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 C).

The virS/virR *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, an ~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 (qRT-PCR) confirmed that, compared with 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 (Fig. 11C and D).

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 *Experimental procedures*. 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 Fig. 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-haemolytic when grown on blood agar plates [CN3685 produces PFO to induce β-haemolysis (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 WT *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 PstI-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 B). 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*

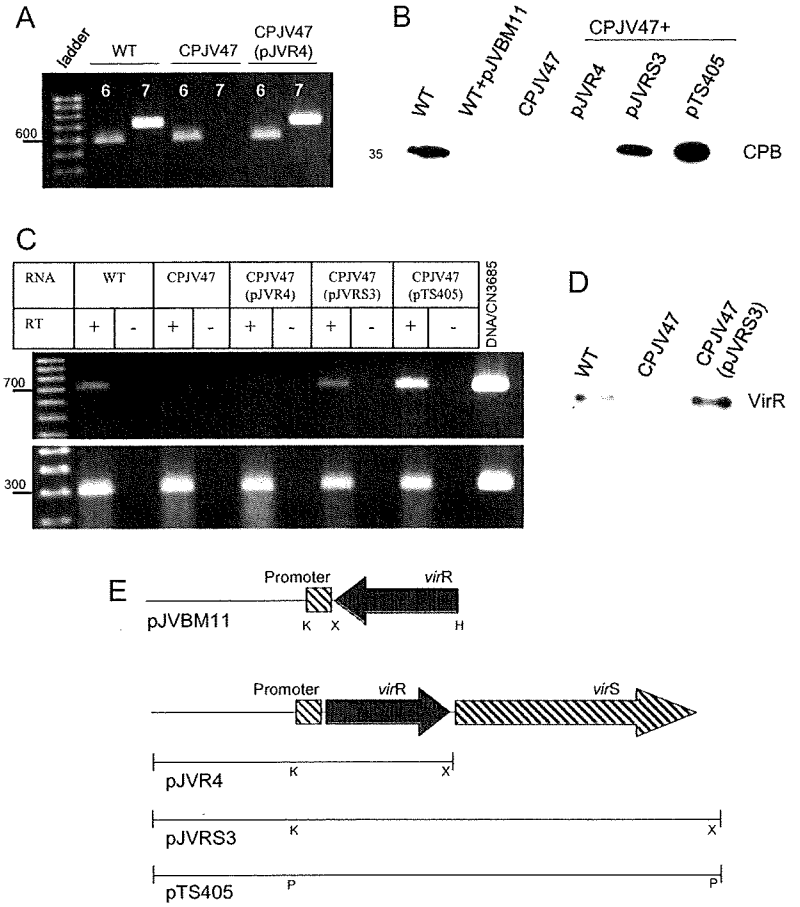

Fig. 10. The *virS*/*virR* two-component regulatory system regulates the Caco-2 cell-induced increase in CPB secretion.
A. Inactivation of the *virR* gene. PCR was performed with primers that amplify (6) an ~590 bp product or (7) ~710 bp *virR* WT gene using DNA from CN3685 (WT), CPJV47 or CPJV47(pJVR4). A 100 bp ladder is shown on the 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 analysed by Western blot using a mouse monoclonal anti-CPB (migration of the 35 kDa CPB protein is noted on the 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) the *virR* gene or (bottom) the housekeeping *polC* gene. Where indicated, retrotranscriptase (RT) was (+) or was not (–) added into the reaction tubes. Reactions containing DNA from the WT strain were included. A 100 bp ladder is shown on the left and the location of 700 (top) and 300 bp (bottom) 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 *virR* antisense vector (pJVBM11) or the plasmids pJVR4, pJVRS3 and pTS405. Note that pJVBM11 has the *virR* gene in the antisense orientation with respect to the *virR* promoter. pJVR3 only encodes the CN3685 *virR* gene, pJVRS3 encodes the *virS*/*virR* operon from CN3685 and pTS405 encodes the *virS*/*virR* operon from Strain 13. K, KpnI; X, XbaI; H, HindIII, P, PstI.

production of potent toxins to cause animal and human disease (Songer, 1996; Voth and Ballard, 2005; McClane *et al.*, 2006). 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 (Sayeed *et al.*, 2005; Voth and Ballard, 2005; Fisher *et al.*, 2006; Amimoto *et al.*, 2007; Fernandez-Miyakawa *et al.*, 2007), 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* versus *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

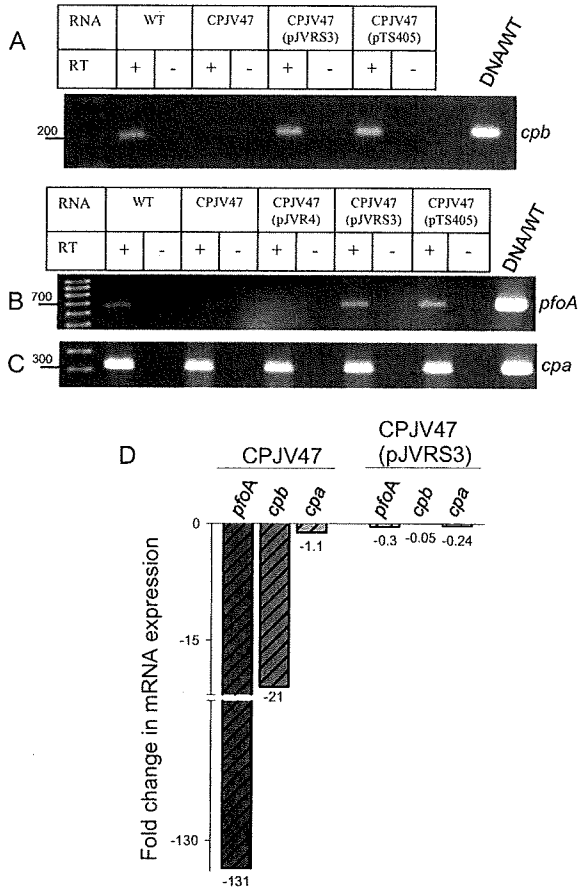

Fig. 11. The *vir*S/*vir*R two-component regulatory system controls the Caco-2-induced increase in transcription of *cpb* and *pfo*A. Caco-2 cells were infected with CN3685 (WT), CPJV47, CPJV47(pJVR4), CPJV47(pJVRS3) or CPJV47(pTS405) for 1 h at 37°C. Bacteria-containing supernatants were obtained to isolate the RNA as described in *Experimental procedures*.
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 were included. A 100 bp ladder is shown on the left, with location of 200 (A), 700 (B) or 300 bp (C) markers is noted. Shown are representative figures of at least three independent experiments.
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* genes. 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.

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 (Gui *et al.*, 2002; Matsuda *et al.*, 2007). 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. This phenomenon is not 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 signalling 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 pre-treated with pronase, which might unmask a signalling 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*, *cpb2*, *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* 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, κ toxin (collagenase) and CPB2 (Lyristis *et al.*, 1994; Ba-Thein *et al.*, 1996; Ohtani *et al.*, 2003). 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 (Lyristis *et al.*, 1994; Ohtani *et al.*, 2003; Cheung *et al.*, 2004; Okumura *et al.*, 2008). 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 (Banu *et al.*, 2000; Okumura *et al.*, 2008).

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 (Fig. 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 *virS*/*virR* operon, indicating that a functional *virS*/*virR* 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* versus *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 WT type C isolate compared with 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 signalling that activates the VirS sensor. VirS/VirR signalling 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* versus *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 signalling 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 signalling 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 signalling that triggers upregulation of toxin production, this factor could be present on the host surface and then be released 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 signalling model for direct interactions with a bacterial surface factor or for cleavage by a *C. perfringens* exoenzyme. Further study of the signalling process behind upregulated toxin production is currently underway to better address these uncertainties.

Experimental procedures

Bacterial strains and culture conditions

Clostridium 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 PBS (pH 7.4) before delicate re-suspension 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 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^{-1}) and streptomycin (100 μg ml^{-1}). 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^{-1}) and streptomycin (100 μg ml^{-1}). Rat1-R12 fibroblasts were maintained in DMEM with 10% tet-off-certified FBS, penicillin (100 U ml^{-1}), geneticin (100 μg ml^{-1}) and streptomycin

Table 2. *C. perfringens* type C isolates, mutants and plasmids used in this study.

Strain	Description	Origin or reference
JGS1495	*cpb*$^+$, *cpb2*$^+$, *plc*$^+$, *pfo*$^+$ and *tpeL*$^+$	Piglet with necrotic enteritis (Fisher *et al.*, 2006)
CN3685	*cpb*$^+$, *plc*$^+$, *pfo*$^+$ and *tpeL*$^+$	Sheep with struck (Fisher *et al.*, 2006)
CN5383	*cpb*$^+$, *plc*$^+$, *pfo*$^+$ and *tpeL*$^+$	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 *vir*R 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 PstI 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

(100 μg ml^{-1}). Each cell line was normally harvested with 0.25% trypsin (Gibco), re-suspended 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^{-1}) or 24-well microplates (containing 7×10^5 cells well^{-1}). 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 moi of 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.

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	AAAAAAGCTTATAATTATCCTTATACTCCAAAGCTGTGCGCCCAGATAGGGTG	This study
luxS-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAAGCTTCTAACTTACCTTTCTTTGT	This study
luxS-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGAGTATCGATAGAGGAAAGTGTCT	This study

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 *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^{-1}) 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^{-1}) 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 at 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.

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 cut-off (Millipore, Bedford, MA, USA). 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.

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 on 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% w/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 a monoclonal anti-ETX antibody (Sayeed *et al.*, 2005). Bound antibody was then detected after incubation with a horseradish peroxidase (HRP)-conjugated secondary anti-species-specific antibody and addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce). Where noted, purified CPB, CPB2 or ETX was added as a Western blot control; those purified toxins were obtained as previously described (Fisher *et al.*, 2005; Sayeed *et al.*, 2007; Vidal *et al.*, 2008).

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 analyse by Western blot the secretion of CPA and PFO toxins in the presence or absence of Caco-2 cells, the sterile culture supernatants were dialysed overnight at 4°C versus 10 mM Tris, pH 7.5. The dialysed supernatants were then lyophilized (using a Labconco freeze-dry system) and re-suspended 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% w/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.

Hb release assay to quantify secreted PFO activity. Horse red blood cell (RBC, Becton-Dickinson Laboratories) were washed three times with sterile PBS and re-suspended 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) was 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 [(absorbance at 570 nm) (A_{570})] was then measured using a microplate reader (Dinex technologies).

ELISA to quantify secreted CPA and CPB levels. *C. perfringens* α 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).

After overnight incubation at 4°C, plates were washed three 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 10 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 colour reaction was stopped with sulfuric acid (0.18 M) and the sample absorbance at 450 nm (A_{450}) 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 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 quantification of mRNA expression was normalized to the constitutive expression of the housekeeping *polC* gene and calculated by the comparative C_T ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001).

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

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 at 7000 *g* to remove cellular material and filtered-sterilized.

Clostridium 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 analysed by Western blot for β-toxin production using a mouse monoclonal anti-CPB antibody, as described earlier.

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^{-1}), 80% (containing 5.5×10^5 cells well^{-1}), 50% (containing 4×10^5 cells well^{-1}), 30% (containing 2.5×10^5 cells well^{-1}) 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 at 8000 *g* at 4°C for 30 min. The supernatants were then filter-sterilized and analysed by Western blot for CPB levels using a mouse monoclonal anti-CPB antibody, as described earlier.

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.

Clostridium 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 analysed by Western blot for CPB levels using a mouse monoclonal anti-CPB antibody, as described earlier.

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% 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 at 8000 *g* at 4°C for 30 min and filter-sterilized. The sterile supernatants were analysed for CPB levels by Western blotting using a mouse monoclonal anti-CPB antibody, as described earlier.

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: (i) 0.25% trypsin or a 0.2% EDTA solution for 15 min, (ii) phospholipase C in the form of CPA (1×10^{-3} or 1×10^{-5} U ml^{-1}) for 1 h, or (iii) pronase (Roche) 100 µg ml^{-1} for 20 min. All cell treatments were performed at

37°C. Treated cells were washed three times with sterile PBS and re-suspended 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^{-1}) were infected with CN3685 or JGS1495 (moi = 20) and incubated for the indicated time at 37°C. The supernatant was obtained and analysed by Western blot for CPB levels as described earlier.

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 4 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^{-1}) 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 (which 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 analysed 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 upregulation, 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 luxS *null mutant using TargeTron® technology*

To inactivate the *lux*S gene, the WT *lux*S gene sequence was first entered into the Intron prediction program (http://www.sigma-genosys.com/targetron/). This program predicted a sense insertion site, with a low e-score (0.170), between the nucleotides 295 and 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 HindIII and BsrGI 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 WT *C. perfringens* type C isolate CN3685, as described previously (Chen *et al.*, 2005). Transformants were plated onto BHI agar plates containing 15 μg ml^{-1} chloramphenicol. Colonies were then PCR screened using *lux*S-specific primers that supported PCR amplification of an ~300 bp product from the WT *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 (Fig. S3A). All PCR-screened reactions were performed using Taq 2× Master Mix (New England Biolabs) and primers final concentration of 1 μM in a Techne (Burkhardtsdorf, Germany) thermocycler.

Inactivation of the virR *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 an ~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^{-1} 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 an ~590 bp product or primers that amplify the entire ~710 bp *vir*R WT gene. A mutant, CPJV47, which had lost the ~710 bp PCR product (Fig. 9A), was then grown in blood agar plates to test for PFO-induced β-haemolysis.

Complementation of virR *mutant*

To complement the *vir*R mutant (CPJV47), the entire *virS/virR* 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 (Fig. 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 (Fig. 9D). Plasmid pTS405, which encodes the *virS/virR* operon, was constructed by digesting Strain 13 DNA with PstI 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 virR *antisense vector*

To knockdown the *vir*R gene with an antisense mRNA, a PCR product containing the *vir*R promoter was cloned between the KpnI–XbaI sites of pJIR750. The *vir*R gene was then amplified using the primers virRanti-L (HindIII) and virRanti-R (XbaI) and

directionally cloned, in the antisense orientation, between the XbaI–HindIII sites of pJIR50, that lie upstream of the *virR* promoter (Fig. 9D). The resulting plasmid, pJVBM11, was electroporated into the WT CN3685.

Southern blot hybridization

Clostridium perfringens genomic DNA from WT CN3685 or CPJV19 was obtained, as described earlier. This DNA was digested with EcoRI and run on a 0.8% agarose gel. After transfer to a nylon membrane (Roche), the blot was probed with a digoxigenin-labelled probe specific for the intron sequence. This probe was prepared using the primer pair luxS-IBS and luxS-EBS1d (Table 3) and labelled with a DIG labelling kit obtained from Roche Applied Science. CSPD substrate (Roche Applied Science) was used for detection of DIG-labelled 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 re-suspended 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 (Cheung and Rood, 2000; McGowan *et al.*, 2002).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Protein secretion and growth of *C. perfringens* type C isolates in the presence of Caco-2 cells.

A and C. Caco-2 cell cultures grown to confluency in 100 mm tissue culture dishes were infected with *C. perfringens* type C isolates JSG1495 (A) or CN3685 (C) for the indicated times at 37°C (moi = 20). For comparison, the same numbers of bacteria were inoculated into TGY or MEM (no Caco-2 cells) and then incubated under the same conditions. As a control, Caco-2 cells were left uninfected but incubated for 3 h in MEM without additives. At the conclusion of the experiment, culture supernatants were filter-sterilized and concentrated 10-fold using 10 kDa cut-off Amicon filter devices. Equal amounts of each concentrated supernatant containing *C. perfringens*-secreted proteins were electrophoresed on a 12% SDS-PAG and stained with Coomassie blue. Numbers on the left are in kDa. Asterisks show the location of the 60 kDa major protein secreted by Caco-2 cells (control lanes).

B. *C. perfringens* strain JGS1495 (1.5×10^7 cfu) was inoculated into FTG, TGY, MEM or Caco-2 cells and then incubated for 2 h at 37°C. Culture supernatant was removed and filter-sterilized. Equal amounts of each supernatant were electrophoresed on a 12% SDS-PAG and transferred to nitrocellulose membrane. Purified, 35 kDa CPB (0.5 μg) was also included (β toxin). Membranes were blocked for 1 h and probed with a mouse monoclonal anti-CPB antibody.

D. Caco-2 cell cultures, TGY or MEM were each inoculated with 1.5×10^7 cfu of *C. perfringens* (Cp) type C isolates CN3685 for 1.5 h, at 37°C (moi = 20). The culture supernatants containing bacteria (10 ml) were aspirated and serially diluted in BHI broth (10 ml final volume) and then plated (1 ml) onto BHI agar plates. The number of bacteria (cfu ml^{-1}) in each culture condition was recorded after 24 h of incubation under anaerobic conditions at 37°C.

Figures shown are representative of at least four independent experiments.

Fig. S2. *cpb* transcription by CN3685 and CPB secretion in the presence of paraformaldehyde (PFA)-fixed Caco-2 cells.

A. *cpb* transcription. Caco-2 cells were infected with CN3685 for 1 h. RNA (50 ng) was isolated and used as template in RT-PCR reactions with primers that amplify an ~200 bp product from the *cpb* gene. Where indicated, retrotranscriptase (RT) was (+) or was not (–) added into the reaction tubes. A reaction containing DNA from CN3685 was included. A 100 bp ladder is shown on the left, with migration of the 200 bp marker noted.

B. CPB secretion in the presence of PFA-fixed Caco-2 cells. MEM cultures, PFA-fixed Caco-2 cells, PBS alone or PFA-Caco-2 cells with PBS were infected with CN3685 during the indicated time at 37°C. Sterile supernatants were obtained and analysed for CPB levels as described above. Migration of the 35 kDa CPB is noted on the blot.

Fig. S3. A *luxS*-controlled quorum-sensing mechanism is not required for CPB secretion *in vitro*.

A. Southern blot showing a single intron insertion in CPJV19. Genomic DNA (3 μg) from CN3685 (WT) or CPJV19 was digested with EcoRI at 37°C and then electrophoresed in a 0.8% agarose gel. After transferring to nylon membrane, the blot was probed with a DIG-labelled probe that detects the sequence of the intron (see *Experimental procedures* for details). Molecular size of markers, in kb, is shown on the left.

B and C. CPB secretion and growth rates. WT or CPJV19 strain was inoculated in TGY and incubated during the indicated time. Sterile supernatants (B) or OD_{600} (C) were obtained in each time point. Supernatants were then electrophoresed on a 12% SDS-PAG and transferred to nitrocellulose membrane. Purified CPB (0.5 μg) was also included (β toxin). Membranes were blocked for 1 h and probed with a mouse monoclonal anti-CPB antibody as described before.

Fig. S4. Caco-2 cells induce upregulated ETX secretion by *C. perfringens* type D. Caco-2 cells, MEM or TGY were infected with *C. perfringens* type D strain NCTC8346 (top) or CN1634 (bottom) for the indicated time and incubated at 37°C (moi = 20). Equal amounts of sterile supernatants were analysed by Western blot using a mouse monoclonal anti-ETX antibody. Migration of the 33 kDa ETX (control) is noted on the blot.