*$^+$, *plc*$^+$, *pfo*$^+$ and *tpe*L$^+$	Human pig bel case, (Fisher *et al.*, 2006).
C. difficile 00030	*tcdA*$^+$, *tcdB*$^+$	Human with colitis, (Curry*et al.*, 2007)
NCTC8346	*etx*$^+$, *plc*$^+$ and *pfo* $^+$	Deceased Sheep, (Sayeed*et al.*, 2005)
CN1634	*etx*$^+$, *plc*$^+$ and *pfo*$^+$	Lamb with dysentery, (Sayeed *et al.*, 2005)
CPJV19	CN3685::*lux*S	This work
CPJV47	CN3685::*vir*R	This work
Plasmids		
pKOR	*E. coli*-based *C. perfringens* suicide plasmid with 590 bp of the virR gene.	(Shimizu *et al.*, 1994)
pJIR750	*E. coli-C. perfringens* shuttle vector	(Bannam and Rood, 1993)
pJVBM11	pJIR750 with *vir*R promoter and the *vir*R gene in antisense orientation, from CN3685.	This work
pJVR4	pJIR750 with *vir*R promoter and the *vir*R gene from CN3685.	This work
pJVRS3	pJIR750 with *vir*R promoter, *vir*R gene and *vir*S gene from CN3685.	This work
pTS405	pJIR418 with a *Pst*I fragment from strain 13 genomic DNA containing *vir*R promoter, *vir*R gene and *vir*S gene.	(Ba-Thein *et al.*, 1996)
pJIR750ai	pJIR750 with *plc* targeted intron in antisense orientation.	(Chen *et al.*, 2005)
pJVLux	pJIR750ai with *lux*S targeted intron in antisense orientation.	This work

Table 3

Primers used in this study.

Primer	Sequence	Source or reference
cpbF	GCGAATATGCTGAATCATCTA	(Garmory *et al.*, 2000)
cpbR	GCAGGAACATTAGTATATCTTC	
cpb2MPF	AGATTTTAAATATGATCCTAACC	(Fisher *et al.*, 2005)
cpb2MPR	CAATACCCTTCACCAAATACTC	
cpaF	GCTAATGTTACTGCCGTTGA	(Garmory *et al.*, 2000)
cpaR	CCTCTGATACATCGTGTAAG	
pfoAF1	ATCCAACCTATGGAAAAGTTTCTGG	(Fisher *et al.*, 2006)
pfoAR1	CCTCCTAAAACTACTGCTGTGAAGG	
polCJVL	AATATATGATACTGAAGAGAGAGTAA	This study
polCJVR	TCTAAATTATCTAAATCTATGTCTACT	
tpeLJVL	TTTTGAAGTTCCACAAGCTCTAATATC	This study
tpeLJVR	CTCCTTTACTGTTAATGAAGCAAATC	
tcdAJVL	AATTTTTACTTTAGAAATGGTTTAC	This study
tcdAJVR	AGAAATATATAACACCATCAATCTC	
virR-L	ATGTTTAGTATTGCCTTATGTGAAGA	This study
virR-R	TTAACA TAT TAA ATC CCC TAA AAG GC	
virR590-L	TTGGATGAAATAGGAGTAGAGT	This study
virR590-R	TCTATGCTTACTTACAGGTA	
virRP-LKpnI	TTGGTACCTTATGTTCATAAAATAGAAAGTGG	This study
virRP-RXbaI	TTTCTAGATAAACATGTCTAATAATCTCCTTT	
virR-RXbaI	TTTCTAGATTAACATATTAAATCCCCTAAAAGGC	This study
virS-RXbaI	TTTCTAGACTAGGCTTCTTTTTCTTGATTTATA	This study
virRanti-L	TTAAGCTTTTTAGTATTGCCTTATGTGAAGAT	This study
virRanti-R	TTTCTAGATAACATATTAAATCCCCTAAAAG	
luxS-L	CCTAAAGGAGATATAGTTTCAAAG	This study
luxS-R	ATGACTCTTAGCTAATTCTAGTGA	
luxS-IBS	AAAAAAGCTTATAATTATCCTTATACTCCAAAGC TGTGCGCCCAGATAGGGTG	This study
luxS-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGT CAAAGCTTCTAACTTACCTTTCTTTGT	This study
luxS-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGAGTATCGA TAGAGGAAAGTGTCT	This study