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Virulence Gene Regulation by the *agr* System in *Clostridium perfringens*[▽]

Kaori Ohtani, Yonghui Yuan, Sufi Hassan, Ruoyu Wang, Yun Wang, and Tohru Shimizu*

Department of Bacteriology, Graduate School of Medical Science, University of Kanazawa, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8203, Japan

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A gram-positive anaerobic pathogen, *Clostridium perfringens*, causes clostridial myonecrosis or gas gangrene in humans by producing numerous extracellular toxins and enzymes that act in concert to degrade host tissue. The *agr* system is known to be important for the regulation of virulence genes in a quorum-sensing manner in *Staphylococcus aureus*. A homologue for *S. aureus agrBD* (*agrBD*$_{Sa}$) was identified in the *C. perfringens* strain 13 genome, and the role of *C. perfringens agrBD* (*agrBD*$_{Cp}$) was examined. The *agrBD*$_{Cp}$ knockout mutant did not express the theta-toxin gene, and transcription of the alpha- and kappa-toxin genes was also significantly decreased in the mutant strain. The mutant strain showed a recovery of toxin production after the addition of the culture supernatant of the wild-type strain, indicating that the *agrBD*$_{Cp}$ mutant lacks a signal molecule in the culture supernatant. An *agr-virR* double-knockout mutant was constructed to examine the role of the VirR/VirS two-component regulatory system, a key virulence regulator, in *agrBD*$_{Cp}$-mediated regulation of toxin production. The double-mutant strain could not be stimulated for toxin production with the wild-type culture supernatant. These results indicate that the *agrBD*$_{Cp}$ system plays an important role in virulence regulation and also suggest that VirR/VirS is required for sensing of the extracellular signal and activation of toxin gene transcription in *C. perfringens*.

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacterium. *C. perfringens* is the causative agent of several human and animal diseases, including clostridial myonecrosis, or gas gangrene (7). *C. perfringens* produces various extracellular enzymes and toxins, including alpha-, theta-, and kappa-toxins encoded by *plc*, *pfoA*, and *colA*, respectively (21). These toxin genes are positively regulated by the two-component VirR/VirS system (25) that is a major regulator of virulence in *C. perfringens*. The VirS is a sensor histidine kinase, and VirR is a response regulator. When VirS senses specific stimuli in the environment, VirS autophosphorylates at a histidine residue and then transfers the phosphate to VirR. Once VirR is activated by phosphorylation, it regulates gene expression. The genomic sequence of *C. perfringens* strain 13 was determined in 2002, and it was found that the genome contains only five genes, including *pfoA* and VR-RNA, that have VirR-binding sites on their promoter regions (24). VR-RNA is known to be a small regulatory RNA and positively regulates *colA* and *plc* transcription (26). Recent microarray analysis suggested that many other genes are regulated by the VirRS-VR-RNA cascade. Thus, a number of virulence-related genes and also some housekeeping genes are included in the VirRS-VR-RNA-regulon (K. Ohtani et al., unpublished data). The *C. perfringens* genome contains many genes for toxins or for enzymes that can degrade host tissue, while the genome lacks many genes related to the synthesis of amino acids. Under infectious conditions, *C. perfringens* might secrete these toxins and enzymes in order to degrade the host tissue. It may then import the resulting amino acids, using them to survive in the host tissue. The VirR/VirS system is therefore very important for the activation of toxin production that results in the degradation of host cells and is critical for the survival of *C. perfringens*, especially within the host. However, it is still unclear what the signal of VirS is and how this signaling system effectively stimulates toxin production.

Many bacteria regulate gene expression in response to cell population density, a phenomenon known as "quorum sensing" (4). Quorum sensing involves the production of extracellular signaling molecules (autoinducers). In general, many known autoinducers of gram-positive bacteria are actively secreted peptides that are processed from larger propeptides. These peptide autoinducers function as ligands for signal receptors such as the two-component sensor histidine kinase (17). In gram-negative bacteria, the *N*-acylhomoserine lactones (AHLs) are well known as autoinducers (14). They diffuse freely in and out of cells and interact directly with intracellular regulatory proteins. AHL accumulates as cells grow, and when it reaches a certain threshold, AHL can efficiently regulate the expression of many genes. In *Vibrio fischeri*, the LuxR protein binds to AHL, and this complex regulates the *lux* operon and many other genes at the transcriptional level (14). Moreover, the *luxS* gene is responsible for the production of another kind of autoinducer, autoinducer 2 (30). Highly conserved *luxS* homologues have been identified in both gram-positive and gram-negative bacteria (2). These quorum-sensing systems play important roles in the regulation of virulence factors and in biofilm formation in various pathogenic bacteria (6, 28, 30).

In *C. perfringens*, the possibility that cell-cell signaling exists has been suggested (8). In a previous report, two types of toxin-negative strains were cross-streaked on a blood agar plate, and one toxin-negative strain recovered its toxin production just after the crossing point of the two strains on the plate (8, 10). This experiment suggested that there is a signal mol-

* Corresponding author. Mailing address: Department of Bacteriology, Graduate School of Medical Science, Kanazawa University, 1-13, Takara-machi Kanazawa, Ishikawa 3920-8640, Japan. Phone: 81-76-265-2200. Fax: 81-76-234-4230. E-mail: tshimizu@med.kanazawa-u.ac.jp.

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TABLE 1. *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Description[a]	Source
C. perfringens strains		
Strain 13	Wild-type strain (type A)	
TS133	Strain 13 *virR*::Tet^r	This study
TS230	Strain 13 Δ*agrBD*::Em^r	This study
TS231	Strain 13 Δ*agrBD*-Δ*virR*::Tet^r Em^r	This study
Plasmids		
pJIR418	*E. coli -C. perfringens* shuttle vector, Cm^r Em^r; pJIR418 Ω (PstI 4.3-kb strain 13 genomic library)	
pTS405	*virR*$^+$ *virS*$^+$ complementation vector; Cm^r	T. Shimizu, unpublished data
pTS1301	pJIR418 Ω(Δpromoter CPE1562-CPE1561-*agrD*-CPE1560)	This study
pTS1302	pJIR418 Ω(Δpromoter *agrD*-CPE1560)	This study
pTS1303	pJIR418 Ω(CPE1563-CPE1562-CPE1561-*agrD* -CPE1560) including promoter	This study
pTS1304	pJIR418 Ω(CPE1563-CPE1562-CPE1561-*agrD*) including promoter	This study
pTS1305	pJIR418 Ω(Δpromoter CPE1562-CPE1561-*agrD*)	This study
pTS1306	pJIR418 Ω(Δpromoter CPE1561-*agrD*)	This study
pTS1307	pJIR418 Ω(Δpromoter *agrD*)	This study
pTS1308	pJIR418 Ω(CPE1562-CPE1561-*agrD*)	This study
pTS1309	pJIR418 Ω(CPE1561-*agrD*)	This study
pTS1310	pJIR418 Ω(*agrD*)	This study
pTS1311	pJIR418 Ω(CPE1563-*agrD*)	This study
pTS1312	pJIR418 Ω(CPE1563-CPE1562-*agrD*)	This study
pTS1313	pJIR418 Ω(CPE1562-*agrD*)	This study
pTS1314	pJIR418 Ω(CPE1562-CPE1561-*agrD*)	This study

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

ecule (called substance A) (9) that stimulates toxin production from outside of the cell. In 2002, cell-cell signaling mediated by *luxS* was reported, and it was concluded that the signal produced actually regulated the transcription of toxin genes (18). However, the mutant strain of *luxS* still retained toxin production; therefore, it was concluded that the *luxS* signaling system might be different from that mediated by substance A and thus that there may be a different cell-cell signaling system in *C. perfringens*.

In gram-positive bacteria, a secreted peptide regulates gene expression in the quorum-sensing manner described above (17). In the case of *S. aureus*, the autoinducer propeptide (AIP) acts as a signal to stimulate gene expression. This peptide contains an intramolecular thiolactone ring. The *agrD* gene is a structural gene for AIP, and AgrB is a protein that is required for modification of the AgrD propeptide. In the genome of *S. aureus*, the genes of a two-component system, *agrA* and *agrC*, lie next to the *agrBD* genes. The AgrA protein is a response regulator, and AgrC is a sensor histidine kinase. The AIP, synthesized from the AgrD protein, is secreted and accumulates in the supernatant. Once AIP reaches a certain threshold level, it activates its receptor, AgrC sensor histidine kinase, which then activates AgrA by phosphotransfer. Finally, AgrA activates the transcription of the regulatory RNA, RNAIII, that regulates the expression of various virulence genes of *S. aureus* (5, 15, 17). This signaling system is highly conserved among many gram-positive bacteria (12, 16, 20).

In the present study we identified an *agrBD* gene in *C. perfringens* ($agrBD_{Cp}$) that is homologous to the *agrBD* gene of *S. aureus* ($agrBD_{Sa}$). Functional genetic analysis revealed that $agrBD_{Cp}$ is involved in the positive regulation of alpha-, kappa-, and theta-toxin genes through a cell-cell signaling mechanism that involves a two-component VirR/VirS system.

MATERIALS AND METHODS

Strains, media, and culture conditions. The *C. perfringens* strains 13 (13) and TS133 (23), as well as the other strains used in the present study (Table 1), were cultured in Gifu anaerobic medium (GAM) or TSF (tryptone, 40 g/liter; soytone, 4 g/liter; fructose, 5 g/liter [pH 5.7]) (9) medium at 37°C under anaerobic conditions as described previously (23). *Escherichia coli* strain DH5α was cultured under standard conditions (22). The plasmid pUC19 was used for general cloning in *E. coli*, and pJIR418 (27) was used as an *E. coli-C. perfringens* shuttle vector. The plasmid pTS405 was used as a complementation vector for *virR/virS* genes (19).

DNA manipulation. General recombinant DNA techniques were performed as described in Sambrook et al. (22) unless otherwise noted. *C. perfringens* strains were transformed by an electroporation-mediated transformation as previously described (23). Deletion endpoints were confirmed by nucleotide sequencing using reverse or universal primers, a BigDye terminator reaction kit, and an ABI 310 sequencer (Applied Biosystems, Tokyo, Japan).

Northern and Southern hybridization. Total RNA from *C. perfringens* was extracted according to a method described previously (1). Northern hybridization was also carried out as described previously (3, 11) except that DNA fragments were labeled with an AlkPhos-direct kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and signals were detected by CDPstar chemiluminescence. Southern hybridization was also performed using the same labeling and detection procedures.

Culture supernatant replacement experiments. *C. perfringens* was cultured in TSF medium to stationary phase (optical density at 600 nm of 5.0) as a primary culture, and then these bacteria were inoculated at 5% concentration. The culture was continued to various growth stages and centrifuged at 15,000 rpm for 5 min, and then the culture supernatant was collected. To prepare recipient cells, *C. perfringens* was cultured in TSF medium for 5 h at 37°C and centrifuged at 15,000 rpm for 5 min, followed by removal of the supernatant from the cells. Recipient cells were suspended with the appropriate culture supernatant and incubated at 37°C for 15 min. Total RNA was then isolated from the incubated cells.

Construction of plasmids for allelic replacements. Specific mutants were constructed by using PCR, and the sequences of all primers used for PCR are shown in Table 2. The fragment upstream of the *agr* region was amplified by PCR using primers 1 and 2 and inserted into the HincII site of pUC118. The fragment downstream of the *agr* region was amplified by PCR using primers 23 and 25 and inserted into the SmaI site of the plasmid containing the upstream region.

TABLE 2. Primers used in this study

Primer	Sequence	Description
1	GAACATATGTTTGCATGGAGG	To make plasmid for allelic replacement
2	CAAGCTCTGGGGCACTAGTT	To make plasmid for allelic replacement
3	ATTGTAAAGAGTGAAGGGAG	To construct pTS1303,1304
4	AAAGTTGGACAATCTATCCTA	To construct pTS1301
5	AGGATAGATTGTCCAACTTT	To construct pTS1308-1310
6	TTTATGGGTAACTATGATGT	To construct pTS1305
7	ACTTGTTCCTATCATATGTA	To make CPE1563 probe
8	ATTCTTCCTCCGCTGTCACT	To construct pTS1314
9	AGTGACAGCGGAGGAAGAAT	To make CPE1562 probe
10	ATGGTATTCATACAATATTG	To construct pTS1306
11	TTTAAACCTTCACATAAA	To make CPE1562 probe
12	TAGGTATTCCATCTACTAT	Sequence primer
13	TTTTTCAGCTATTAACTTCGA	To construct pTS1313,1314
14	TTTACAGCAAGCATACTTA	To construct pTS1310,1311, to make CPE1561 probe
15	TTCTGGAGGAGCACATTCAG	To construct pTS1302
16	TCCTTAGAGTCATACATTGC	To make CPE1561 probe
18	TTGTTAAAAACTATAGATTCTT	To check mutation, *agrD* Northern
19	GGCCGGTTTAAAACCTACCT	To check mutation, agrD Northern
20	TATACTAGATTAGAGAGGGAGAAT	To make CPE1560 probe
21	CTCTTCTCCTCCATATCTAGC	To make CPE1560 probe
22	ACTTCAGCTAAGCTATGCTG	To construct pTS1302
23	AAGGTCATAGGTGTTGTATAGC	To make plasmid for allelic replacement
24	TAACAGTACGTGTTCCAAAC	To construct pTS1301,1303
25	AGATGGGGCGGTAGACGTAG	To make plasmid for allelic replacement

Reverse PCR was performed using primers 2 and 23 and the erythromycin resistance gene was cloned into the region deleted by reverse PCR.

Construction of deletion strains. The resulting plasmid for allelic replacement of *agr* operon was transformed into wild-type strain 13. Transformants were screened on a blood agar plate containing erythromycin (25 μg/ml). A hemolysis-negative colony was picked up and Southern analysis was performed to confirm the null mutation of the *agr* region in TS230.

To construct double-knockout mutants, an internal PCR fragment of the *virR* gene was inserted into pUC18 containing the *tetA* gene. The resulting plasmid was transformed into TS230 and screened on an agar plate containing 25 μg of erythromycin/ml and 2.5 μg of tetracycline/ml. The single-crossover mutation of *virR* in TS231 was confirmed by PCR using the appropriate primer set.

Construction of deletion mutants. To construct the pTS1304 deletion mutant containing the genomic fragment stretching from CPE1563 to $agrD_{Cp}$, PCR was performed using the primers listed in Table 2. This PCR fragment was inserted into the HincII site of pUC118, and the resulting plasmid was used as a template for further PCR. Each fragment amplified by PCR was self-ligated and transformed into *E. coli* DH5α. The inserted fragments containing various *agr* genomic regions were then subcloned into pJIR418. To construct the pTS1313 deletion mutant, PCR was performed using pTS1312 as a template.

RESULTS AND DISCUSSION

Identification of an *agrBD* homologue in *C. perfringens*. The *agr* operon of *S. aureus* is known to mediate a quorum-sensing system (17). It has been reported that there is a homologue of this *agr* system in *C. perfringens* SM101 and ATCC 13124 genomes (29). However, the function of the *agr* system in *C. perfringens* has not been determined. To investigate the function of the *agr* system in *C. perfringens*, we searched for homologues of the *agr* operon in the genome of *C. perfringens* strain 13. We found that the amino acid sequence deduced from CPE1561 showed a 29% identity and 50% similarity with the $AgrB^{Sa}$ protein of *S. aureus*. The *agrB* gene encodes an integral membrane protein that modifies the AIP produced by AgrD protein. Downstream of CPE1561 ($agrB_{Cp}$), there was a small open reading frame (ORF) that was not assigned as an ORF when the *C. perfringens* genome sequence was determined (Fig. 1A). The protein from this ORF (designated CPE1560a) was similar to the AgrD peptide of *S. aureus* (32% identity and 46% similarity in a 43-amino-acid-overlap region), which is a propeptide for AIP. Next to the $agrBD_{Sa}$ gene in *S. aureus*, there are genes for a two-component system (*agrA* and *agrC*) that can act as a receptor for AIP and induce gene expression. However, in the *C. perfringens* strain 13 genome, a similar two-component system could not be found in the vicinity of the *agrBD* gene (Fig. 1A). AIPs in *S. aureus* show a variety of amino acid sequences, but the central cysteine, which is important for the formation of a thiolactone ring with the C-terminal amino acid, is conserved in all of them (15). The amino acid sequence of the *C. perfringens* AgrD ($AgrD_{Cp}$) is completely different from that of AIPs, with the exception that this same central cysteine is conserved (Fig. 1B). However, the predicted peptide sequences are conserved in all three *C. perfringens* whose genome sequences are available (Fig. 1B).

To examine the mRNA corresponding to the $agrBD_{Cp}$ region, Northern analysis was performed using gene probes for $agrD_{Cp}$, CPE1561 ($agrB_{Cp}$), CPE1562, CPE1563, CPE1564, and CPE1560. The mRNA obtained from the CPE1561 region was ~2.5 kb in length (Fig. 1C). This length is consistent with the total length of the CPE1561 operon calculated from genome information. Thus, the CPE1561 operon encodes $agrD_{Cp}$, CPE1562, and CPE1563. These data also suggest that CPE1564 and CPE1560 must be independently transcribed, since mRNAs of different sizes were detected by Northern hybridization using gene probes for CPE1564 and CPE1560 (data not shown). The $agrD_{Cp}$ gene is included in the operon, but a second, small independent mRNA was also identified that corresponds to the $agrD_{Cp}$ gene (Fig. 1C). This mRNA is transcribed at a high level up to the stationary phase of growth (data not shown). The length of the $agrD_{Cp}$ mRNA was calculated as 0.45 kb (Fig. 1C). This 0.45-kb mRNA was also de-

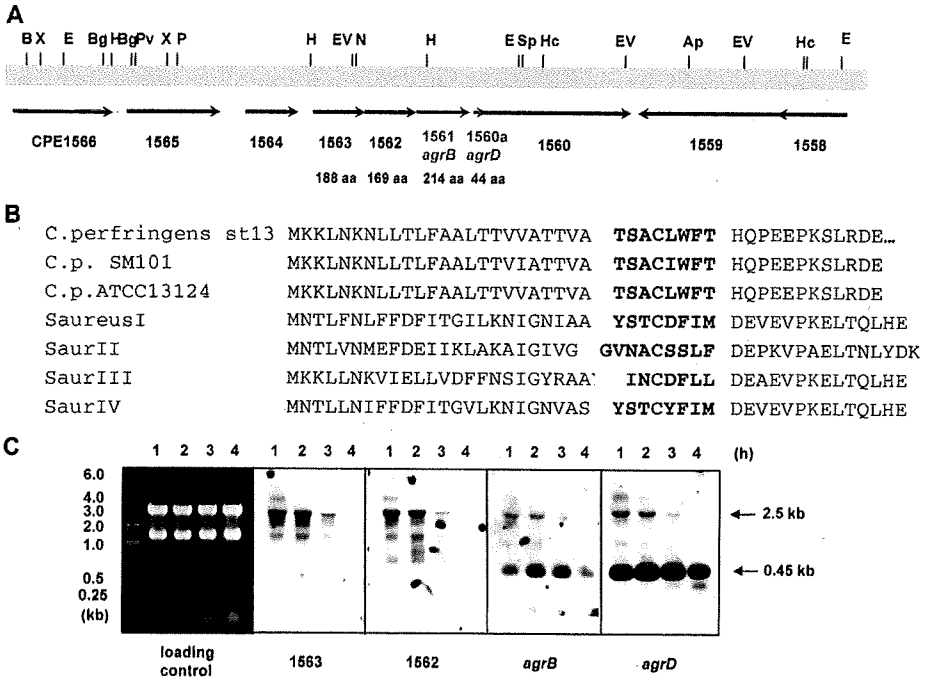

FIG. 1. Analysis of the *agr* region in *C. perfringens*. (*A*) Gene map of the *agr* region in *C. perfringens*. (*B*) Alignment of the deduced amino acid sequence of $AgrD_{Cp}$ in *C. perfringens* and *S. aureus* AIPs. Conserved residues are indicated in red, and the deduced sequence of the mature peptides is in boldface. (C) Northern analysis of the $agrBD_{Cp}$ region. RNA was isolated from strain 13 after culture for 1, 2, 3, and 4 h.

tected by using the CPE1561 ($agrB_{Cp}$) probe, probably because the transcription start site of this mRNA exists in the coding region of CPE1561.

Effect of $agrBD_{Cp}$ on toxin gene expression. To examine the role of the $agrBD_{Cp}$ region in detail, an $agrBD_{Cp}$-null mutant strain and its complement strain were constructed as described in Materials and Methods. The resulting mutant strain (TS230) lacked PfoA-hemolytic activity on blood agar plates (see Fig. 4). Transcription of $agrD_{Cp}$ was completely absent in TS230 but was recovered in the strains that carry pTS1303 and pTS1304 (Fig. 2). In the TS230/pTS1304, an extra band was detected above the $agrD_{Cp}$ mRNA; this band presumably originated from a readthrough transcription occurring in the recombinant plasmid (Fig. 2). The transcription of *pfoA* in TS230/pJIR418 was very low, and the *plc* and *colA* mRNA levels were significantly decreased (Fig. 2). In the TS230 strain that was complemented with a plasmid containing the intact 2.5-kb $agrBD_{Cp}$ operon (TS230/pTS1304), transcription of the toxin genes increased to almost the same level as that in the wild-type strain (Fig. 2). Since the level of toxin gene transcription was practically the same between the TS230/pTS1304 strain complemented with the 2.5-kb operon and the TS230/pTS1303 strain complemented with the 2.5-kb operon and the downstream CPE1560 (Fig. 2), it was concluded that CPE1560 does not have a significant effect on toxin gene expression. From these data it was concluded that the $agrBD_{Cp}$ operon is responsible for the transcriptional activation of toxin genes in *C. perfringens*.

Function of each gene in the operon. In *S. aureus*, $agrBD_{Sa}$ and a two-component regulatory system are all included in a single operon. However, in the case of *C. perfringens* there is no

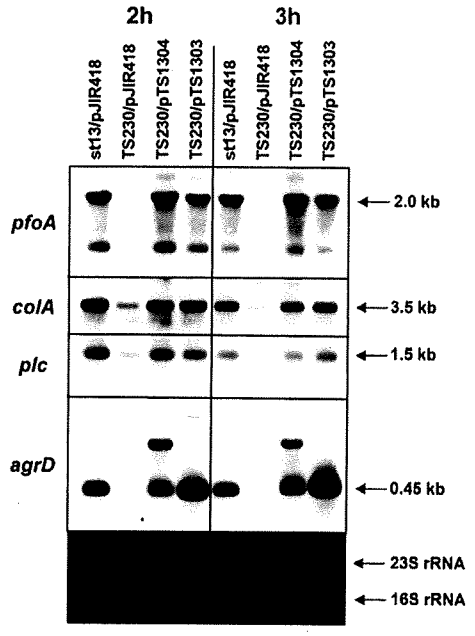

FIG. 2. Northern analysis of the $agrBD_{Cp}$ mutant and complemented strains. An $agrBD_{Cp}$-null mutant (TS230) was constructed by a double-crossing-over method, and the $agrBD_{Cp}$ region was complemented by transformation with pTS1304 and pTS1303. Total RNA was prepared from 2- and 3-h-cultured cells, and 10 μg of total RNA was used for Northern analysis. The internal regions of *pfoA*, *colA*, *plc*, and *agrD* were used as probes.

apparent two-component system in the vicinity of $agrBD_{Cp}$ in the genome. Instead, two other hypothetical genes exist upstream of CPE1561 ($agrB_{Cp}$) and compose a 2.5-kb operon together with $agrBD_{Cp}$ (Fig. 1A). It was therefore considered a possibility that these genes might also have a regulatory effect on toxin gene expression. To analyze the effect of these genes on toxin transcription, plasmids encoding various deletions in these genes were constructed and transformed into the $agrBD_{Cp}$ mutant TS230 (Fig. 3). Deletion plasmids containing both an intact $agrD_{Cp}$ and the CPE1561 gene (pTS1303, pTS1308, pTS1309, and pTS1314) could restore transcription of the toxin genes, whereas plasmids that do not contain the CPE1561 gene (pTS1302, pTS1307, pTS1310, pTS1311, pTS1312, and pTS1313) could not recover toxin gene transcription even when the plasmids contained an intact $agrD_{Cp}$ gene (Fig. 3). Plasmids that contain both the $agrD_{Cp}$ and CPE1561 genes but that do not contain a potential promoter region located upstream of CPE1563 (pTS1301, pTS1302, pTS1305, and pTS1306) also could not activate transcription of the toxin genes (Northern blot data not shown). These experiments suggest that at least CPE1561 ($agrB_{Cp}$) and $agrD_{Cp}$ appear to be essential to the regulatory function of this operon and that transcription is started from a position upstream of CPE1563. Interestingly, in TS230/pTS1308 and TS230/pTS1309 (the plasmids that contain CPE1561 and $agrD_{Cp}$ but not CPE1563) toxin genes are transcribed, but the level of transcription is much weaker than that in TS230/pTS1304 (the plasmid containing all of the genes). However, transcription of the toxin genes in the mutant strain with pTS1314 (ΔCPE1562) was at almost the same level as that in the TS230/pTS1304 strain. These data indicated that CPE1561 ($agrB_{Cp}$) and $agrD_{Cp}$ are essential genes for toxin gene activation but that CPE1563 is required for complete activation.

Activation of toxin production by the toxin-negative strain TS133. We examined whether TS230 can recover its hemolytic activity by exposure to a signal molecule produced from another toxin-negative *virR* mutant strain, TS133. First, TS133 was streaked on a blood agar plate, and then TS230 was streaked at a right angle to TS133 at various distances (Fig. 4). As the two strains became closer, hemolysis from TS230 became stronger. This finding suggested that TS133 secreted a signal molecule that activated toxin production and that TS230 recovered its toxin production by absorbing this molecule from TS133. However, this signal molecule did not appear to diffuse widely in the agar medium because hemolysis of TS230 only occurred when the distance between TS230 and TS133 was quite short (Fig. 4).

Production of the signal molecule and its putative sensor protein. In *S. aureus*, AIP is produced from the $agrBD_{Sa}$ region and is secreted from the cell into the culture supernatant, where it regulates gene expression via a two-component system consisting of AgrA and AgrC (17). To determine whether $agrBD_{Cp}$ is related to the signaling component that is secreted from *C. perfringens* cells, we assayed the ability of $agrBD_{Cp}$ to modulate toxin expression. The culture supernatant was collected from the wild-type *C. perfringens* strain 13 or the $agrBD_{Cp}$ mutant TS230 at early log phase (optical density at 600 nm of 0.5) and was then added to TS230 cells. The cells were incubated at 37°C for 15 min, and total RNA was prepared and analyzed by Northern analysis. The transcription of toxin genes was significantly increased in the TS230 cells only when the wild-type supernatant was added (Fig. 5A), suggesting that the TS230 cells lacked the ability to produce the signal molecule and release it into the supernatant. To further confirm that the signal molecule in the supernatant of strain 13 was produced from the $agrBD_{Cp}$ region, the supernatant was collected from a TS230 mutant strain that had been complemented with an intact $agrBD_{Cp}$ (TS230/pTS1304). When this supernatant was tested on TS230 cells, the expression of toxin genes, especially that of *pfoA*, was strongly induced (Fig. 5A). These data clearly indicate that the $agrBD_{Cp}$ gene is responsible for the production of an extracellular autoinducible signal molecule that controls the expression of toxin genes in *C. perfringens*.

In *C. perfringens*, the VirR/VirS-VR-RNA system is known as a global regulator and can regulate the expression of many toxin genes, including *plc*, *pfoA*, and *colA*; however, the signal that activates the sensor protein VirS has not been identified. Since the $agrBD_{Cp}$ locus controls the expression of a subset of toxin genes similar to that of the VirR/VirS-VR-RNA system, it seemed highly probable that VirS is a sensor protein for the signal molecule produced from the $agrBD_{Cp}$ region. To examine this hypothesis, an $agrBD_{Cp}$-*virR/virS* double-knockout mutant was constructed (designated TS231), and the effect of the wild-type supernatant on toxin transcription in the double mutant was examined. The transcription of *pfoA* in the TS231 mutant was not activated by the wild-type supernatant (Fig. 5B). In contrast, when TS231 was complemented with the plasmid pTS405, which contains the intact *virR/virS* genes, the resulting strain (TS231/pTS405) could sense the extracellular signal, and the transcription of toxin genes was significantly induced by the addition of wild-type or TS230/pTS1304 supernatants (Fig. 5C). In addition, the transcription of *plc* and *colA* in TS231/pJIR418 or TS231/pTS1304 was also upregulated by addition of the wild-type supernatant (Fig. 5C). It was suggested from these data that VirR/VirS is important for sensing of the extracellular signal and activation of toxin gene transcription in *C. perfringens*. However, it remains possible that another two-component system or another protein plays a role in the sensing of this signal, and thus further experiments are needed to elucidate the relationship between the signal molecule from $agrBD_{Cp}$ and the VirS sensor protein.

Regulation between *agr* and *virR/virS*. In *S. aureus*, the *agr* signaling system results in a positive-feedback loop, and the expression of both $agrBD_{Sa}$ for AIP production and *agrA/agrC* for AIP sensing are positively regulated in an operon (15). To examine the regulatory mechanism of the *agr* system in *C. perfringens*, Northern analysis was performed by using TS133 and TS230. At first, RNA was isolated from the wild-type strain (strain 13), TS133, and its complement strain TS133/pTS405, which were cultured for 2 h and 3 h. As in previous experiments, transcription of *pfoA* was absent in TS133 but recovered in TS133/pTS405 (Fig. 6A). In contrast, the transcriptional levels of $agrD_{Cp}$ and the 2.5-kb operon in the three strains were almost the same at 2 h under a *virR/virS*-negative background (Fig. 6A), although the level of $agrD_{Cp}$ transcript was slightly decreased in TS133/pJIR418 at 3 h, which was thought to be not significant.

Next, Northern analysis was performed using strains 13/pJIR418, TS230/pJIR418, and TS230/pTS1304 to check the *virR/virS* transcription under $agrBD_{Cp}$-negative conditions. As shown in Fig. 6B, the transcription of the *virR/virS* operon was

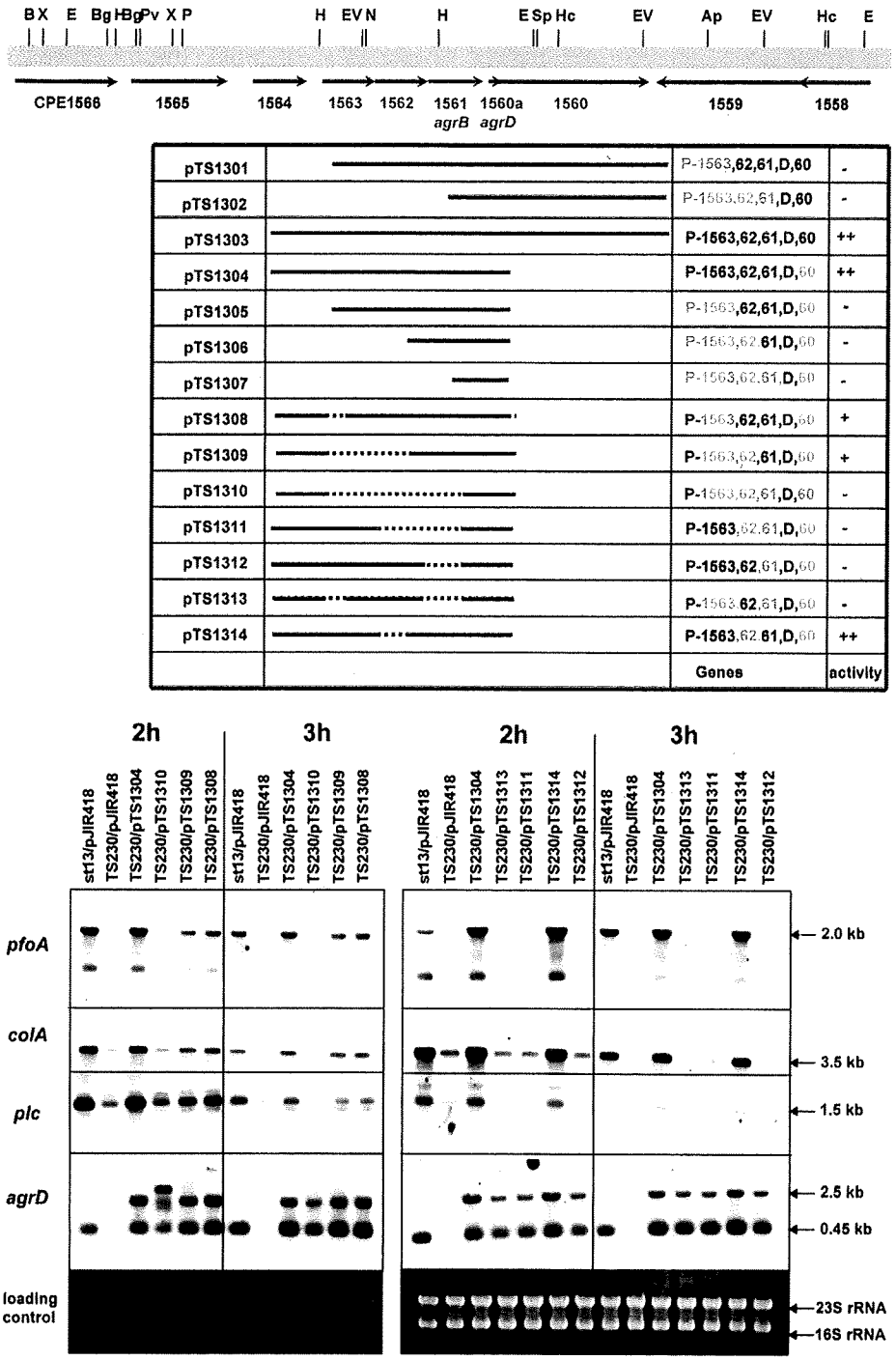

FIG. 3. Deletion analysis of the *agr* region. To determine the role of each gene in the operon, deletion plasmids were constructed and transformed into the $agrBD_{Cp}$-null mutant, TS230. Each strain was cultured, and RNA was isolated after 2 and 3 h of culture. The RNA was used for Northern analysis of the indicated toxin genes. In the deletion table, "−" indicates no activity, "++" indicates the plasmid has activity to induce the expression of toxin genes, and "+" indicates the plasmids have activity but that the activity is lower than that of pTS1304. The internal regions of *pfoA*, *colA*, *plc*, and *agrD* were used as probes.

too faint to confirm its regulation, but the mRNA level was almost the same in all three strains. These results suggested that the *agr* regulatory system involving the $agrBD_{Cp}$ and *virR/virS* operons in *C. perfringens* is not completely analogous to the *agr* regulation system in *S. aureus*.

Effect of a stationary culture supernatant on *pfoA* transcription. To further analyze the mechanism by which the extracellular signal in the culture supernatant of *C. perfringens* regulates toxin gene expression, the effect of addition of the *C. perfringens* culture supernatant on *pfoA* expression was exam-

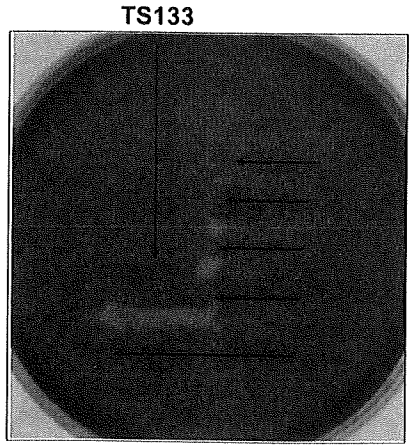

FIG. 4. Cross-streaking of TS230 and TS133. The *virR* mutant strain, TS133, was streaked onto a blood agar plate, and then several streaks of TS230 were made at a right angle to TS133. The distance between the two strains was decreased with each successive streak.

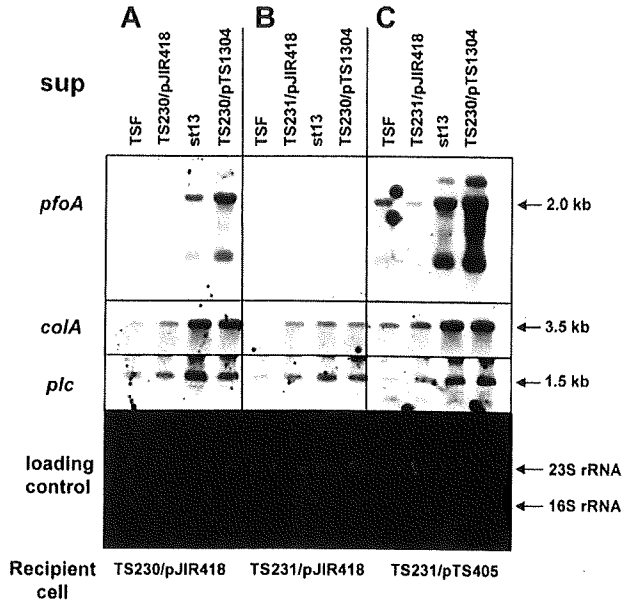

FIG. 5. Effect of the wild-type supernatant on the expression of toxin genes in TS230 and TS231. The culture supernatant was collected from strain 13 and added to the indicated strains to check the effect of the supernatant on sensor protein activity. The supernatants were collected from the wild-type strain, strain 13/pJIR418, and strain TS230/pTS1304 after culture for 1.5 h. Total RNA was prepared 15 min after addition of the supernatant. (A) The supernatant (sup) was added onto the *agr*-null mutant, TS230. (B) The supernatant (sup) was added onto the *agr*-null *virR* mutant, TS231/pJIR418. (C) The supernatant (sup) was added onto TS231 that contains an intact *virR/virS*, TS231/pTS405.

ined in more detail by Northern analysis. Although the expression of *plc* and *colA* was also partially regulated by the extracellular signal molecule in the supernatant, we focused on the regulation of *pfoA* in this analysis, since *pfoA* appears to be the main target of this system. First, the supernatant was removed from wild-type strain 13 cells that were cultured to various growth stages (Fig. 7A, 2 to 8 h). These cells were used as recipient cells and were resuspended in fresh TSF medium. As a control, cells were resuspended in the supernatant that had been removed. After 15 min of incubation in the added medium or supernatant, total RNA was prepared from the recipient cells. In the control experiment (see the "sup" lane in Fig. 7A), maximum transcription of *pfoA* was observed when the supernatant from a 2-h cell culture was added. However, *pfoA* transcription in the recipient cells was clearly observed within 15 min after the supernatant was replaced with fresh TSF medium (Fig. 7A, lane TSF). Surprisingly, the transcription of *pfoA* occurred even in the 8-h-cultured recipient cells after replacement of the supernatant with fresh medium (Fig. 7A). Furthermore, the transcription of *pfoA* in the 3- to 8-h-cultured recipient cells (lane TSF; 3 to 8 h of culture) was at a much higher level than that observed in the recipient cells cultured for 2 h in the presence of a 2-h culture supernatant (lane 2h sup). These data suggest that there is another signaling molecule in the supernatant that negatively controls *pfoA* expression, especially at the stationary phase, because removal of the culture supernatant and re-addition of fresh medium leads to activation of *pfoA* transcription in the 3-h (mid log)- to 8-h (stationary)-cultured recipient cells. Furthermore, these data presumably suggest that the amount of signal molecule that binds to recipient cells is sufficient to activate *pfoA* transcription. Moreover, through the removal of the stationary-phase supernatant, the concentration of the inhibitory substance might decrease, and the remaining activator bound to cells could stimulate *pfoA* transcription.

To confirm this hypothesis, the supernatant from the stationary phase was diluted with TSF medium and added to TS230 recipient cells. As predicted, diluted supernatant from the stationary phase could activate *pfoA* transcription, with a maximum activation observed at a fourfold dilution (Fig. 7B). These data suggest that there may be an inhibitory molecule in the supernatant from the stationary phase that represses *pfoA* expression but that this inhibition is abrogated when the hypothetical inhibitor is diluted. The proportions of activator concentration and inhibitory molecule might be important for determining the transcriptional level of the *pfoA* gene. Thus, in *C. perfringens*, a gradual accumulation of the inhibitor might occur over the culture period and, when the concentration of the inhibitor reaches a certain threshold, it may completely stop the transcription of *pfoA*. This mechanism could explain the decrease in toxin production at the stationary phase of growth in *C. perfringens*.

In the present study, we examined novel regulatory genes ($agrBD_{Cp}$) for toxin production in *C. perfringens*. These genes are highly similar to the *agr* system in *S. aureus*, and we have shown that the $agrBD_{Cp}$ locus is responsible for the production of an extracellular signal molecule that stimulates the expression of toxin genes in *C. perfringens*. We also found that the two-component VirR/VirS system appears to be required for the regulation by the signaling molecule produced by $agrBD_{Cp}$.

In *C. perfringens* the functions of $agrBD_{Cp}$, the VirR/VirS system, and VR-RNA seem to be quite similar to those of *S. aureus* $agrBD_{Sa}$, AgrA/AgrC, and RNAIII, respectively. Consequently, the two bacteria might have evolved similar regulatory systems to control their pathogenicity toward humans.

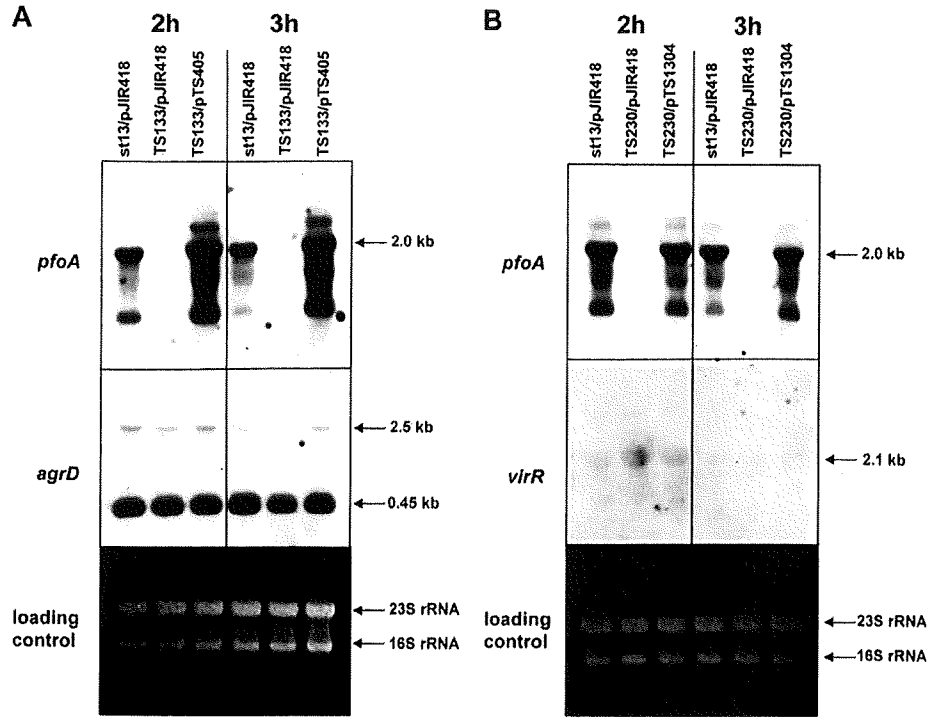

FIG. 6. Regulatory relationship between $agrBD_{Cp}$ and *virR/virS*. (A) Regulation of $agrBD_{Cp}$ by *virR/virS*. Total RNA was isolated from 2- and 3-h-cultured strain 13/pJIR418, TS133/pJIR418, and TS133/pTS405. (B) Regulation of *virR/virS* by $agrBD_{Cp}$. Total RNA was isolated from strain 13/pJIR418, TS230/pJIR418, and TS230/pTS1304. A 10-μg portion of total RNA was used for Northern analysis.

However, the genes involved in the regulation of toxin genes are scattered around the genome of *C. perfringens*, whereas the genes involved in the *agr* system are located in a cluster on the *S. aureus* chromosome (17).

It is noteworthy that toxin gene expression in *C. perfringens* reaches a maximum during the log phase of growth and completely stops at the stationary phase, whereas in many other pathogenic bacteria, toxin gene expression commonly starts at the stationary phase. Induction of toxin gene expression at the stationary phase is mainly mediated by a quorum-sensing mechanism. In contrast, the $agrBD_{Cp}$ system of *C. perfringens* induces the expression of toxin genes in the early stages of cell growth. For this expression pattern, there may be other unique systems that ensure the specific expression of toxin genes at the early stages of cell growth. From the data in the present study, we predict that there might exist in *C. perfringens* a system whereby inhibitory molecules are secreted into the medium. However, these molecules would stop toxin gene expression only upon reaching a critical level at the stationary phase. The balance between the $agrBD_{Cp}$ activator system and a second,

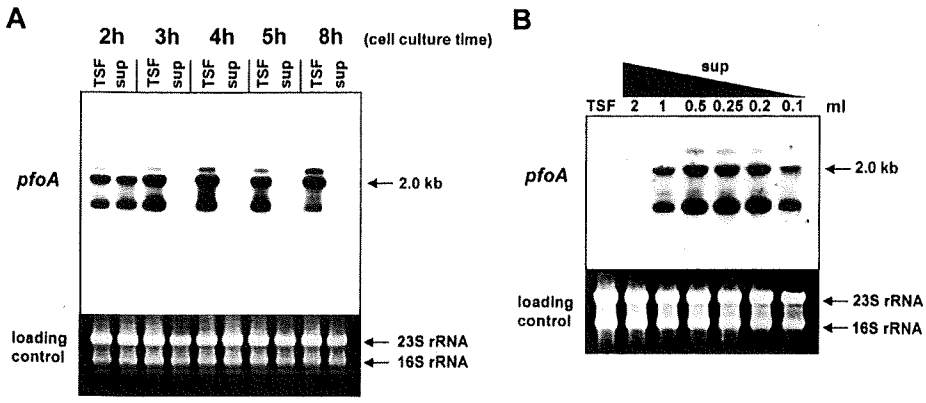

FIG. 7. Effect of the supernatant on toxin gene expression. (A) The supernatant was removed from the various time points of the culture. The cells from each time point were incubated with TSF at 37°C. As a control, the removed supernatant was added again to the same cells. RNA was isolated after 15 min of incubation. Lanes: TSF, TSF control; sup, culture supernatant. (B) The supernatant from strain 13 after 6 h of culture was diluted with TSF medium and added to TS230 cells. RNA was isolated after a 15-min incubation.

as-yet-undefined inhibitory system may be important for the proper control of gene expression in *C. perfringens*.

The unique regulation of toxin expression in *C. perfringens* is consistent with the requirement of *C. perfringens* to secrete various tissue-degrading toxins and enzymes at an early stage of infection. These secreted products enable the organism to acquire essential nutrients from the host (resulting in gas gangrene) that are required for the survival and growth of the bacteria. Genomic analysis has shown that *C. perfringens* lacks many genes related to amino acid biosynthesis, with the exception of genes for the three amino acids cysteine, serine, and glycine. Thus, in order to survive, especially in a host environment, *C. perfringens* may require a well-coordinated system to secrete numerous toxins and enzymes for the degradation of host cells and for the effective import of nutrients from the environment. Therefore, it is very important to precisely elucidate how these extracellular regulatory systems control the virulence of *C. perfringens*. Elucidation of these regulatory systems may lead to an understanding of the relationship between *C. perfringens* and other bacteria that coexist in the intestine or in wounds and, furthermore, to the identification of new therapeutic targets for the treatment of life-threatening diseases caused by *C. perfringens*.

ACKNOWLEDGMENTS

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REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905–11910.
2. **Bassler, B. L.** 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr. Opin. Microbiol. **2:**582–587.
3. **Ba-Thein, W., M. Lyristis, K. Ohtani, I. T. Nisbet, H. Hayashi, J. I. Rood, and T. Shimizu.** 1996. The *virR/virS* locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. J. Bacteriol. **178:**2514–2520.
4. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
5. **George, E. A., and T. W. Muir.** 2007. Molecular mechanisms of *agr* quorum sensing in virulent *staphylococci*. Chembiochem. **8:**847–855.
6. **Gobbetti, M., M. De Angelis, R. Di Cagno, F. Minervini, and A. Limitone.** 2007. Cell-cell communication in food related bacteria. Int. J. Food Microbiol. **120:**34–45.
7. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66–98.
8. **Higashi, Y., M. Chazono, K. Inoue, Y. Yanagase, T. Amano, and K. Shimada.** 1973. Complementation of theta toxinogenecity between mutants of two groups of *Clostridium perfringens*. Biken J. **16:**1–9.
9. **Imagawa, T., and Y. Higashi.** 1992. An activity which restores theta toxin activity in some theta toxin-deficient mutants of *Clostridium perfringens*. Microbiol. Immunol. **36:**523–527.
10. **Imagawa, T., T. Tatsuki, Y. Higashi, and T. Amano.** 1981. Complementation characteristics of newly isolated mutants from two groups of strains of *Clostridium perfringens*. Biken J. **24:**13–21.
11. **Kobayashi, T., T. Shimizu, and H. Hayashi.** 1995. Transcriptional analysis of the β-galactosidase gene (*pbg*) in *Clostridium perfringens*. FEMS Microbiol. Lett. **133:**65–69.
12. **Lyon, G. J., and R. P. Novick.** 2004. Peptide signaling in *Staphylococcus aureus* and other gram-positive bacteria. Peptides **25:**1389–1403.
13. **Mahony, D. E., and T. J. Moore.** 1976. Stable L-forms of *Clostridium perfringens* and their growth on glass surfaces. Can. J. Microbiol. **22:**953–959.
14. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165–199.
15. **Muir, T. W.** 2003. Turning virulence on and off in *staphylococci*. J. Pept. Sci. **9:**612–619.
16. **Nakayama, J., A. D. Akkermans, and W. M. De Vos.** 2003. High-throughput PCR screening of genes for three-component regulatory system putatively involved in quorum sensing from low-G+C gram-positive bacteria. Biosci. Biotechnol. Biochem. **67:**480–489.
17. **Novick, R. P., and T. W. Muir.** 1999. Virulence gene regulation by peptides in staphylococci and other gram-positive bacteria. Curr. Opin. Microbiol. **2:**40–45.
18. **Ohtani, K., H. Hayashi, and T. Shimizu.** 2002. The *luxS* gene is involved in cell-cell signaling for toxin production in *Clostridium perfringens*. Mol. Microbiol. **44:**171–179.
19. **Okumura, K., K. Ohtani, H. Hayashi, and T. Shimizu.** 2008. Characterization of genes regulated directly by the VirR/VirS system in *Clostridium perfringens*. J. Bacteriol. **190:**7719–7727.
20. **Rieu, A., S. Weidmann, D. Garmyn, P. Piveteau, and J. Guzzo.** 2007. Agr system of *Listeria monocytogenes* EGD-e: role in adherence and differential expression pattern. Appl. Environ. Microbiol. **73:**6125–6133.
21. **Rood, J. I.** 1998. Virulence genes of *Clostridium perfringens*. Annu. Rev. Microbiol. **52:**333–360.
22. **Sambrook, J., E. F. Fritch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. **Shimizu, T., W. Ba-Thein, M. Tamaki, and H. Hayashi.** 1994. The *virR* gene, a member of a class of two-component response regulators, regulates the production of the perfringolysin O, collagenase, and hemagglutinin in *Clostridium perfringens*. J. Bacteriol. **176:**1616–1623.
24. **Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. USA **99:**996–1001.
25. **Shimizu, T., A. Okabe, and J. I. Rood.** 1997. Regulation of toxin production in *Clostridium perfringens*, p. 451–470. *In* J. I. Rood, G. Songer, B. A. McClane, and R. W. Titball (ed.), The clostridia: molecular biology and pathogenesis. Academic Press, London, England.
26. **Shimizu, T., H. Yaguchi, K. Ohtani, S. Banu, and H. Hayashi.** 2002. Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. Mol. Microbiol. **43:**257–265.
27. **Sloan, J., T. A. Warner, P. T. Scott, T. L. Bannam, D. I. Berryman, and J. I. Rood.** 1992. Construction of a sequenced *Clostridium perfringens-Escherichia coli* shuttle plasmid. Plasmid **27:**207–219.
28. **Waters, C. M., and B. L. Bassler.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. **21:**319–346.
29. **Wuster, A., and M. M. Babu.** 2008. Conservation and evolutionary dynamics of the *agr* cell-to-cell communication system across firmicutes. J. Bacteriol. **190:**743–746.
30. **Xavier, K. B., and B. L. Bassler.** 2003. LuxS quorum sensing: more than just a numbers game. Curr. Opin. Microbiol. **6:**191–197.

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

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

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

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

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

Summary

***Clostridium perfringens* type C isolates cause necrotizing enteritis in humans and domestic animals. *In vitro*, type C isolates often produce β toxin (CPB), β2 toxin (CPB2), α toxin (CPA), perfringolysin O (PFO) and TpeL during (or after) late log-phase growth. In contrast, the current study found that many type C isolates respond to close contact with enterocyte-like Caco-2 cells by producing all toxins, except TpeL, much more rapidly than occurs during *in vitro* growth. This *in vivo* effect involves rapid transcriptional upregulation of the *cpb*, *cpb2*, *pfo*A and *plc* toxin genes. Rapid Caco-2 cell-induced upregulation of CPB and PFO production involves the VirS/VirR two-component system, since upregulated *in vivo* transcription of the *pfo*A and *cpb* genes was blocked by inactivating the *vir*R gene and was reversible by complementation to restore VirR expression. However, the *lux*S quorum-sensing system is not required for the rapid upregulation of type C toxin production induced by contact with Caco-2 cells. These results provide the first indication of host cell: pathogen cross-talk affecting toxin production kinetics by any pathogenic *Clostridium* spp., identify *in vivo* versus *in vitro* differences in *C. perfringens* toxin expression, and implicate VirS/VirR as a possible contributor to some *C. perfringens* enteric diseases.**

Received 13 January, 2009; revised 24 April, 2009; accepted 28 April, 2009. *For correspondence. E-mail bamcc@pitt.edu; Tel. (+1) 412 6489022; Fax (+1) 412 6241401.

Introduction

The anaerobic, spore-forming bacterium *Clostridium perfringens* is one of the most important Gram-positive pathogens of humans and animals (McClane *et al.*, 2006). *C. perfringens* diseases include numerous gastrointestinal syndromes and enterotoxaemias, as well as gas gangrene and other histotoxic infections. The virulence of this bacterium is directly related to its production of potent toxins. Differential production of four lethal typing toxins [α, β, ε and/or ι] is used to classify *C. perfringens* isolates into five pathogenic types (A–E). Each *C. perfringens* type is associated with certain human or animal diseases (Smedley *et al.*, 2004; McClane *et al.*, 2006).

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

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

In humans, *C. perfringens* type C isolates cause enteritis necroticans, an often rapidly fatal disease that involves vomiting, diarrhoea, severe abdominal pain and the presence of blood in the stools (McClane *et al.*, 2004). This

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

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

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

Regulation of toxin production by *C. perfringens* vegetative cells has only been studied for gangrene-producing type A isolates growing *in vitro*. A two-component regulatory system named VirS/VirR, comprised of (respectively) a membrane sensor and transcriptional regulator (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994), is encoded by the *vir*S and *vir*R genes that form an operon transcribed as a single 2.1 kb mRNA molecule (Ba-Thein *et al.*, 1996). The VirS/VirR two-component regulatory system helps to govern *in vitro* transcription of the chromosomal *plc*, *pfo*A and *col*A toxin genes (encoding CPA, PFO and collagenase respectively) and the plasmid-borne *cpb2* gene encoding CPB2 toxin (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994; Ba-Thein *et al.*, 1996; Ohtani *et al.*, 2003). In gangrene-producing type A strain 13, PLC and PFO toxin regulation also involves the *lux*S quorum-sensing mechanism (Ohtani *et al.*, 2002).

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

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

Results

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

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

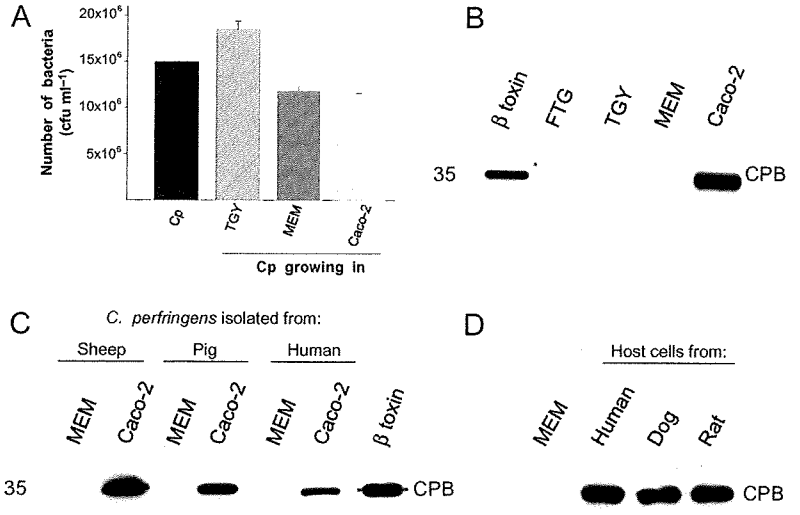


Fig. 1. CPB secretion is upregulated during infection of mammalian Caco-2 cells.
A. Cell culture dishes containing Caco-2 cell cultures, TGY or MEM were each inoculated with 1.5×10^7 cfu of *C. perfringens* (Cp) type C isolates JGS1495 and incubated for 3 h at 37°C (moi = 20). The culture supernatants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

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

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

The presence of Caco-2 cells and other mammalian cells causes rapid upregulated secretion of CPB

Since the Fig. S1 results indicated that some of the increased protein secretion stimulated by type C infection of Caco-2 cells included proteins matching the size of

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

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

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

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

In addition to CPB, *C. perfringens* type C isolates commonly secrete CPB2, CPA and PFO into the medium during late log-phase growth in TGY broth (Petit *et al.*, 1999; Smedley *et al.*, 2004; Fisher *et al.*, 2006). CPA is a lethal toxin with phospholipase C and sphingomyelinase activities, while PFO is a lethal, cholesterol-dependent cytolysin (Tweten, 1988a; Titball *et al.*, 1999); CPB2 action is unknown.

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

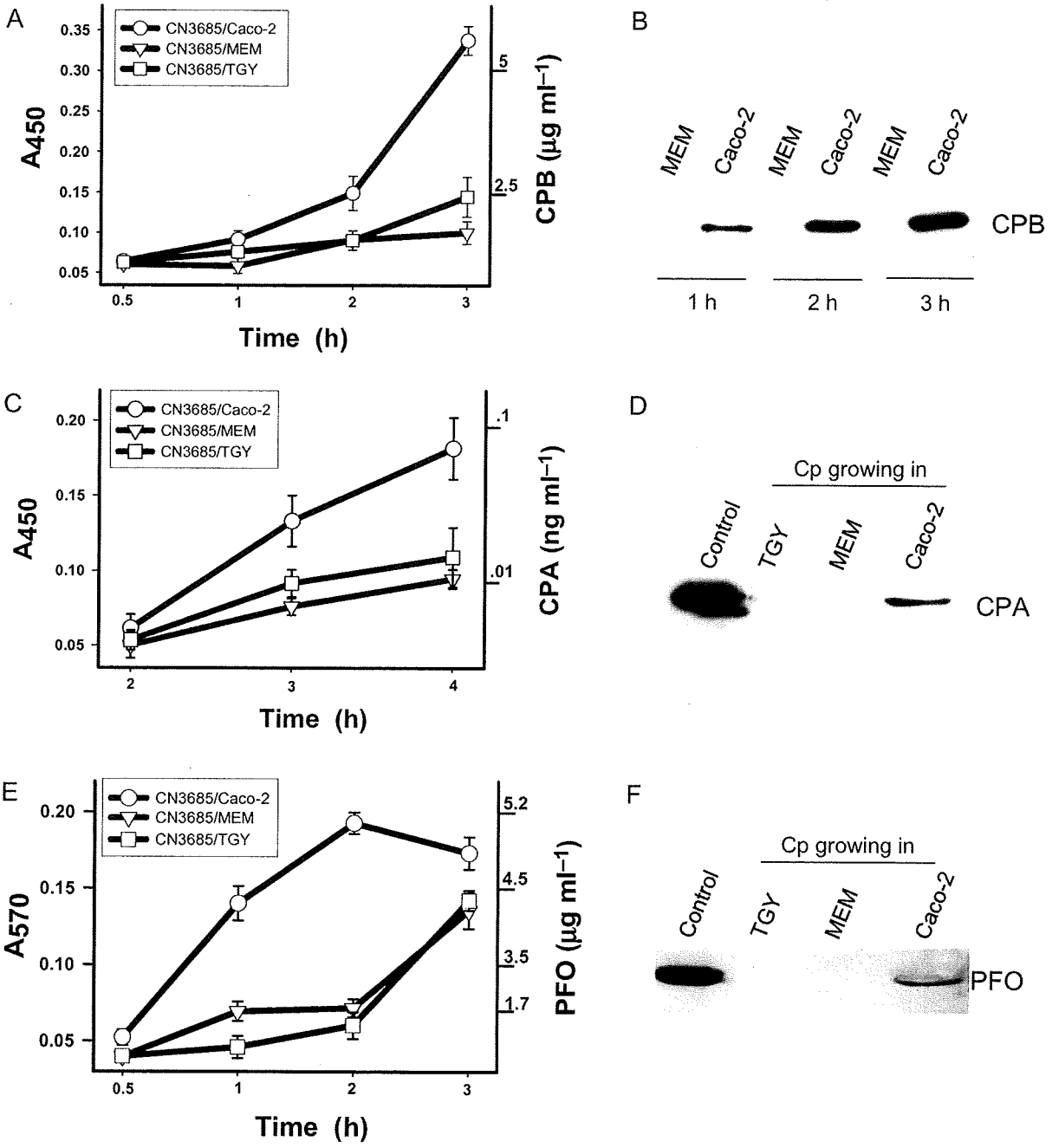
Similarly, Western blot analyses demonstrated that, compared with MEM or TGY cultures, the presence of Caco-2 cells induces a more rapid secretion of PFO by type C isolates (Fig. 2F). The ability of *C. perfringens* supernatants to lyse horse erythrocytes and release haemoglobin (Hb) is specifically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

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

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

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

Using 20, 50 or 100 ng levels of RNA extracted from Caco-2 cell cultures that had been infected for only 2 h with type C strain JGS1495, RT-PCR studies detected transcripts encoding CPB, CPB2, PFO and CPA (Fig. 4B,



C, D and E respectively). In contrast, no mRNA encoding any of those toxins could be detected by RT-PCR using the same levels of RNA extracted from JGS1495 grown for 2 h in either TGY or MEM (no Caco-2 cells). The type C isolate CN3685 also quickly upregulated transcription of the *cpb* gene in the presence of Caco-2 cells (Fig. S2A).

The Fig. 4 results indicated that the rapid upregulated secretion of toxins involves stimulation of toxin gene transcription soon after type C isolates encounter Caco-2 cells. To explore whether this stimulation of toxin gene expression simply reflects a general host cell-mediated increase in transcription of all *C. perfringens* genes, primers were designed to amplify the alpha subunit of the DNA polymerase III gene (*polC*), which is a *C. perfringens* type A housekeeping gene (Myers *et al.*, 2006). As shown in Fig. 4A, the *polC* gene could be PCR-amplified using

Fig. 2. Levels of CPB, CPA and PFO toxins secreted in the presence of Caco-2 cells.
A and C. ELISA analyses. *Clostridium perfringens* type C strain CN3685 was inoculated into Caco-2 cells (CN3685/Caco-2), MEM (CN3685/MEM) or TGY (CN3685/TGY) (moi = 20) and incubated for the indicated time at 37°C. Supernatants were removed and sterilized by filtration. Sterile supernatants and purified CPB (A) or purified CPA (C) were coated in separate wells of an ELISA microplate overnight at 4°C. The wells were incubated with a mouse monoclonal anti-CPB (A) or anti-CPA antibody (C) followed by a HRP-conjugated anti-mouse antibody. The bound antibody was detected with a TMB substrate solution and the colour reaction stopped with sulfuric acid (0.18 M). Absorbance at 570 nm (A_{450}) was determined using an ELISA reader.
Western blot analyses (right panels).
B. CPB secretion. Caco-2 cells or MEM cultures were infected with CN3685 for 1, 2 or 3 h and incubated at 37°C. Equal amounts of sterile supernatants were analysed by Western blot using a mouse monoclonal anti-CPB antibody.
D and F. Secretion of 47 kDa CPA (D) and 54 kDa PFO (F) by Caco-2 cells grown in 100 mm culture dishes prior to infection for 3 h at 37°C with *C. perfringens* type C isolate JGS1495 (moi = 20). For comparison, culture dishes containing TGY or MEM (no cells) were similarly inoculated and then incubated under the same conditions. For (D) and (F), supernatants were removed, filter-sterilized and concentrated 200-fold (see *Experimental procedures*). Control sample is an infected 8 h TGY culture that was similarly concentrated. The same amounts of each concentrated supernatant were subjected to SDS-PAGE on a 12% acrylamide gel and then analysed by Western blotting using a mouse monoclonal anti-CPA (D) antibody or a rabbit-raised polyclonal anti-PFO antibody (F) as described.
E. Haemoglobin (Hb) release assay for PFO activity. Supernatants obtained as above or purified PFO were incubated (1:1) with a 1% suspension of horse red blood cells for 30 min at 37°C. PFO-induced Hb release was detected by obtaining the absorbance at 570 nm (A_{570}).

template DNA extracted from either *C. perfringens* type C strain JGS1495 (Fig. 4A, line 1) or CN3685 (result not shown). Moreover, RT-PCR detected mRNA from the *polC* gene in *C. perfringens* cultures growing for 2 h in either TGY, MEM or in the presence of Caco-2 cells (Fig. 4A).

Early transcription of the tpeL toxin gene is not stimulated by the presence of Caco-2 cells

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

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

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

The results presented above indicate that rapid host cell-stimulated secretion of most toxins made by *C. perfringens* type C isolates is triggered by an unknown host factor present during infection. Since some host proteins

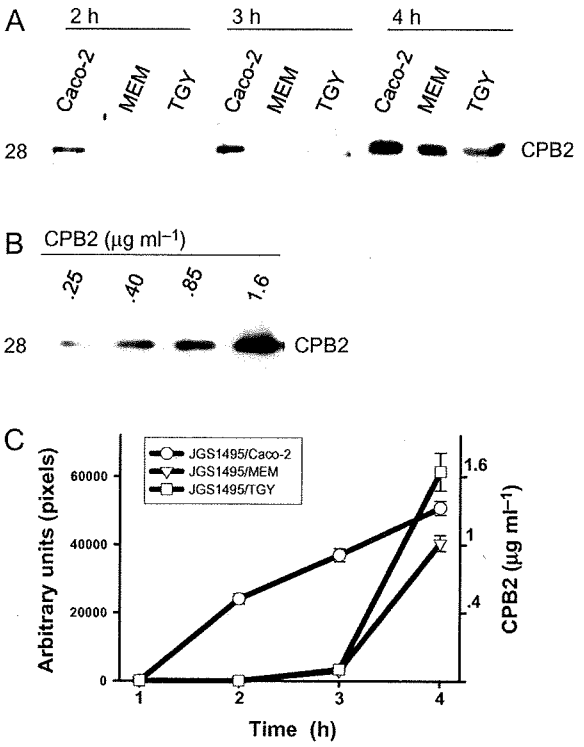


Fig. 3. Levels of CPB2 secreted in the presence of enterocyte-like Caco-2 cells. *Clostridium perfringens* type C strain JGS1495 was inoculated into Caco-2 cell cultures (JGS1495/Caco-2), MEM (JGS1495/MEM) or TGY (JGS1495/TGY) (moi = 20) and incubated for the indicated time at 37°C. Supernatants were removed and sterilized.
A and B. (A) Equal amounts (25 μl) of each supernatant or different amount of purified CPB2 (B) were subjected to SDS-PAGE on a 12% acrylamide gel and proteins were then transferred to nitrocellulose membrane. Membranes were probed by Western blot using a polyclonal anti-CPB2 antibody. CPB2 molecular weight is shown on the left of each figure in kDa.
C. Quantification of CPB2 secretion. Experiments described above were repeated three times. Western blots were then scanned and their pixel intensity [(A) *x*-axis or (B) *y*-axis right] was quantified and graphically integrated against a standard curve of purified CPB2 to determine CPB2 levels.

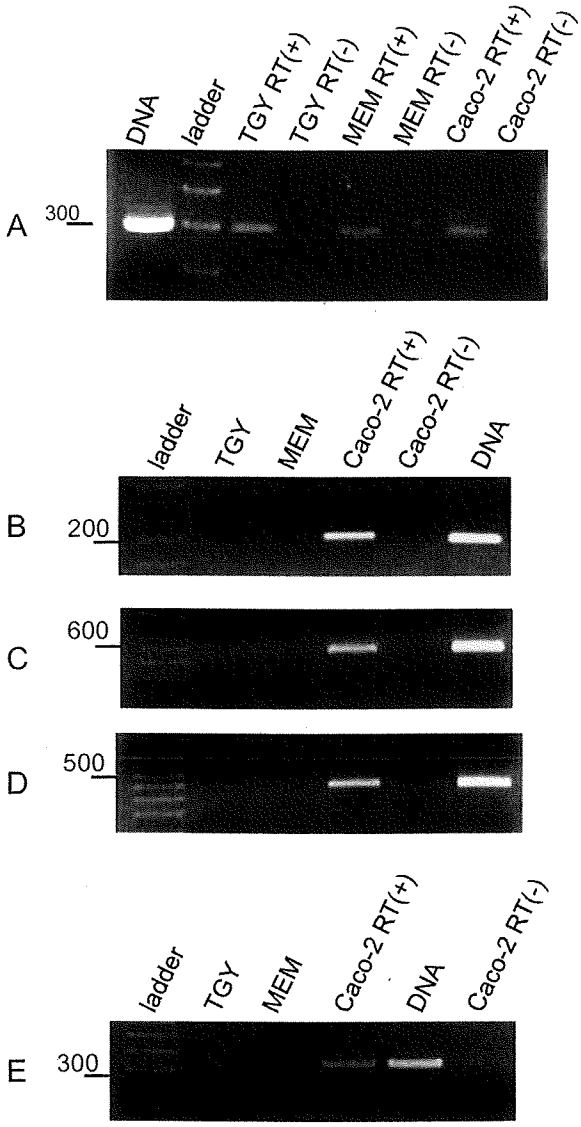


Fig. 4. The transcription of *cpb*, *cbp2*, *plc* and *pfo*A toxin genes is quickly upregulated during Caco-2 infection. Type C strain JGS1495 was inoculated into TGY, MEM or Caco-2 cell cultures (moi = 20) and then incubated for 2 h at 37°C. Bacteria were then collected and pelleted by centrifugation. Total RNA was extracted and treated with DNase I. RT-PCR reactions were performed with 20–100 ng of RNA (results shown are for 100 ng) and using a specific pair of primers to amplify (A) the housekeeping *pol*C gene, (B) *cpb*, (C) *cpb2*, (D) *pfo*A or (E) *plc* toxin genes. Where indicated, reverse transcriptase (RT) was (+) or was not (–) added into reaction tubes as a control to confirm RT-PCR signals were from RNA rather than DNA. DNA from JGS1495 strain was also included, as a control reaction. Molecular markers shown are a 100 bp increment ladder with selected marker size, in bp, shown on the left of each gel. For all panels, products were electrophoresed on a 2% agarose gel and then stained with ethidium bromide for visualization. Shown are representative figures of three independent experiments.

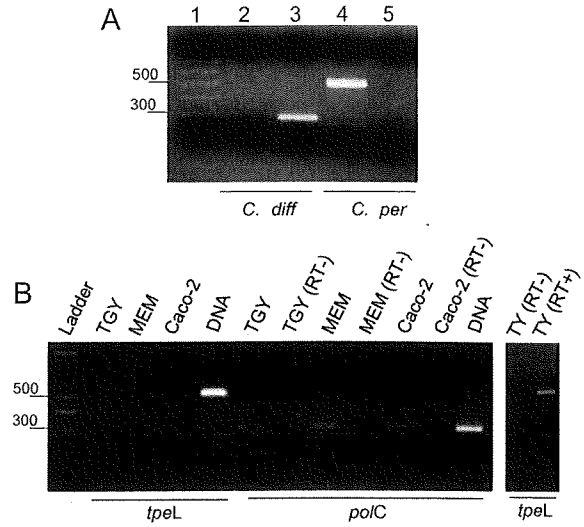


Fig. 5. Transcription of the *C. perfringens tpe*L toxin gene is not upregulated early during infection of Caco-2 cell cultures.
A. PCR was performed using primers to specifically amplify either the *C. perfringens tpe*L gene (lanes 2 and 4) or a 3′ fragment of the *C. difficile tcd*A gene (lanes 3 and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

(mostly with molecular masses > 60 kDa) are present in the supernatant of non-infected Caco-2 cells (Fig. S1A and C, control lanes), we next evaluated whether supernatants of non-infected Caco-2 cell cultures, referred to here as conditioned medium (CM), are sufficient to stimulate the secretion of *C. perfringens* type C toxins.

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

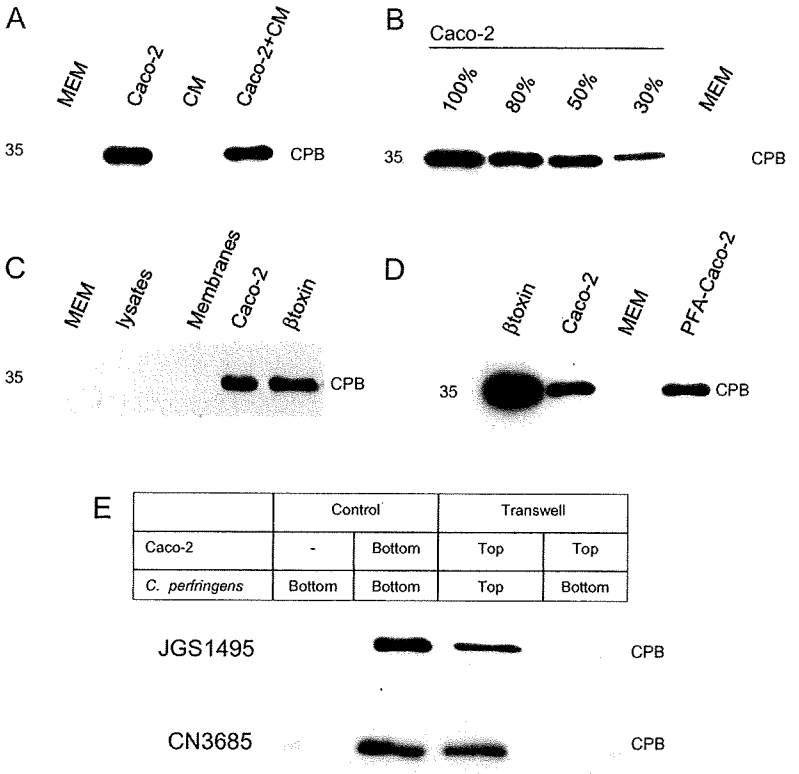


Fig. 6. Upregulation of CPB secretion requires close contact between *C. perfringens* and host cells.
A. Conditioned medium (CM) does not stimulate CPB secretion. *C. perfringens* type C strain JGS1495 was inoculated into a well of a 24-well tissue culture microplate containing: 1 ml of CM alone, 1 ml of MEM alone, Caco-2 cell cultures treated with 1 ml of CM, or Caco-2 cells in 1 ml of fresh (non-conditioned) MEM. After 3 h of incubation at 37°C, supernatants were analysed by Western blot for CPB production using a mouse monoclonal anti-CPB antibody (expected migration of a 35 kDa protein indicated on the left of each blot in (A)–(D).
B. CPB secretion increases in proportion to the number of host cells in a culture. *C. perfringens* type C strain JGS1495 (1.5×10^7 cfu) was inoculated into Caco-2 cell cultures which were at the following confluency: 30%, 50%, 80% or 100% and then incubated for 3 h at 37°C. A mock infection without cells (MEM) was also included. Equal amounts of each sterilized supernatant were electrophoresed, transferred to nitrocellulose membranes and analysed as described above.
C. Living cells, but not Caco-2 cell membranes or cell lysates, stimulated CPB secretion. JGS1495 was inoculated (2.5×10^8 cfu) into 100 mm tissue culture dishes containing 12 ml of MEM without additives, MEM without additives but with Caco-2 cell lysates, MEM without additives but with the Caco-2 cell membrane fraction or MEM plus confluent living Caco-2 cells. After 3 h of infection at 37°C, supernatants were analysed by Western blot for CPB levels as described.
D. Fixed Caco-2 cells stimulate CPB secretion. JGS1495 was inoculated into MEM (no cells), Caco-2 cell cultures or plates with paraformaldehyde (PFA)-fixed Caco-2 cells and incubated for 3 h at 37°C. Supernatants were filter-sterilized and then analysed by Western blot for CPB levels. Purified CPB (0.5 μg) was included (β toxin).
E. *C. perfringens* type C isolates JGS1495 or CN3685 was inoculated (3×10^7 cfu) into (i) wells of a 12-well culture microplate containing MEM [no cells (–)] or a Caco-2 cell culture or (ii) the Transwell filter chamber (Top) containing a confluent culture of Caco-2 cells or (iii) the well (Bottom) in which a Transwell filter chamber containing Caco-2 cells was installed (i.e. bacteria and cells are present in the same well but physically separated). The infected cultures were incubated for 3 h at 37°C and the supernatants were collected and filter-sterilized. The sterile supernatants were analysed by Western blot as above. Shown are representative results from at least four independent experiments.

shown). These results could suggest that a soluble factor secreted by Caco-2 cells is not responsible for stimulating rapid toxin secretion by *C. perfringens* type C isolates.

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

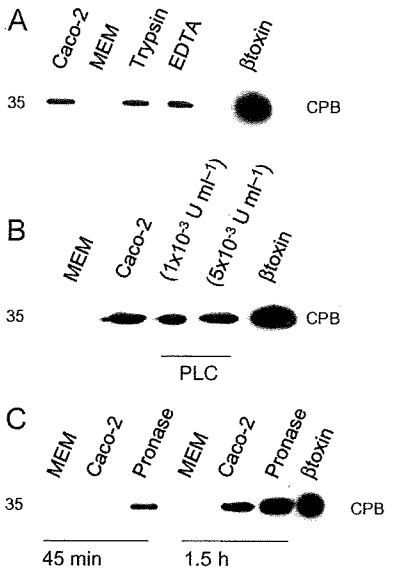


Fig. 7. Treatment of Caco-2 cell cultures with pronase, but not trypsin or PLC, enhances CPB secretion.
A. Caco-2 cells were treated with trypsin or EDTA for 15 min at 37°C. Detached cells were washed and re-suspended in MEM to a final cell density of 7×10^5 cells ml^{-1}. Caco-2 cell cultures, MEM, trypsin-detached or EDTA-detached Caco-2 cells were then infected, in a 24-well microplate, with CN3685 for 1.5 h at 37°C. The supernatants were analysed for CPB levels by Western blot.
B. Caco-2 cells were treated with 1×10^{-3} or 5×10^{-3} U ml^{-1} of phospholipase C (PLC) in the form of CPA for 1 h at 37°C. Cells were then washed and added with MEM. Untreated Caco-2 cell cultures, MEM or PLC-treated Caco-2 cells were infected with CN3685 for 1.5 h at 37°C. The supernatants were analysed for CPB levels by Western blot.
C. Caco-2 cells were treated with pronase (100 μg ml^{-1}) for 20 min at 37°C. Detached cells were thoroughly washed and re-suspended in MEM to a final cell density of 7×10^5 cells ml^{-1}. MEM, Caco-2 cell cultures or pronase-treated Caco-2 cells were then infected, in a 24-well microplate, with CN3685 for 45 min or 1.5 h at 37°C. The supernatants were analysed for CPB levels by Western blot. Expected migration of a 35 kDa protein indicated on the left of each blot.

pre-treatment of Caco-2 cells with Pronase enhanced the rapid secretion of CPB (Fig. 7C).

To further explore whether Caco-2 cell-associated surface components are sufficient for stimulating toxin secretion, *C. perfringens* type C strain JGS1495 was incubated for 3 h with MEM containing either the insoluble membrane fraction of Caco-2 cells or Caco-2 cell lysates. Neither those isolated Caco-2 cell membranes nor Caco-2 cell lysates stimulated CPB secretion like intact, living Caco-2 cells (Fig. 6C), which could suggest that intact host cells, if not viable host cells, are needed to trigger rapid CPB secretion by type C isolates.

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

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

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

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

We next investigated whether the rapid host cell-induced stimulation of toxin production and secretion by *C. perfringens* type C isolates have cytotoxic consequences for Caco-2 cells. To test this, Caco-2 cells were infected with *C. perfringens* type C strain CN3685 for 1 h. As a control, bacteria were similarly grown for 1 h in MEM (no Caco-2 cells). Both supernatants containing secreted proteins were collected, filter-sterilized and added to fresh Caco-2 cell cultures for 2 h. As shown in Fig. 8B, the supernatant from bacteria that had been incubated for 1 h in MEM (without Caco-2 cells) did not damage Caco-2 cells

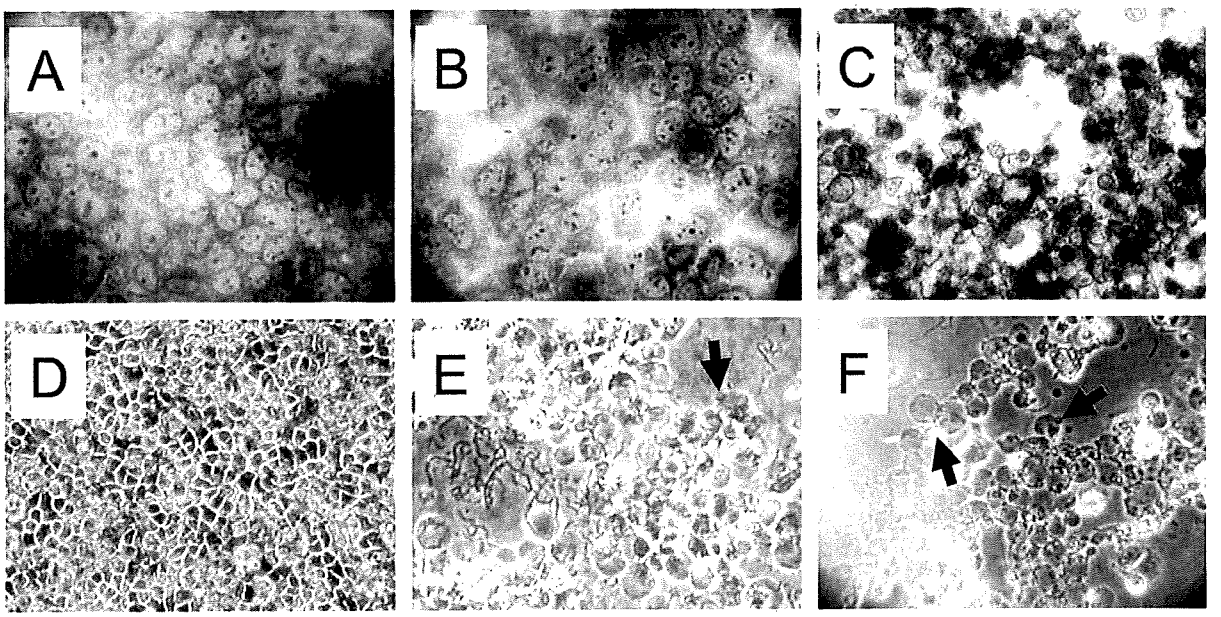


Fig. 8. *C. perfringens* type C strains are cytotoxic for Caco-2 cells.
A–C. Cytotoxic consequences of host cell-induced stimulation of toxin secretion. *C. perfringens* type C strain CN3685 was inoculated into tissue culture wells containing MEM (no cells) or Caco-2 cells for 1 h. The culture supernatant was removed and filter-sterilized. The sterilized supernatant from inoculated MEM alone (no Caco-2 cells) (B) or infected Caco-2 cell cultures (C) was individually added to fresh confluent Caco-2 cells and incubated for 5 h at 37°C. Then, treated or untreated Caco-2 cells (A) were washed three times with pre-warmed PBS, fixed with 70% methanol and stained with Giemsa stain. Slides were analysed at a magnification of 40× with standard bright-field light microscopy.
D–F. Cytotoxicity induced by *C. perfringens* type C infection. Phase-contrast microscopy of confluent Caco-2 cell cultures left uninfected (D) or infected with CN3685 (E) or JGS1495 (F) (moi = 20) for 5 h at 37°C. Arrows show membrane blebs.

within 5 h. However, by that same time, the supernatant collected from infected Caco-2 cell cultures had induced morphological damage, which was characterized by cell rounding, cellular contraction and membrane blebbing (Fig. 8C). After 6 h of incubation, this treated Caco-2 cell monolayer had detached from the glass substratum.

To better characterize this cytotoxicity phenotype, a Caco-2 cell monolayer was infected with either *C. perfringens* type C strain CN3685 or JGS1495 at a multiplicity of infection (moi) = 20 and cell morphology changes were then followed (with cytotoxicity scored as described in *Experimental procedures*). Interestingly, as early as 2 h post infection, strain CN3685 induced swelling of Caco-2 cells; by 3 h post infection, this effect turned into a striking cytotoxic phenotype characterized by rounding, detachment of cells from the glass and formation of some membrane blebs (Table 1 and Fig. 8E). A similar cytotoxic phenotype was induced by strains JGS1495 or CN5383, although those strains induced cytotoxicity more slowly than CN3685. Morphological damage in JGS1495- or CN5383-infected Caco-2 cells cultures was observed by 3.5 h post infection and rounding of the cell monolayer with membrane blebbing was seen by 5 h post infection (Table 1 and Fig. 8F). Together, these results indicate that *C. perfringens* type C strains produce toxins that

Table 1. Morphological changes induced on Caco-2 cells by *C. perfringens* strains.

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

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

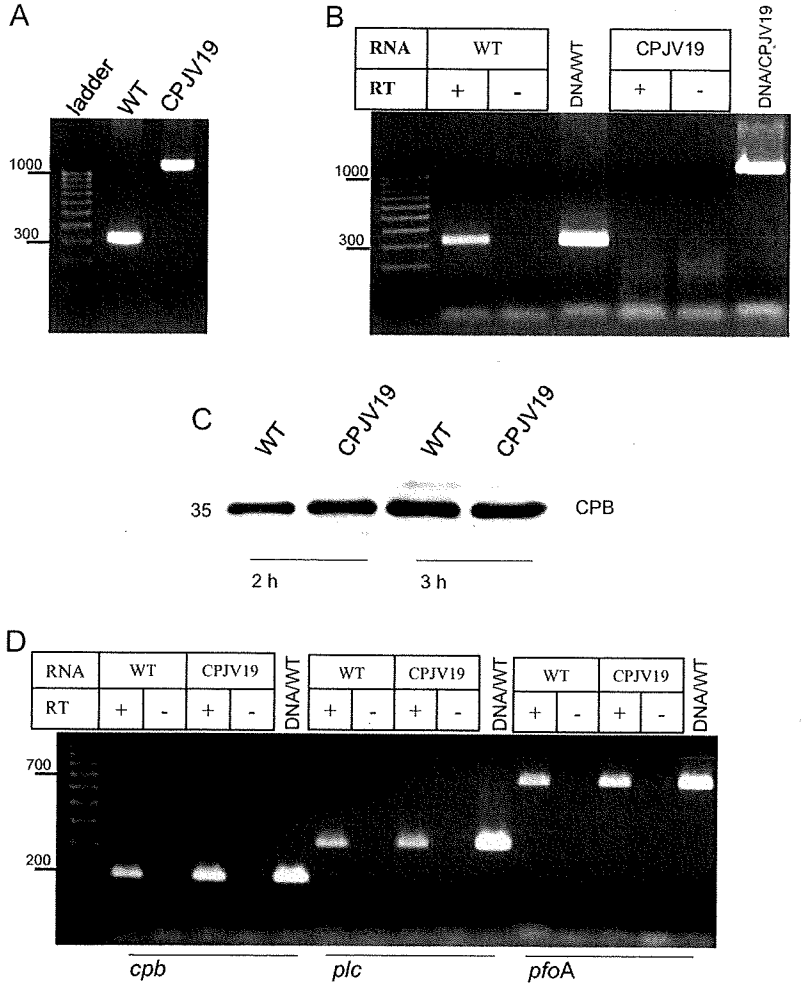


Fig. 9. *lux*S-controlled quorum-sensing mechanism is not required for Caco-2 cell-induced upregulation of toxin production by CN3685.
A. PCR showing an intron insertion in the *lux*S gene. Primers LuxS-L and LuxS-R amplified a 300 bp product of the *lux*S gene using DNA isolated from CN3685 (WT) or 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