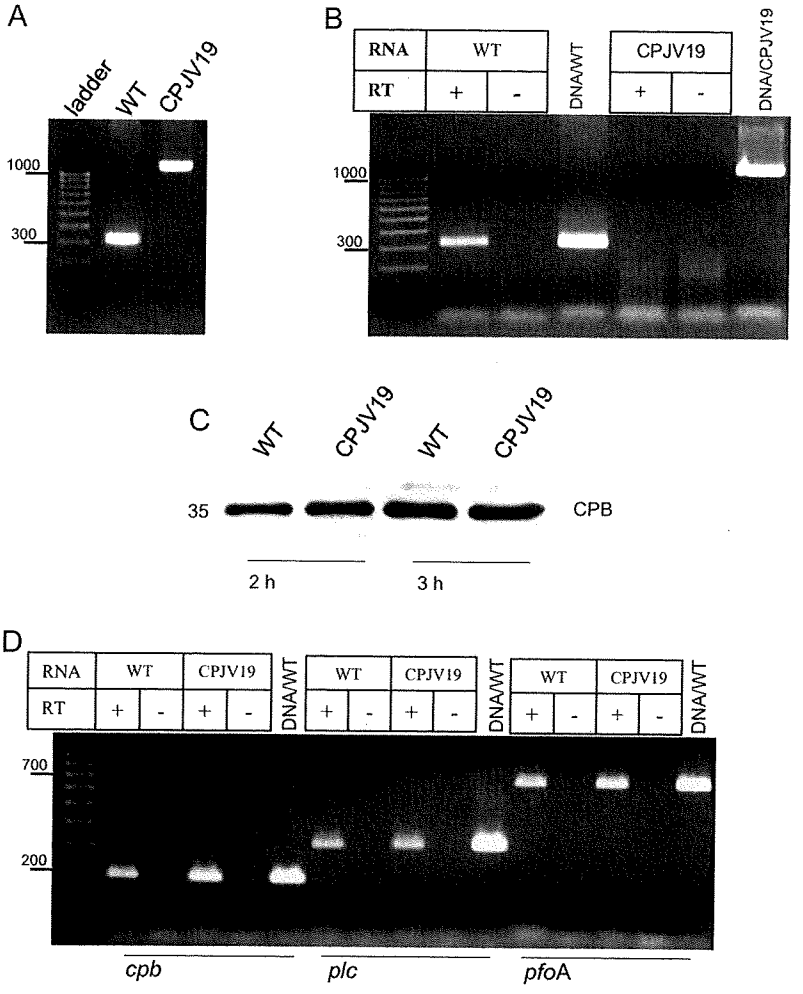

Fig. 9. *lux*S-controlled quorum-sensing mechanism is not required for Caco-2 cell-induced upregulation of toxin production by CN3685.
A. PCR showing an intron insertion in the *lux*S gene. Primers LuxS-L and LuxS-R amplified a 300 bp product of the *lux*S gene using DNA isolated from CN3685 (WT) or 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 on the left is a 100 bp ladder; selected marker size, in bp, are noted on the 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 or 3 h (moi = 20). Sterile culture supernatants were obtained and analysed 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 on the left and selected marker size, in bp, are noted on the left of the gel.
Figures shown are representatives of at least three independent experiments.

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 luxS*-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 (Chen *et al.*, 2005; Li and McClane, 2008; Sayeed *et al.*, 2008). A Group II intron (~900 bp) was inserted, in the sense orientation, between nucleotides 295 and 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 (WT) *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 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*

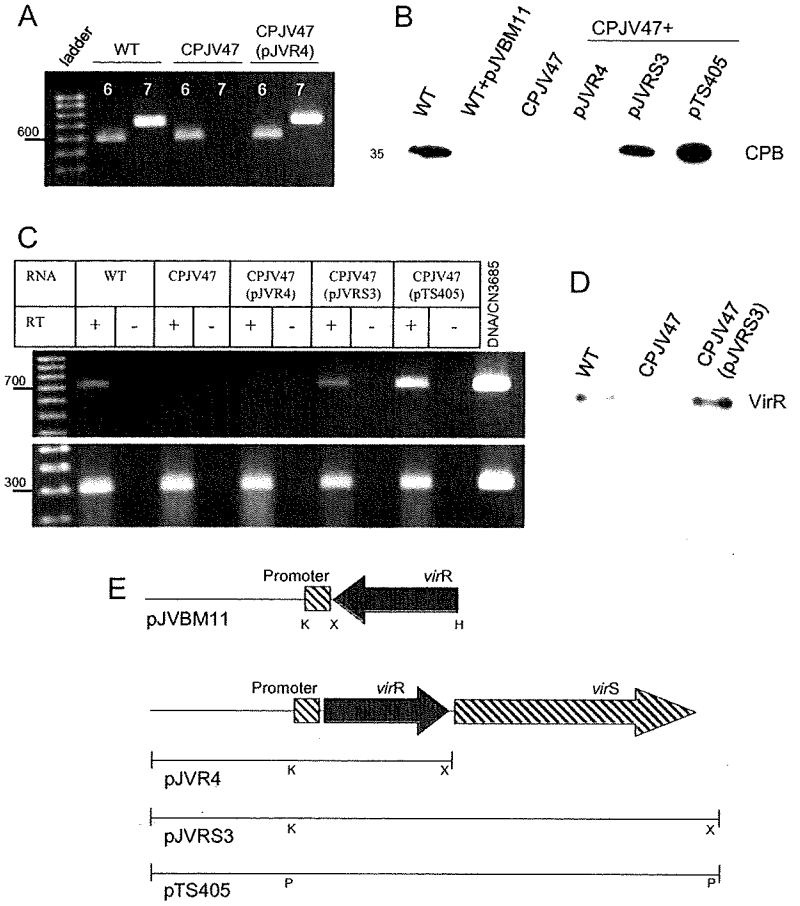

Fig. 10. The *vir*S/*vir*R two-component regulatory system regulates the Caco-2 cell-induced increase in CPB secretion.
A. Inactivation of the *vir*R gene. PCR was performed with primers that amplify (6) an ~590 bp product or (7) ~710 bp *vir*R WT gene using DNA from CN3685 (WT), CPJV47 or CPJV47(pJVR4). A 100 bp ladder is shown 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 *vir*R gene or (bottom) 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 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 *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, 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

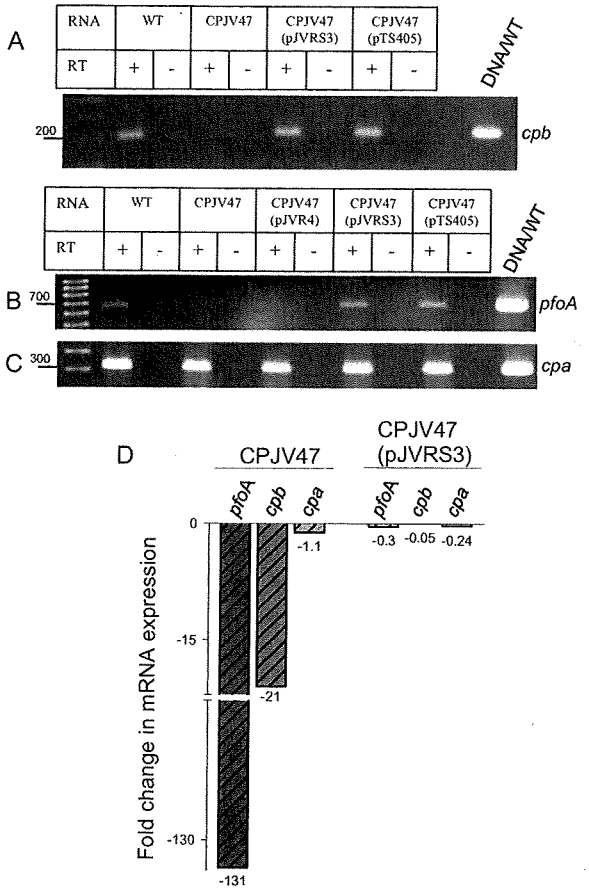

Fig. 11. The *vir*S/*vir*R two-component regulatory system controls the Caco-2-induced increase in transcription of *cpb* and *pfo*A.
Caco-2 cells were infected with CN3685 (WT), CPJV47, CPJV47(pJVR4), CPJV47(pJVRS3) or CPJV47(pTS405) for 1 h at 37°C. Bacteria-containing supernatants were obtained to 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, *cpb2* 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::*luxS*	This work
CPJV47	CN3685::*virR*	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 *virR* promoter and the *virR* gene in antisense orientation, from CN3685	This work
pJVR4	pJIR750 with *virR* promoter and the *virR* gene from CN3685	This work
pJVRS3	pJIR750 with *virR* promoter, *virR* gene and *virS* gene from CN3685	This work
pTS405	pJIR418 with a PstI fragment from strain 13 genomic DNA containing *virR* promoter, *virR* gene and *virS* gene	Ba-Thein *et al.* (1996)
pJIR750ai	pJIR750 with *plc* targeted intron in antisense orientation	Chen *et al.* (2005)
pJVLux	pJIR750ai with *luxS* 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*/*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 (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*/*vir*R 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 *vir*R 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 *lux*S-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.

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Characterization of Genes Regulated Directly by the VirR/VirS System in *Clostridium perfringens*[▽]

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Analysis of the complete sequence of the genome of *Clostridium perfringens* strain 13 resulted in identification of five genes, including *pfoA* (encoding theta toxin) and *vrr* (encoding VirR/VirS-regulated RNA), with consensus VirR-binding sequences upstream of the open reading frame (ORF), suggesting that expression of these genes may be regulated directly by the two-component VirR/VirS system. To test this possibility, we examined VirR/VirS system-mediated transcriptional regulation of three genes, *virT*, *ccp* (encoding alpha-clostripain), and *virU*, with the novel VirR-binding sequences. Northern analysis revealed that the steady-state levels (increases or decreases in the amounts of RNA expressed) of *virT*, *ccp*, and *virU* mRNAs were lower in a *virR* mutant strain than in the wild-type strain, as were the levels of the *pfoA* and *vrr* transcripts. The consensus VirR-binding sites were located similarly relative to the transcription start sites in the *virT*, *ccp*, and *virU* promoters. Mutation and overexpression analyses with *virT* and *virU* revealed that the *virT* gene product has a negative effect on expression of *pfoA* and *ccp*, whereas the *virU* gene product positively affects expression of *pfoA*, *virT*, *ccp*, and *vrr*. Nonsense and frameshift mutations in the *virT* or *virU* putative ORF did not affect the regulatory functions, suggesting that *virT* and *virU* may encode RNA regulators rather than proteins. These results suggest that a complex regulatory network, perhaps involving several regulatory RNA molecules, governs the expression of the VirR/VirS regulon in *C. perfringens*.

The gram-positive anaerobic bacterium *Clostridium perfringens* produces numerous extracellular toxins that are believed to play important roles in the pathogenicity of various diseases, including gas gangrene, which is also known as clostridial myonecrosis (9, 21). Because the toxins are thought to act synergistically in the development of gas gangrene (2), knowledge of the mechanisms that regulate expression of toxin genes is critical for understanding the pathogenesis of myonecrosis.

Bacterial two-component systems, consisting of a sensor histidine kinase and a response regulator, enable bacteria to respond to various environmental conditions through a phosphorelay between the sensor and the regulator. The two-component VirR/VirS system comprising the VirR response regulator and the VirS sensor protein is known to be involved in global regulation of the production of theta-toxin (also known as perfringolysin O), kappa-toxin (or collagenase), alpha-toxin (or phospholipase C), sialidase, protease, and hemagglutinin in *C. perfringens* (13, 24). The VirR/VirS system regulates the mRNA levels of *plc* (alpha-toxin), *pfoA* (theta-toxin), and *colA* (kappa-toxin) (4). Primer extension analysis revealed both VirR/VirS-dependent and independent promoters for *pfoA* and *colA* and a single VirR/VirS-dependent promoter for *plc* (4). The absence of a consensus binding site for phosphorylated VirR protein in the promoters of the *colA* and *plc* genes (4) suggests that complex regulatory networks might be involved in *C. perfringens* toxin production (26).

Four targets of the VirR/VirS system have been identified through differential display analyses. The VirR/VirS system was found to promote expression of *ptp* (encoding protein tyrosine phosphatase), *cpd* (encoding 2′,3′-cyclic nucleotide phosphodiesterase), and *hyp7* (encoding a hypothetical 7-kDa protein) (3) and to inhibit expression of the *ycgJ-metB-cysK-luxS* (*ygaG*) operon (3, 20). It was suggested previously that *hyp7* acts as a secondary regulator that positively regulates the levels of *colA* and *plc* mRNAs but not the level of *pfoA* mRNA (3). However, we reported previously that VirR/VirS-regulated RNA (VR-RNA) (encoded by *vrr*) transcribed from the Hyp7 coding region is a regulatory RNA that mediates the signal from the VirR/VirS system to control the expression of *colA*, *plc*, *ptp*, *cpd*, and *ycgJ-metB-cysK-luxS*, whereas *pfoA* is regulated directly by the VirR/VirS system (28). The VirR/VirS–VR-RNA cascade was also found to affect levels of plasmid-borne *cpb2* (encoding beta2 toxin) and *cna* (encoding a possible collagen adhesin) mRNAs positively and negatively, respectively (19).

Two repeated sequences have been found upstream of the *pfoA* promoter (4), and it was reported previously that the VirR protein binds independently to these two repeats (CCC AGTTNTNCAC) (6). Interestingly, a monomeric repeat similar but not identical to the *pfoA* VirR-binding site has also been found in the promoter of *vrr*, the gene encoding VR-RNA (28). A CCAGTTNNNCAC core motif was highly conserved in both genes. These findings suggest that the VirR protein may bind to the *vrr* promoter, activating transcription of VR-RNA, which in turn activates *colA* and *plc* transcription,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
C. perfringens strains		
13	Wild-type strain (type A)	14
TS133	Strain 13 *virR*::Tet^r	24
TS140	Strain 13 Δ*vrr* Em^r	28
TS190	Strain 13 *virT*::Em^r	This study
E. coli DH5α	*supE*44 Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Takara Bio Inc.
Plasmids		
pBT405	pJIR418 Ω(PstI 4.3-kb strain 13 genomic library) (*virR*$^+$ *virS*$^+$ complementation vector), Amp^r	Shimizu, unpublished data
pJIR418	*E. coli-C. perfringens* shuttle vector, Cm^r Em^r	29
pSB1031	pJIR418 Ω(PCR-amplified 637-bp fragment) (*vrr*$^+$ complementation vector)	4
pTS930	pJIR418 Ω(PCR-amplified 1,400-bp fragment) (*virT*$^+$ complementation vector)	This study
pTS931	pJIR418 Ω(PCR-amplified 539-bp fragment) (*virU*$^+$ complementation vector)	This study
pTS932	pTS930 (585T→A)	This study
pTS933	pTS931 (182A→T)	This study
pTS934	pUC118 Ω(PCR-amplified 334-bp fragment) (*virT*$^+$ suicide vector), Em^r	This study
pTS935	pTS930 (1-bp frameshift at position 33)	This study
pTS936	pTS931 (1-bp frameshift at position 15)	This study
pUC19	Cloning vector, Amp^r *lacZ′* pMB 1 *ori*	Takara Bio Inc.
pUC118	Cloning vector, Amp^r *lacZ′* pMB 1 *ori*	Takara Bio Inc.

[a] Tet^r, resistance to tetracycline; Em^r, resistance to erythromycin, Cm^r, resistance to chloramphenicol; Amp^r, resistance to ampicillin.

thus forming the basis for a regulatory cascade in the VirR/VirS regulon (28).

The complete genomic sequence of *C. perfringens* strain 13 has been reported (25). On the basis of sequence similarities with other known virulence genes, more than 20 candidate virulence genes were identified. By screening the genome for the previously identified VirR-binding consensus sequence, we identified genes potentially regulated by the VirR/VirS system. Five genes were found to have the consensus VirR-binding site in their putative promoter regions (25). In the present study, regulation of expression of these novel target genes was analyzed to improve our understanding of the VirR/VirS regulon in *C. perfringens*.

MATERIALS AND METHODS

Strains, plasmids, medium, and culture conditions. The strains and plasmids used in this study are listed in Table 1. All *C. perfringens* strains were cultured in Gifu anaerobic medium (Nissui, Japan) at 37°C under anaerobic conditions as described previously (24). A single-crossover mutation was introduced into the *virT* gene of *C. perfringens* strain 13 with a pUC19-based suicide vector containing a 334-bp internal PCR fragment of *virT* and the *ermB* gene from pJIR418 (29). *Escherichia coli* DH5α was cultured as described previously (22). Plasmids pUC19 and pUC118 were used for cloning in *E. coli*, and pJIR418 (29) was used as an *E. coli-C. perfringens* shuttle vector.

DNA manipulation. Recombinant DNA was manipulated as described previously (22), unless otherwise noted. *C. perfringens* strains were transformed by electroporation as previously described (24).

Northern hybridization. Total RNA from *C. perfringens* was extracted and Northern blotting was performed as previously described (1) with an AlkPhos-direct kit and CDP-*star* chemiluminescence (GE Healthcare). DNA probes were prepared from genomic DNA of *C. perfringens* strain 13 by performing PCR with the appropriate primer sets (Table 2). In some situations, the signal densities of the mRNA bands were measured with a densitometer. All Northern hybridization experiments were performed at least three times, and the reproducibility was confirmed. The results described below are representative results from the repeated experiments.

Primer extension analysis. Primer extension was carried out as described previously (17) by using an Amersham 5′ oligolabeling fluorescence kit and the Promega primer extension system. Oligonucleotide primers 0845-PE, 0846-PE, and 0920-PE used to determine the transcription start sites of CPE0845 (*virT*), CPE0846 (*ccp*), and CPE0920 (*virU*), respectively, are shown in Table 2. Signals were detected with a FluorImager analyzer (GE Healthcare).

Assays for perfringolysin O and alpha-clostripain. The perfringolysin O activity in the *C. perfringens* culture supernatant was measured by the horse erythrocyte hemolysis method described previously (2). *C. perfringens* cells were cultured for 3 h to mid-log phase (see Fig. 2A) and collected by centrifugation. The supernatant was used for the hemolytic assay. Hemolytic activity was expressed as the reciprocal of the dilution that resulted in 50% hemolysis of 0.5% horse erythrocytes. The proteolytic activity of alpha-clostripain in the culture supernatant was determined with azocasein (Sigma Aldrich Japan) and the cysteine protease-specific inhibitors leupeptin and antipain (Wako Pure Chemicals) as previously described (11, 27). In brief, *C. perfringens* cells were cultured for 2 h to early log phase (see Fig. 2A) and collected by centrifugation, and 500 μl of the supernatant was mixed with an equal volume of an azocasein solution (5 mg/ml azocasein in 25 mM Tris-HCl [pH 7.5]–5 mM dithiothreitol) with or without 10 μM leupeptin or antipain. The mixture was incubated for 2 h at 37°C with gentle shaking, intact azocasein was removed by 3% trichloroacetic acid precipitation, and the absorbance at 450 nm of the supernatant was determined.

Site-directed mutagenesis and frameshift mutagenesis. Site-directed mutagenesis of the *virT* gene harbored by pTS930 was performed with an LA PCR in vitro mutagenesis kit (Takara Bio) with the mutagenic primer virT-NM (Table 2) to obtain pTS932. The *virU* gene on pTS931 was mutated by using a QuikChange site-directed mutagenesis kit (Stratagene) with primers virU-NM-F and virU-NM-R (Table 2) to obtain pTS933. Similarly, 1-base deletion frameshift mutations at positions 33 and 15 in the *virT* and *virU* coding regions, respectively, in the complemented plasmid vectors pTS930 and pTS931 (Table 1), respectively, were obtained by using a QuikChange site-directed mutagenesis kit (Stratagene) with primers virT-FM-F and virT-FM-R for *virT* and primers virU-FM-F and virU-FM-R for *virU* to construct pTS935 and pTS936 (Table 1). All procedures were performed according to the manufacturers' instructions.

RESULTS

Screening for VirR-binding sites in the *C. perfringens* genome. We scanned the genomic sequence of *C. perfringens* (25) for VirR-binding sites (CCAGTTNNNCAC) located upstream of the open reading frame (ORF). Only five genes, *pfoA*, CPE0845 (*virT*), CPE0846 (*ccp*), CPE0920 (*virU*), and *vrr*, were found to have sequences similar to the VirR-binding site in their putative promoter regions (Fig. 1) (25). With the exception of *virT* and *ccp*, which are located back to back and are

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′to 3′)	Use
virT-F	GAAGGTAAGTACCAAGATGA	Northern blotting
virT-R	TGATATTGCCACCCCAACTT	Northern blotting
ccp-F	GAGGCCGAAAAGACTGAAGG	Northern blotting
ccp-R	GCCCAATGTGGTATTGCTTG	Northern blotting
virU-F	CGATTCGTTTTTGATAGAAATGG	Northern blotting
virU-R	TTTATTTCTAGTTTTTCCTTTGATGA	Northern blotting
vrr-F	GACCAGTTACGCACAAAC	Northern blotting
vrr-R	GGACAGTTCTATTTCTAGG	Northern blotting
pfoA-F	GCAAGTATTGCAATGGCTTT	Northern blotting
pfoA-R	GTAAGTAATACTAGATCCAGGGT	Northern blotting
colA-F	GGTCTAGAGGATAGTGGAAGA	Northern blotting
colA-R	GTTCCTCTCATATCGTAAGT	Northern blotting
0845-PE	TTTATCAATGGGTAACGTAAAGAAAGTACCAGATA	Primer extension
0846-PE	GTTGAAACTCCTCCTAATAGAACGCTACCAATGGT	Primer extension
0920-PE	TCGGTATAATAAAAATGATATTGTTACTAATATG	Primer extension
virT-NM	GAAAGTACCAGATTAAATAGAAATAG	Site-directed mutagenesis
virU-NM-1	TTGGTATTATATATTAATATTTTCGTC	Site-directed mutagenesis
virU-NM-2	GACGAAAATATTAATATATAATACCAA	Site-directed mutagenesis
virT-FM-F	ATCGCTTTAAGTTTATCATTTCTATTTTATCTGGT	Site-directed mutagenesis
virT-FM-R	ACCAGATAAAATAGAAATGATAAACTTAAAGCGAT	Site-directed mutagenesis
virU-FM-F	GATATGAAAGACGAAAAATTAATATAAAATACCAA	Site-directed mutagenesis
virU-FM-R	TTGGTATTTTATATTAATTTTTCGTCTTTCATATC	Site-directed mutagenesis

transcribed on opposite strands, these five genes are not clustered (Fig. 1). The deduced amino acid sequence of the putative protein encoded by *ccp* was highly similar to that of alpha-clostripain, a cysteine proteinase from *Clostridium histolyticum* (7). The expression of *ccp* was previously shown to be positively regulated by the VirR/VirS system (27). The putative proteins encoded by *virT* and *virU* showed no significant similarity to known proteins, and their functions remain unclear. Identification of the previously uncharacterized putative VirR-binding sites in *virT*, *ccp*, and *virU* led to an investigation of whether these three genes, like *pfoA* and *vrr*, are targets for direct regulation by the VirR/VirS system.

Transcriptional regulation of *virT*, *ccp*, and *virU* by the VirR/VirS system. We performed Northern analyses of RNA of *C. perfringens* strains with different mutant backgrounds at different growth stages to look for changes in the steady-state levels

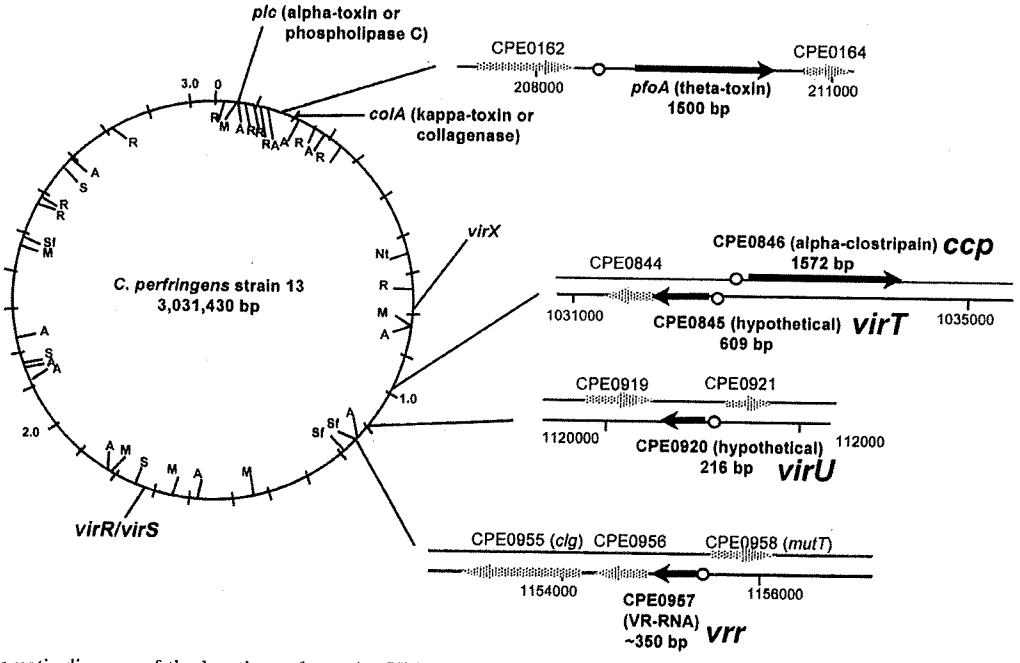

FIG. 1. Schematic diagram of the locations of putative VirR-binding sites in five genes (*pfoA*, *virT*, *ccp*, *virU*, and *vrr*) on the chromosome of *C. perfringens* wild-type strain 13. The solid and cross-hatched arrows represent genes with VirR-binding sites and their flanking genes, respectively. Open circles indicate putative VirR-binding sites. The chromosomal locations of other genes mentioned in this paper are also indicated. The nucleotide numbers are the numbers for the chromosomal sequence of *C. perfringens* strain 13 (GenBank accession number BA000016).

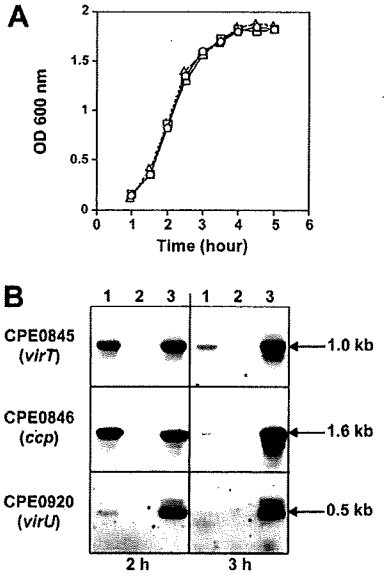

FIG. 2. Growth curves and Northern blot analyses for the *virR* mutant (TS133) of *C. perfringens*. (A) Growth curves for *C. perfringens* strains with a 1% inoculum. All three *C. perfringens* strains grew with a doubling time of ~20 min in Gifu anaerobic medium. ○, strain 13(pJIR418); □, strain TS133(pJIR418) (*virR*); △, strain TS133 (pBT405) (*virR*$^+$ *virS*$^+$). OD 600 nm, optical density at 600 nm. (B) Total RNA was prepared from each culture at the indicated times (2 and 3 h). Either 10 μg (for *virT* and *ccp*) or 40 μg (for *virU*) of total RNA was resolved by agarose electrophoresis, blotted onto a nylon membrane, and hybridized with probes for *virT* (CPE0845), *ccp* (CPE0846), and *virU* (CPE0920). Lane 1, strain 13(pJIR418); lane 2, strain TS133 (pJIR418); lane 3, strain TS133(pBT405).

of *virT*, *ccp*, and *virU* that confirmed that that there is regulation by the VirR/VirS system. In *C. perfringens* wild-type strain 13, a 1.0-kb transcript of *virT* was clearly detected after 2 h of growth (early to mid-exponential phase) (Fig. 2B, upper left panel), and the level had decreased after 3 h of incubation (late exponential phase) (Fig. 2B, upper right panel). Similar patterns were observed for expression of the 1.6-kb alpha-clostripain (*ccp*) transcript (Fig. 2B, center panels) and the 0.5-kb *virU* transcript (Fig. 2B, lower panels) in the wild-type background. Even with 40 μg of total RNA blotted onto the membrane, the *virU* transcript signal was very low for strain 13, indicating that the level of expression of *virU* was significantly lower than the level of expression of *virT* or *ccp*. The time points of accumulation for *virT*, *ccp*, and *virU* mRNAs were similar to those for *pfoA* and *vrr*; all of the molecules were most abundant during the early exponential to mid-exponential growth phase (3, 4). Importantly, transcripts of *virT*, *ccp*, and *virU* either were undetectable or the bands were very weak for the *virR* mutant strain TS133 (Fig. 2B, lane 2). Expression of these genes was increased by transformation of intact *virR* and *virS* genes into TS133 (Fig. 2B, lane 3). These data clearly indicate that the steady-state RNA levels for *virT*, *ccp*, and *virU* were regulated positively by the VirR/VirS system in *C. perfringens* in a manner similar to the manner of regulation of *pfoA* and *vrr* (3, 4, 28). The higher levels of the *virT*, *ccp*, and *virU* transcripts in the complemented strains were likely due to the high copy number of the pBT405 complementation plasmid.

Promoter analysis of the VirR/VirS-regulated genes. To analyze the promoter regions of the three VirR/VirS-regulated genes, we identified transcription initiation sites for *virT*, *ccp*, and *virU* by performing a primer extension experiment with wild-type and *virR* mutant RNA templates. The *virT*-specific primer generated a single extension product with wild-type strain 13 RNA, whereas no product was obtained with the *virR* mutant strain TS133 (Fig. 3A, left panel). Similarly, both the *ccp* and *virU* gene-specific primers (Fig. 3A, middle and right panels, respectively) yielded single extension products with the wild-type RNA template, whereas no products were obtained with *virR* mutant strain RNA. These results indicate that transcription initiation from single sites in *virT*, *ccp*, and *virU* is dependent on the VirR/VirS system because little mRNA for these genes was present in the *virR* mutant strain.

The length of each primer extension product was used to assign the position 1 site of transcription initiation for *virT*, *ccp*, and *virU* and to identify conserved elements (−35 and −10) in the promoter of each gene (Fig. 3B). We compared the locations of the VirR-binding sequences (CCAGTTWTNCA), the consensus promoter sequences (−35 and −10), and the mRNA start sites with those determined for *pfoA* and *vrr* in previous studies (4, 28) (Fig. 3B) and found that the relative distances between these elements were highly conserved in the five promoter regions. In particular, all five genes have a 42-bp interval between the VirR-binding sequences and the mRNA start site (Fig. 3B). The locations of the consensus promoter sequences (−35 and −10) were almost identical in all five genes.

In previous studies, two repeated sequences (VB1 and VB2) in the promoter region of *pfoA* (Fig. 3B) were identified as independent binding sites for the VirR protein (5, 6). The promoter regions of *vrr*, *virT*, *ccp*, and *virU* contained sequences similar to the VB2 sequence of *pfoA* (Fig. 3B). Although more divergent than the similarities between these four genes and *pfoA* in the VB2 consensus sequence, sequences similar to VB1 were found in these four genes (Fig. 3B), suggesting that the VB1 region may also be important for regulation of transcript by the VirR protein. The promoter structures of these five VirR-regulated genes are highly conserved and are distinct from the promoter structures of previously analyzed VirR/VirS-regulated genes (4, 19). The ability of the VirR protein to bind to the conserved sequences upstream of *virT*, *ccp*, and *virU* was confirmed in a previous study (5). Moreover, VirR has been reported to bind to some VirR boxes found in genes of two other strains of *C. perfringens*, ATCC13124 and SM101 (16), which suggests that the VirR-dependent regulatory system is present in various types of *C. perfringens* strains. Binding of a glutathione *S*-transferase–VirR fusion protein to the VirR-binding sequence was also examined using gel mobility shift assays, and this analysis confirmed that the VirR protein bound specifically to the conserved sequences in the promoter regions of *pfoA*, *vrr*, *virT*, *ccp*, and *virU* (data not shown).

Functional analysis of the *virT* and *virU* genes. We were unable to predict the putative function of either *virT* or *virU* using the results of computer-based searches for sequence similarities. To explore the functional roles of these genes, we

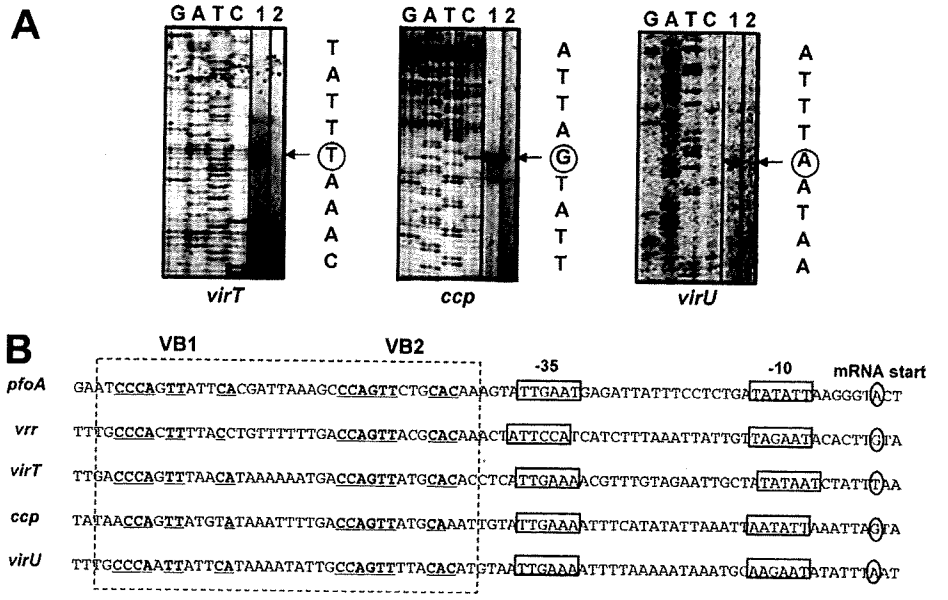

FIG. 3. Identification of the transcription initiation sites of the *virT*, *ccp*, and *virU* genes in *C. perfringens*. (A) Primer extension products derived using the oligonucleotide primers listed in Table 2 with template RNA prepared from 2-h cultures of *C. perfringens* strains (lane 1, wild-type strain 13; lane 2, *virR* strain TS133) were separated by electrophoresis on acrylamide gels. Sequencing reactions with the same primers and appropriate DNA templates were run on the same gel. The positions of extended products obtained with the *virT*, *ccp*, and *virU* primers are indicated by arrows, and the putative mRNA start sites are indicated by circles. (B) Deduced promoter sequences (−35 and −10) and consensus VirR-binding sites of the five VirR/VirS-regulated genes. The putative promoter sequences and mRNA start sites are indicated by boxes and circles, respectively. The deduced VirR-binding sequence of each gene is indicated by a dotted box, and conserved nucleotides are underlined. The promoter sequences of the theta-toxin (*pfoA*) and VR-RNA (*vrr*) genes are aligned, and the VB1 and VB2 regions are shown.

constructed *virT* isogenic mutants of strain 13 (see Materials and Methods). The resulting *virT* mutation in strain TS190, which was confirmed by Southern hybridization with a *virT* gene probe (data not shown), was used to examine expression of VirR/VirS-regulated genes. Compared to wild-type strain 13, in mutant strain TS190 there were at least 2.5-fold increases in the levels of both *pfoA* and *ccp* mRNAs during the logarithmic growth phase (2 and 3 h) (Fig. 4A). When mutant strain TS190 was complemented with the *virT*$^+$ pTS930 plasmid [resulting in TS190(pTS930)], the level of each of the transcripts was reduced to the wild-type level, although complementation was not complete until the 3-h time point for unknown reasons (Fig. 4A). These data suggest that the *virT* gene product acts as a negative regulator of *pfoA* and *ccp* in the wild-type strain. However, no significant change in *plc*, *colA*, *vrr*, or *virU* expression was observed in TS190 (Fig. 4A), indicating that the negative effect of the *virT* gene product was specific to *pfoA* and *ccp*.

For unknown reasons, repeated attempts to construct a strain 13 *virU* mutant using single-crossover or double-crossover recombination methods failed. As an alternative approach to test the function of *virU*, we introduced a *virU* overexpression plasmid, pTS931, into wild-type strain 13 to measure the effect of *virU* on the steady-state levels of putative VirR/VirS target genes. In 3-h cultures, the *pfoA*, *ccp*, *vrr*, and *virT* mRNA levels were all increased in response to the higher number of *virU* copies (Fig. 4B), suggesting that the *virU* gene encodes a positive regulator of *pfoA*, *ccp*, *vrr*, and *virT* expression. Although the abundance of *plc* and *colA* transcripts also increased slightly following transformation of pTS931 into strain 13 (Fig. 4B), we believe that this may have been a secondary effect of increased VR-RNA (Fig. 4B, *vrr* panels), which is known to enhance expression of *plc* and *colA*.

In addition to measuring changes in the levels of the *pfoA* and *ccp* mRNAs, we also measured the activities of the secreted gene products, perfringolysin O and alpha-clostripain, respectively, in supernatants from cultures of wild-type and mutant *C. perfringens* strains. Perfringolysin O activity was measured by determining the hemolytic activities of the culture supernatants with horse erythrocytes. The hemolytic activities of *virT* mutant strain TS190, TS190 with the plasmid expressing *virT* (pTS930), and wild-type strain 13 with the *virU* overexpression plasmid (pTS931) were 3.9-, 1.2- and 4.3-fold higher, respectively, than the hemolytic activity of wild-type strain 13 (Table 3). These results, which correspond well with the results of the Northern analyses, indicate that the activity encoded by wild-type *virU* stimulates the production of perfringolysin O, whereas the wild-type *virT* gene product inhibits the production of perfringolysin O.

Azocasein, a colorimetric substrate of alpha-clostripain and other cysteine proteinases, was used to measure alpha-clostripain activity specifically in culture supernatants containing two inhibitors of cysteine proteinases, leupeptin and antipain. In the presence of these inhibitors, the proteolytic activity of each strain, as measured by absorbance at 450 nm, decreased between 25 and 60% (Fig. 5A). The difference in activity represents alpha-clostripain-specific proteolysis of azocasein (Fig. 5B). The alpha-clostripain activity was increased in the *virT* mutant strain TS190, and when the mutation was complemented with pTS930 (*virT*$^+$), the activity decreased to a level

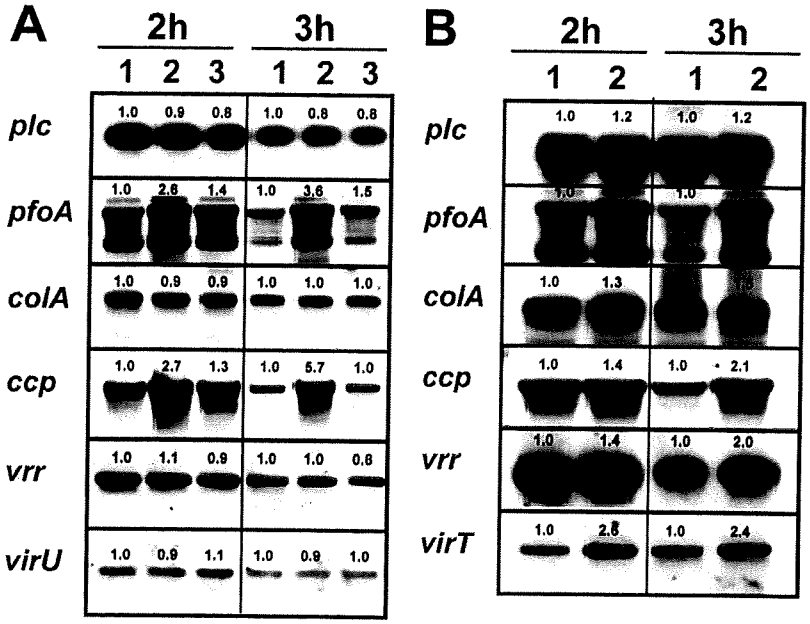

FIG. 4. Northern blot analyses of the *virT* mutant and wild-type strains transformed with the *virU*$^+$ expression plasmid (pTS931). Total RNA was prepared from each culture at the times indicated (2 and 3 h), and 10 μg of each RNA preparation (40 μg for hybridization with *virU* probe) was resolved by agarose electrophoresis, blotted onto nylon membranes, and hybridized with *plc*, *pfoA*, *colA*, *ccp*, *vrr*, *virT*, and *virU* gene probes as indicated. The band densities relative to those for wild-type strain 13 are indicated above the bands. (A) Lane 1, wild-type strain 13(pJIR418); lane 2, strain TS190(pJIR418) (Δ*virT*); lane 3, strain TS190(pTS930) (Δ*virT virT*$^+$). (B) Lane 1, wild-type strain 13(pJIR418); lane 2, wild-type strain 13(pTS931) (*virU*$^+$).

similar to the level in the wild-type strain (Fig. 5B), indicating that expression of alpha-clostripain is negatively regulated by *virT*. Similarly, the alpha-clostripain activity was higher in the *virU*-overexpressing strain than in the wild-type strain (Fig. 5B), suggesting that the *virU* gene product stimulates alpha-clostripain production. The similarities between the changes in perfringolysin O and alpha-clostripain activities and the changes in the levels of mRNAs for these genes in different genetic backgrounds (wild-type strain versus *virT* mutant or *virU*-overexpressing strain) suggest that the *virT* gene product negatively controls expression of *pfoA* and *ccp* and that the *virU* gene product enhances expression of *pfoA* and *ccp* in *C. perfringens*.

Mutational analyses of *virT* and *virU*. It has been reported that in *C. perfringens* regulatory RNA molecules control expression of several genes, including toxin genes (17, 28). Con-

TABLE 3. Perfringolysin O activities of various *C. perfringens* strains

Strain	Genotype	Perfringolysin O titer (log_2)[a]	Difference (fold) compared to strain 13
13	Wild type	7.4 ± 0.5	1
TS133	Strain 13 *virR*::Tet^r	2.0 ± 0.1	<0.1
TS190	Strain 13 Δ*virT*	9.4 ± 0.5	3.9
TS190(pTS930)	Strain 13 Δ*virT* (*virT*$^+$)	7.7 ± 0.4	1.2
13(pTS931)	Strain 13 (*virU*$^+$)	9.5 ± 0.7	4.3

[a] Each value was calculated by using the results of triplicate independent experiments, and the values are means ± standard deviations.

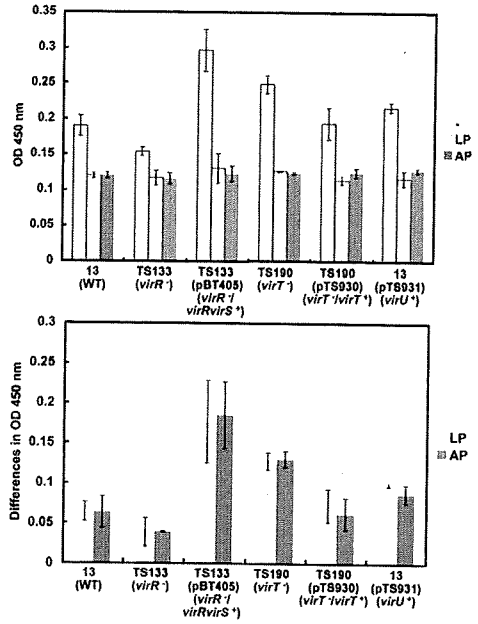

FIG. 5. Alpha-clostripain activities of various *C. perfringens* strains. (A) The alpha-clostripain activity of each *C. perfringens* strain (indicated at the bottom) was determined with azocasein as a substrate under conditions with no inhibitor (−), with leupeptin (LP), or with antipain (AP). For each strain and treatment combination, the mean absorbance and standard deviation (error bar) calculated from three independent experiments are shown. (B) The difference in mean proteolytic activity between assays without inhibitors and assays with the leupeptin or antipain inhibitor (shown in panel A), which represents alpha-clostripain-specific proteolytic activity, was plotted for each *C. perfringens* strain. WT, wild type; OD 450 nm, optical density at 450 nm.

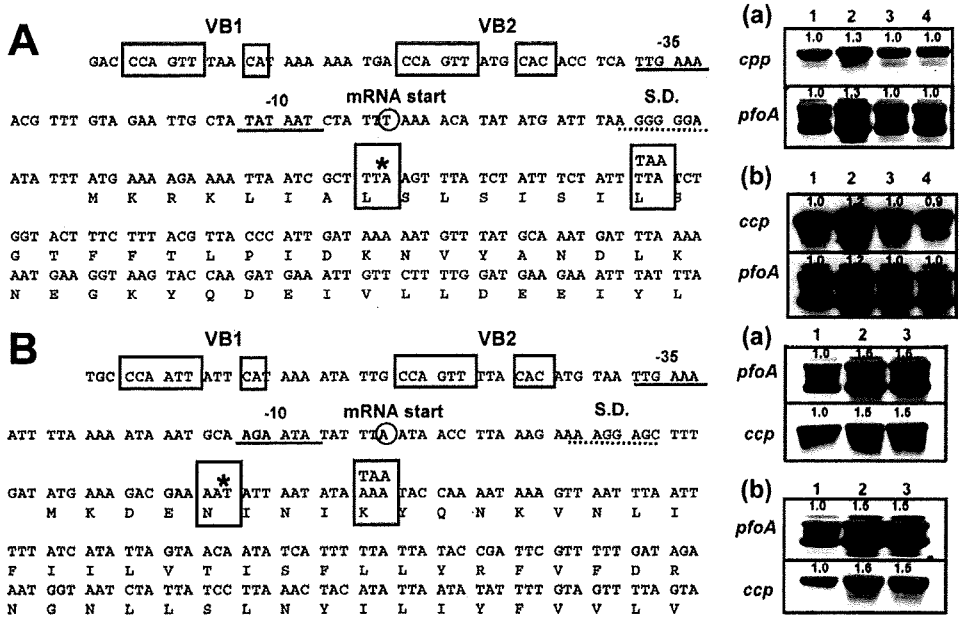

FIG. 6. Site-directed mutagenesis of the *virT* (A) and *virU* (B) genes and the effects of the mutations on steady-state levels of VirR-regulated gene mRNAs. The nonsense and frameshift codons engineered in the *virT* and *virU* coding regions (left panels) are indicated by large boxes. The positions of the nonsense mutation (NM) and frameshift mutation (FM) are indicated by TAA and an asterisk, respectively. Plasmids carrying mutated *virT* and *virU* genes (designated pTS932 and pTS935 for *virT* and pTS933 and pTS935 for *virU*) were transformed into different *C. perfringens* strains. The band densities relative to those for wild-type strain 13 are indicated above the bands. (A) (Panel a) Lane 1, wild-type strain 13(pJIR418); lane 2, strain TS190(pJIR418) (Δ*virT*); lane 3, strain TS190(pTS930 (Δ*virT virT*$^{+}$); lane 4, strain TS190(pTS932) (Δ*virT virT*^{NM}). (Panel b) Lane 1, wild-type strain 13(pJIR418); lane 2, strain TS190(pJIR418) (Δ*virT*); lane 3, strain TS190(pTS930) (Δ*virT virT*$^{+}$); lane 4, strain TS190(pTS935) (Δ*virT virT*^{FM}). (B) (Panel a) Lane 1, wild-type strain 13(pJIR418); lane 2, wild-type strain 13(pTS931) (*virU*$^{+}$); lane 3, wild-type strain 13(pTS933) (*virU*^{NM}). (Panel b) Lane 1, wild-type strain 13(pJIR418); lane 2, wild-type strain 13(pTS931) (*virU*$^{+}$); lane 3, wild-type strain 13(pTS9360 (*virU*^{FM}). Northern analyses were performed with the indicated probes (right panels).

sidering the relatively small size of the *virT* and *virU* ORFs (609 and 216 bp, respectively), it is possible that these genes, like *vrr* and *virX*, encode regulatory RNAs. To test this hypothesis, nonsense mutations were introduced into the protein-encoding regions of *virT* and *virU* with plasmid vectors (pTS932 and pTS933, respectively), and then differences between the *ccp* and *pfoA* steady-state mRNA levels in samples of wild-type and mutant cell total RNAs were determined (Fig. 6). When the *virT* nonsense mutant gene in pTS932 was introduced into the isogenic *virT* mutant strain TS190, the relative levels of *ccp* and *pfoA* transcripts in TS190(pTS932) were not different from the levels in TS190(pTS930) containing an intact *virT* gene (Fig. 6A, panel a, lanes 3 and 4). Similarly, a nonsense mutation was constructed in the coding region of *virU* in plasmid pTS933, which was transformed into wild-type strain 13 (Fig. 6B). The results indicated that there was a difference in the level of *vrr*, *pfoA*, or *ccp* mRNA between wild-type strain 13 overexpressing intact *virU* (pTS931) and wild-type strain 13 overexpressing mutated *virU* (pTS933) (Fig. 6B, panel a, lanes 2 and 3). Furthermore, one-base deletions at positions 33 and 15 in the *virT* and *virU* coding regions (Fig. 6) were introduced to generate frameshift mutations, resulting in plasmids pTS935 and pTS936, respectively. Plasmid pTS935 was transformed into the isogenic *virT* mutant strain TS190 and wild-type strain 13, and the mRNA levels for *pfoA* and *ccp* were determined by Northern hybridization. As shown in Fig. 6, the relative levels of *ccp* and *pfoA* transcripts in TS190(pTS935) with frameshifted *virT* were not different from the levels in TS190 (pTS930) containing an intact *virT* gene. Similarly, the transcript levels of *ccp* and *pfoA* were not different in strain 13 harboring pTS936 (with frameshifted *virU*) and strain 13 harboring pTS931. Taken together, these results indicate that there was no difference in the regulatory functions of *virT* and *virU* whether the ORFs were intact or not intact. These data strongly suggest that both the *virT* and *virU* genes encode regulatory RNA molecules, not proteins, that regulate the VirR/VirS regulon. The promoters of *pfoA*, *virT*, *ccp*, *vrr*, and *virU* were screened, and no conserved sequence motifs (besides the VirR consensus sequence) were found, indicating that it is unlikely that there are other regulatory RNAs or proteins that are shared by these VirR/VirS-regulated genes.

DISCUSSION

A genome-wide search for promoter-proximal VirR-binding sites previously identified the theta-toxin-encoding gene *pfoA* (6) and the *vrr* gene, which encodes regulatory VR-RNA (28), and also identified three new genes, *virT*, *ccp*, and *virU*, as potential members of the VirR/VirS regulon (25). To test the veracity of this in silico identification, genetic and molecular analyses of *virT*, *ccp*, and *virU* functions and regulation by the VirR/VirS system were performed.

Comparative Northern analyses of wild-type and *virR* mutant strains of *C. perfringens* revealed that *virT*, *ccp*, and *virU*