

# Epsilon-Toxin Production by *Clostridium perfringens* Type D Strain CN3718 Is Dependent upon the *agr* Operon but Not the VirS/VirR Two-Component Regulatory System

Jianming Chen,<sup>a</sup> Julian I. Rood,<sup>b</sup> and Bruce A. McClane<sup>a,b</sup>

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA,<sup>a</sup> and Australian Research Council, Centre of Excellence in Structural and Functional Genomics, Department of Microbiology, Monash University, Clayton, Victoria, Australia<sup>b</sup>

**ABSTRACT** *Clostridium perfringens* type B and D strains cause enterotoxemias and enteritis in livestock after proliferating in the intestines and producing epsilon-toxin (ETX), alpha-toxin (CPA), and, usually, perfringolysin O (PFO). Although ETX is one of the most potent bacterial toxins, the regulation of ETX production by type B or D strains remains poorly understood. The present work determined that the type D strain CN3718 upregulates production of ETX upon close contact with enterocyte-like Caco-2 cells. This host cell-induced upregulation of ETX expression was mediated at the transcriptional level. Using an isogenic *agrB* null mutant and complemented strain, the *agr* operon was shown to be required when CN3718 produces ETX in broth culture or, via a secreted signal consistent with a quorum-sensing (QS) effect, upregulates ETX production upon contact with host cells. These findings provide the first insights into the regulation of ETX production, as well as additional evidence that the Agr-like QS system functions as a global regulator of *C. perfringens* toxin production. Since it was proposed previously that the Agr-like QS system regulates *C. perfringens* gene expression via the VirS/VirR two-component regulatory system, an isogenic *virR* null mutant of CN3718 was constructed to evaluate the importance of VirS/VirR for CN3718 toxin production. This mutation affected production of CPA and PFO, but not ETX, by CN3718. These results provide the first indication that *C. perfringens* toxin expression regulation by the Agr-like quorum-sensing system may not always act via the VirS/VirR two-component system.

**IMPORTANCE** Mechanisms by which *Clostridium perfringens* type B and D strains regulate production of epsilon-toxin (ETX), a CDC class B select toxin, are poorly understood. Production of several other toxins expressed by *C. perfringens* is wholly or partially regulated by both the Agr-like quorum-sensing (QS) system and the VirS/VirR two-component regulatory system, so the present study tested whether ETX expression by type D strain CN3718 also requires these regulatory systems. The *agr* operon was shown to be essential for signaling CN3718 to produce ETX in broth culture or to upregulate ETX production upon close contact with enterocyte-like Caco-2 cells, which may have pathogenic relevance since ETX is produced intestinally. However, ETX production remained at wild-type levels after inactivation of the VirS/VirR system in CN3718. These findings provide the first information regarding regulation of ETX production and suggest Agr-like QS toxin production regulation in *C. perfringens* does not always require the VirS/VirR system.

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Address correspondence to Bruce A. McClane, bamcc@pitt.edu.

The Gram-positive, spore-forming, anaerobic bacterium *Clostridium perfringens* is an important pathogen of humans and livestock, causing clostridial myonecrosis and numerous diseases originating in the intestines (1, 2). *C. perfringens* virulence is largely dependent upon prolific toxin production, with this bacterium capable of producing at least 17 different toxins. However, only portions of this toxin arsenal are produced by individual strains, which allows for a toxinotyping classification (A to E) system based upon the production of alpha-toxin (CPA), beta-toxin, epsilon-toxin (ETX), and iota-toxin (1, 2).

By definition, *C. perfringens* type D strains must produce alpha-toxin (CPA) and epsilon-toxin (ETX). Some type D strains also produce additional toxins, such as perfringolysin O (PFO),

that are not used for toxin typing (3). *C. perfringens* type D isolates cause often fatal enterotoxemias in several livestock species, as well as acute or chronic enteritis in goats (1, 4). During type D enterotoxemias, toxins are produced in the intestines and then absorbed through the intestinal mucosa into the circulation, where they spread to other internal organs (1). This enterotoxemia then causes edema in several organs, notably the brain, kidneys, lungs, and liver.

An important feature of *C. perfringens* pathogenicity is the correlation between different toxin types and pathologies, strongly suggesting that particular toxins are important for certain diseases. ETX, an ~30-kDa pore-forming protein, is considered a major virulence factor of both type B and D strains (3, 5). Ranking

as the third most potent clostridial toxin after the botulinum and tetanus toxins, ETX is listed as a class B CDC select toxin. Epsilon-toxin is normally produced as an inactive ~33-kDa prototoxin, but after proteolytic hydrolysis by intestinal proteases (such as trypsin and chymotrypsin) or lambda-toxin produced by *C. perfringens*, this prototoxin is converted into a fully active toxin (6, 7).

Intensive studies over the past 20 years have provided an understanding of the structure, mode of action, and genetics of many *C. perfringens* toxins (8, 9). However, detailed information about the regulation of expression for several *C. perfringens* toxins remains rudimentary, at best. In particular, little or no information is available regarding the regulation of toxin production by *C. perfringens* type D strains.

A common trait of bacteria is their adaptive ability to environmental changes. Quorum sensing (QS) is used by many bacteria to control gene expression in a cell density-dependent manner that is often influenced by environmental fluctuations (10, 11). QS systems typically utilize extracellular signaling molecules named autoinducers; in Gram-positive bacteria, these QS autoinducers are usually secreted peptides processed from larger propeptides (12). The Agr QS system has been found exclusively amongst certain Gram-positive bacteria (12–15) but is best studied in *Staphylococcus aureus* (12), where the signaling molecule is referred to as autoinducing peptide (AIP). AIP is encoded by the *agrD* gene, while the *S. aureus* *agr* operon also encodes the AgrB protease, which is required for modification of the AgrD propeptide to mature AIP. Once AIP reaches a threshold level, it activates the AgrC sensor histidine kinase. The activated AgrC sensor then phosphorylates AgrA, a transcriptional regulator, which increases the transcription of a regulatory RNA named RNAIII. Increased levels of RNAIII then modulate the expression of various *S. aureus* virulence genes.

Recently, evidence was reported for an Agr-like quorum sensing system in *C. perfringens* (13, 14). Interestingly, while the *agrB* and *agrD* genes are present, no *agrA* or *agrC* orthologs have been identified in this bacterium. The *agr* locus, apparently acting via an Agr-like QS system, was recently implicated in positive control of PFO and CPA production by *C. perfringens* type A strains (13, 14) and was also shown to influence sporulation and the production of beta2-toxin and enterotoxin by *C. perfringens* type A strain F5603, a non-food-borne human gastrointestinal disease isolate (16).

The apparent absence of *agrA* and *agrC* genes from the *C. perfringens* genome suggests that this bacterium uses some other two-component regulatory system to mediate Agr-like QS effects. Consequently, it was proposed that the *C. perfringens* Agr-like QS system interacts with the VirS/VirR two-component system (13), which is comprised of the VirS membrane sensor histidine kinase and the VirR response regulator (17–19). Consistent with this hypothesis, both the Agr-like QS system and VirS/VirR system are known to positively regulate several toxins produced by *C. perfringens* type A, including CPA, beta2-toxin, and PFO (13, 14, 16–18).

Since little is known about the regulation of ETX production, the present study investigated whether toxin, particularly ETX, production by *C. perfringens* type D isolate CN3718 is regulated by the Agr-like QS system, the VirS/VirR two-component regulatory system, or both systems. These studies revealed that ETX production in this strain requires the *agr* operon acting via a diffusible signaling molecule, a result consistent with QS regulation. However, while the VirR/VirS two-component system regulated pro-

duction levels of PFO and CPA production by CN3718, its inactivation surprisingly had no effect on ETX production by this strain. In addition, the Agr-like QS system was shown to upregulate ETX production when CN3718 contacts Caco-2 cells. This rapid host cell-induced stimulation of ETX production had cytotoxic consequences for host MDCK cells, suggesting potential disease relevance.

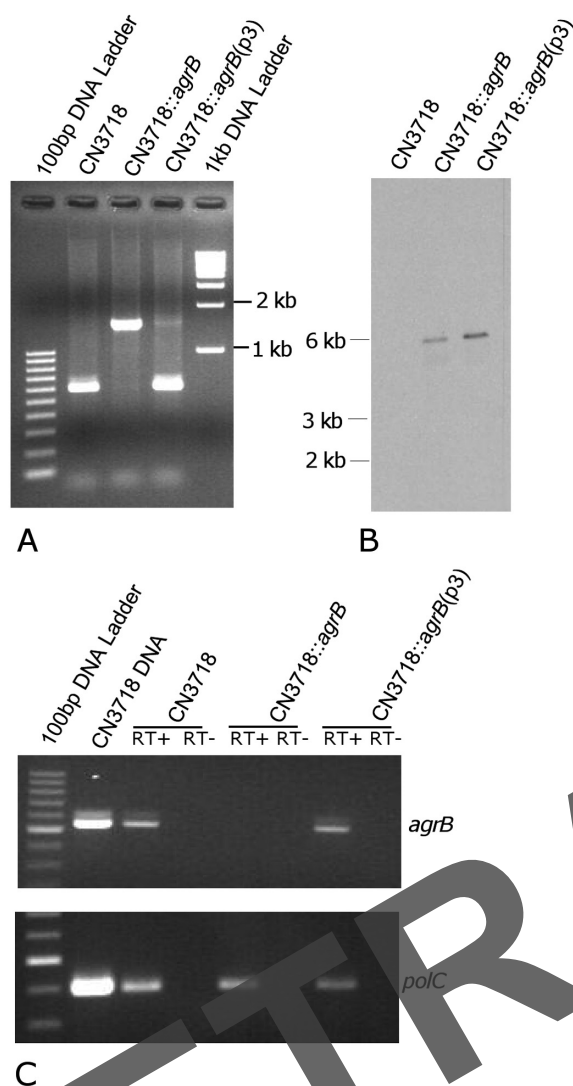
## RESULTS

**Analysis of the *agr* locus in CN3718.** The present study first examined whether type D strain CN3718 carries an *agr* locus similar to that present in type A strain 13 (13, 14). PCR using primers that amplify the ~2.9-kb strain 13 *agr* locus also produced a similar size product using CN3718 DNA (data not shown). When this CN3718 PCR product was sequenced, it revealed the presence of an *agr* locus with 99% identity, at the nucleotide level, to the strain 13 *agr* locus. When translated, this sequence indicated that the CN3718 *agr* locus encodes CPE1562 and CPE1563 hypothetical proteins and an AgrB protein that are each ≥99% identical to the corresponding proteins encoded by the strain 13 *agr* locus. In addition, the predicted amino acid sequence of the AgrD proteins encoded by strains 13 and CN3718 were 100% identical at the amino acid level.

**Construction and genotypic characterization of a CN3718 *agrB* null mutant.** To evaluate whether the *agr* operon is necessary for toxin regulation in CN3718, the *agrB* gene of this *C. perfringens* type D strain was inactivated by using a *Clostridium*-modified targetron vector (20) that inserted, in the sense orientation, a group II intron (~900 bp) between nucleotides 566 and 567 in the *agrB* gene. Confirmation of this *agrB* mutant, named CN3718::*agrB*, was first shown by PCR using primers specific for internal *agrB* sequences (Fig. 1A). In wild-type CN3718, these internal PCR primers specifically amplified a PCR product of ~650 bp. However, consistent with the insertion of the ~900-bp intron into *agrB*, the same primers amplified a PCR product of ~1.6 kb from the putative *agrB* mutant. Southern blot analyses using an intron-specific probe then demonstrated the presence of only a single intron insertion in CN3718::*agrB* (Fig. 1B). As expected, no intron signal was detected using DNA from the wild-type isolate in this Southern blot procedure (Fig. 1B). To complement the CN3718 *agrB* mutant, an *agrB* operon-carrying plasmid named p3 (14) was transformed into the CN3718::*agrB* mutant by electroporation. PCR demonstrated the presence of the wild-type *agrB* gene in the complemented strain, which was named CN3718::*agrB*(p3) (Fig. 1A).

To evaluate whether AgrB expression was silenced in the putative *agrB* null mutant, a reverse transcription-PCR (RT-PCR) assay was performed since no anti-AgrB antibody was available. Wild-type CN3718 and the complemented strain were both able to express *agrB* transcripts. However, no *agrB* transcription was detectable for the *agrB* null mutant (Fig. 1C).

**The *agr* operon positively regulates *in vitro* ETX production by CN3718.** To evaluate whether the *agr* operon, which encodes the *agrB* gene, is involved in regulating ETX production during *in vitro* growth of CN3718 in TGY broth (tryptic soy broth, glucose, yeast extract, with thioglycolate), Western blotting and RT-PCR analyses were performed with the wild-type parent, *agrB* null mutant, and CN3718::*agrB*(p3) complementing strain to compare ETX expression at the protein and transcriptional levels, respectively. When these strains were grown in TGY broth for 4 h (early-



**FIG 1** Construction of a CN3718 *agrB* null mutant by intron-based insertional mutagenesis. (A) PCR analyses using DNA from wild-type CN3718, the *agrB* null mutant (CN3718::*agrB*), or the complemented strain [CN3718::*agrB*(p3)]. (B) Southern blot hybridization of wild-type CN3718, the *agrB* null mutant, and the complemented strain. DNA from each strain was digested with *Eco*RI and then electrophoresed on a 0.8% agarose gel. After transfer onto a nylon membrane, the separated DNA was hybridized with a DIG-labeled intron-specific probe. Sizes of DNA fragments in kilobases (kb) are shown to the left. (C) RT-PCR analyses for *agrB* or *polC* expression by the wild type, *agrB* mutant, and the complemented strain. Sample pellets were collected from 3-h TGY cultures. As indicated, reverse transcriptase (RT) was (+) or was not (–) added to the reaction tubes.

log-phase growth), Western blotting detected ETX production by wild-type CN3718, but not by the *agrB* mutant (Fig. 2A). This loss of ETX production by TGY cultures of the *agrB* mutant was reversible by complementation. In 8-h (late-log-phase growth) TGY cultures, ETX production by the *agrB* mutant was reduced compared to that of the strong ETX production noted for both the wild-type and CN3718::*agrB*(p3) complemented strains (Fig. 2A). By quantitative analysis and statistical comparison, ETX production by the wild type and *agrB* mutant showed a statistically significant difference with both 4-h and 8-h TGY cultures ( $P < 0.01$ ) (Fig. 2B).

RT-PCR studies then indicated that the reduced ETX production by the *agrB* null mutant involves regulation at the transcriptional level (Fig. 2C). In order to determine whether the decreased ETX production by the *agrB* mutant is simply due to impaired bacterial growth, growth curve experiments were performed. Measurement of culture optical density at 600 nm ( $OD_{600}$ ) at different time periods revealed no differences in growth between the wild-type parent, *agrB* mutant, and CN3718::*agrB*(p3) complemented strain (Fig. 2D).

**The *agr* operon also positively regulates *in vitro* CPA and PFO production by CN3718.** To evaluate the role of the *agr* operon in regulating *in vitro* production of CPA and PFO by CN3718, CPA activity was first measured using egg yolk agar plates (Fig. 2E). When grown on these plates, colonies of the wild-type and CN3718::*agrB*(p3) complemented strain were both surrounded by a zone of insoluble diacylglycerol precipitation, which is indicative of lecithin breakdown due to the phospholipase C activity of CPA (21, 22). However, the CN3718::*agrB* mutant failed to produce this characteristic precipitation zone around its colonies, indicating that the *agr* operon can regulate CPA activity by this type D strain.

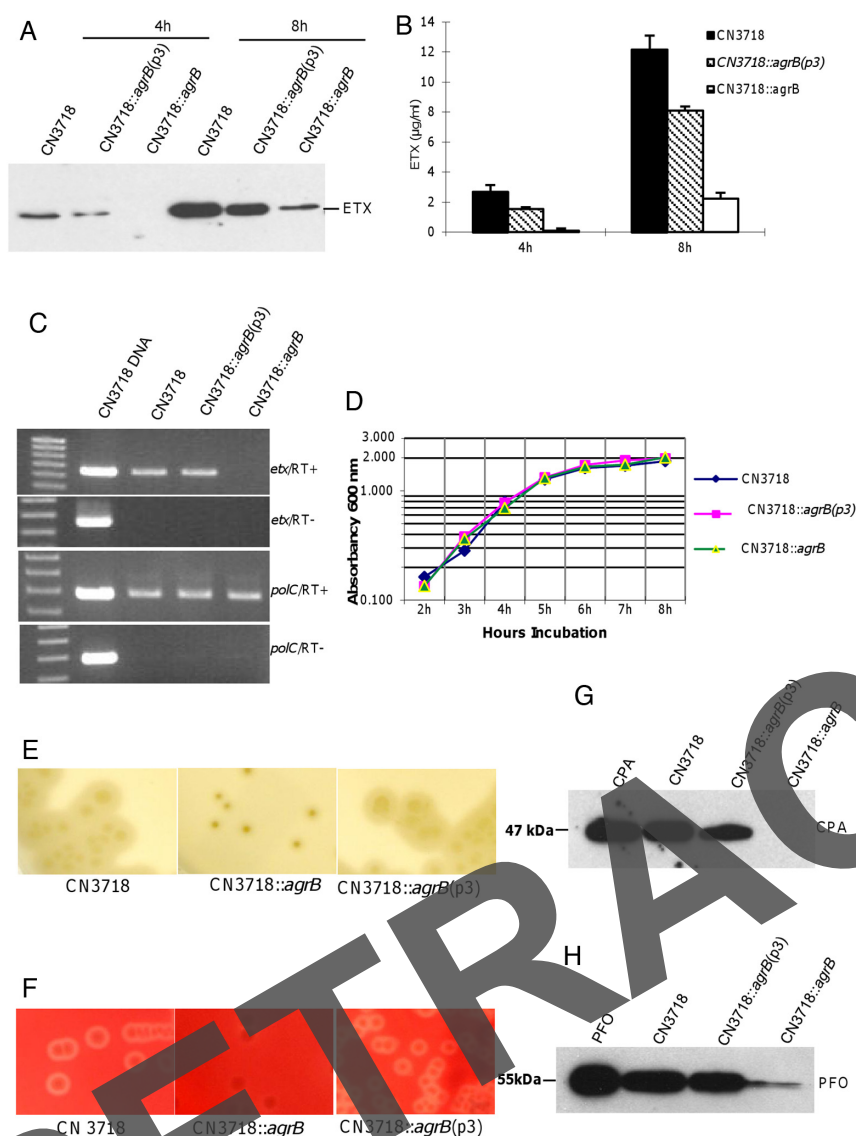
Wild-type CN3718, CN3718::*agrB*, and CN3718::*agrB*(p3) were also grown on sheep blood agar plates to begin evaluating their PFO activity (Fig. 2F). Colonies of wild-type CN3718 and the complemented strain were surrounded by a double zone of hemolysis, including the inner zone of beta-hemolysis indicative of *C. perfringens* PFO activity (21, 23, 24). In contrast, colonies of the *agrB* null mutant were not closely surrounded by this inner beta-hemolytic zone, suggesting their loss of PFO activity.

To specifically demonstrate that the *agr* operon positively regulates the CPA or PFO activity of CN3718, Western blots were performed on the same supernatants used for detecting ETX production in Fig. 2A. These Western blots (Fig. 2G and H) using anti-CPA or anti-PFO specific antibodies showed that CPA and PFO were produced by both wild-type CN3718 and the CN3718::*agrB*(p3) complemented strain. However, CPA was absent and PFO levels sharply decreased in the supernatants of the *agrB* null mutant strain.

**The *agr* operon is necessary for upregulation of ETX production when CN3718 contacts Caco-2 cells.** In previous studies, we reported that contact with enterocyte-like cells induces rapid upregulation of toxin production by *C. perfringens* type C isolates (25). While type C isolates do not produce ETX, they resemble type D strains by causing disease that originates in the intestines, where their vegetative cells contact enterocytes (1). Therefore, we asked whether ETX production might also be upregulated when vegetative cells of the type D strain CN3718 contact enterocyte-like Caco-2 cells.

When CN3718 or the CN3718::*agrB*(p3) complementing strain was used to infect Caco-2 cell cultures under anaerobic conditions, rapid ETX production was detected (Fig. 3A). In contrast, only weak ETX production was observed when these strains were similarly incubated in minimal essential Eagle's medium (MEM) (no Caco-2 cells present) under similar anaerobic conditions. While the presence of Caco-2 cells caused wild-type CN3718 to upregulate ETX production, production of this toxin was barely detectable when the isogenic *agrB* null mutant was used to infect Caco-2 cultures under these conditions. This deficiency was reversible by complementation, indicating that the Agr-like quorum-sensing system is necessary for mediating the upregula-





**FIG 2** AgrB positively regulates toxin production by CN3718 *in vitro*. (A) Western blot analyses of ETX production by wild-type CN3718 (WT), the isogenic *agrB* null mutant (CN3718::*agrB*), or the complemented strain [CN3718::*agrB*(p3)] at 4 h or 8 h. (B) Quantitative analysis of ETX production by the wild type, *agrB* mutant, or complemented strain at 4 h or 8 h in TGY cultures. Shown are mean values  $\pm$  standard deviations (SD) for two repetitions. (C) RT-PCR analyses for *etx* or *polC* gene expression by the wild type, *agrB* mutant, or the complemented strain in 3-h TGY cultures. As indicated, reverse transcriptase (RT) was (+) or was not (–) added to the reaction tubes. (D) Comparison of growth characteristics of wild-type CN3718, the *agrB* mutant, and the complemented strain. The optical density (OD<sub>600</sub>) of each strain growing in TGY medium was measured using a Bio-Rad Smart-spec spectrophotometer. Shown are representative results from two similar repetitions. (E) CPA activity detected using an egg yolk agar plate. Wild-type CN3718 and the complemented strain, but not the *agrB* mutant, showed a characteristic CPA-induced precipitation zone surrounding colonies on egg yolk agar plates. (F) PFO activity detected on sheep blood agar plates. Colonies of the wild-type and complemented strain, but not the *agrB* mutant, were surrounded by the inner zone of beta-hemolysis indicative of PFO activity. (G) Western blot analysis of CPA production by the wild type, *agrB* mutant, and complemented strain after overnight growth in TGY medium. The molecular mass is indicated on the left. Purified CPA was also included as control. (H) Western blot analyses of PFO production by the wild type, *agrB* mutant, or the complemented strain after overnight growth in TGY medium. The molecular mass is indicated on the left. Purified PFO was also included as control.

tion of ETX production that occurs when CN3718 contacts host enterocytes.

An experiment was then performed to begin exploring whether

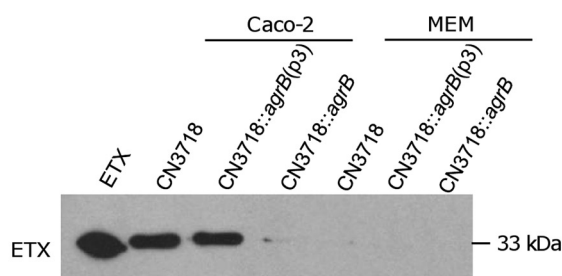
*agrB* mutant to produce ETX, consistent with a QS effect.

**The *agr* operon regulates CN3718 cytotoxicity for MDCK cells.** Since the toxins produced by CN3718 are rapidly upregu-

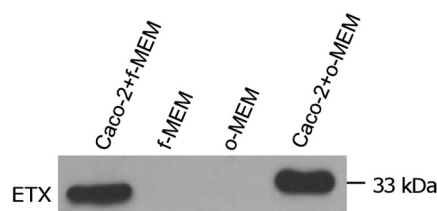
this Caco-2 cell-induced upregulation of ETX production involves secreted factors of Caco-2 cells or direct bacterial contact with Caco-2 cells (Fig. 3B). When MEM from 4-day-old Caco-2 cell cultures was collected and coincubated with washed CN3718 cells for 2 h under anaerobic conditions, no ETX expression was detected. Similar results were observed when CN3718 was grown in only fresh MEM. In contrast, direct contact between CN3718 and Caco-2 cells resulted in ETX production, whether using fresh or 4-day-old MEM (Fig. 3B).

Results from two other experiments further supported a need for direct contact with Caco-2 cells to trigger upregulation of ETX production by CN3718. First, ETX production was not observed if the bacteria and host cells were anaerobically cultured in separate chambers of a Transwell plate (data not shown). However, ETX production was detected when both CN3718 and Caco-2 cells were anaerobically cocultured in the same Transwell chamber (data not shown). Second, filter-sterilized MEM supernatants that had been removed from Caco-2 cells infected with the CN3718 *agrB* mutant were unable to upregulate ETX production (Fig. 3C).

**The *agr* operon mediates control of ETX production via a diffusible molecule.** No ETX production was observed when either CN3718::*agrB* or a CN3718 *etx* null mutant (JIR4982) was incubated alone in Transwells (Fig. 4). To address whether *agr* operon regulation of ETX production by CN3718 involves a secreted signaling molecule, as would be expected if this was a QS effect, these two strains were coincubated in the same Transwell, but separated by a membrane that is impermeable to bacteria yet allows passage of small signaling molecules. Under these conditions, ETX production by the *agrB* mutant was readily detectable when CN3718::*agrB* was inoculated into one Transwell chamber and the CN3718 *etx* null mutant (JIR4982) was inoculated into the other Transwell chamber (Fig. 4). These physical complementation results strongly suggested that toxin regulation by the *agr* operon involves a secreted factor produced by the CN3718 *etx* null mutant, which can diffuse to the medium and function as a signal for the CN3718::



A



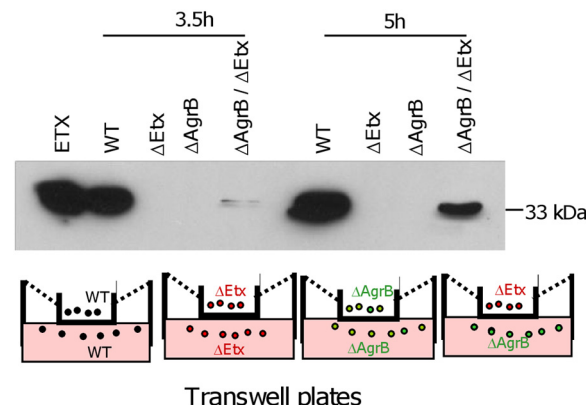
B



C

**FIG 3** Upregulated ETX production by CN3718 during infection of human intestinal Caco-2 cells involves AgrB. (A) ETX production is upregulated when CN3718 contacts Caco-2 cells. Cell culture dishes containing MEM (no cells) or Caco-2 cells were infected for 1 h or 2 h with  $1.5 \times 10^7$  CFU of CN3718, the CN3718::agrB isogenic agrB null mutant, or the CN3718::agrB(p3) complemented strain at 37°C under anaerobic conditions. Culture supernatants were then removed and subjected to Western blotting using an ETX MAb. Purified 33-kDa ETX was also included as a control. (B and C) Evidence that Caco-2 cell-induced upregulation of ETX production by wild-type CN3718 does not involve Caco-2 cell-secreted factors. (B) Fresh MEM (Caco-2 + f-MEM) or filter-sterilized MEM removed from 4-day-old Caco-2 cell cultures (Caco-2 + o-MEM) was added to a dish of confluent Caco-2 cells before infection for 2 h with CN3718. As negative controls, only fresh MEM (f-MEM) or filter-sterilized MEM removed from 4-day-old Caco-2 cell cultures (o-MEM) was added to CN3718 cells in the absence of Caco-2 cells. Culture supernatants were then removed and subjected to ETX Western blotting. (C) Prior to ETX Western blotting, washed CN3718 cells were suspended for 2 h in filter-sterilized MEM culture supernatants removed from Caco-2 cells that had been infected for 5 h with wild-type CN3718 (Caco-2 + f-MEM), filter-sterilized MEM culture supernatants removed from Caco-2 cells that had been infected for 5 h in CN3718::agrB (MEM of Caco-2/CN3718::agrB), MEM culture supernatants removed from Caco-2 cells grown for 5 h in the absence of bacteria (MEM of Caco-2), and filter-sterilized MEM culture supernatants removed from CN3718::agrB that had been grown in the absence of Caco-2 cells (MEM of CN3718::agrB). All cultures were incubated anaerobically.

lated when this *C. perfringens* type D strain directly contacts Caco-2 cells (Fig. 3A and B), an experiment was performed to investigate whether the rapid host cell-induced stimulation of



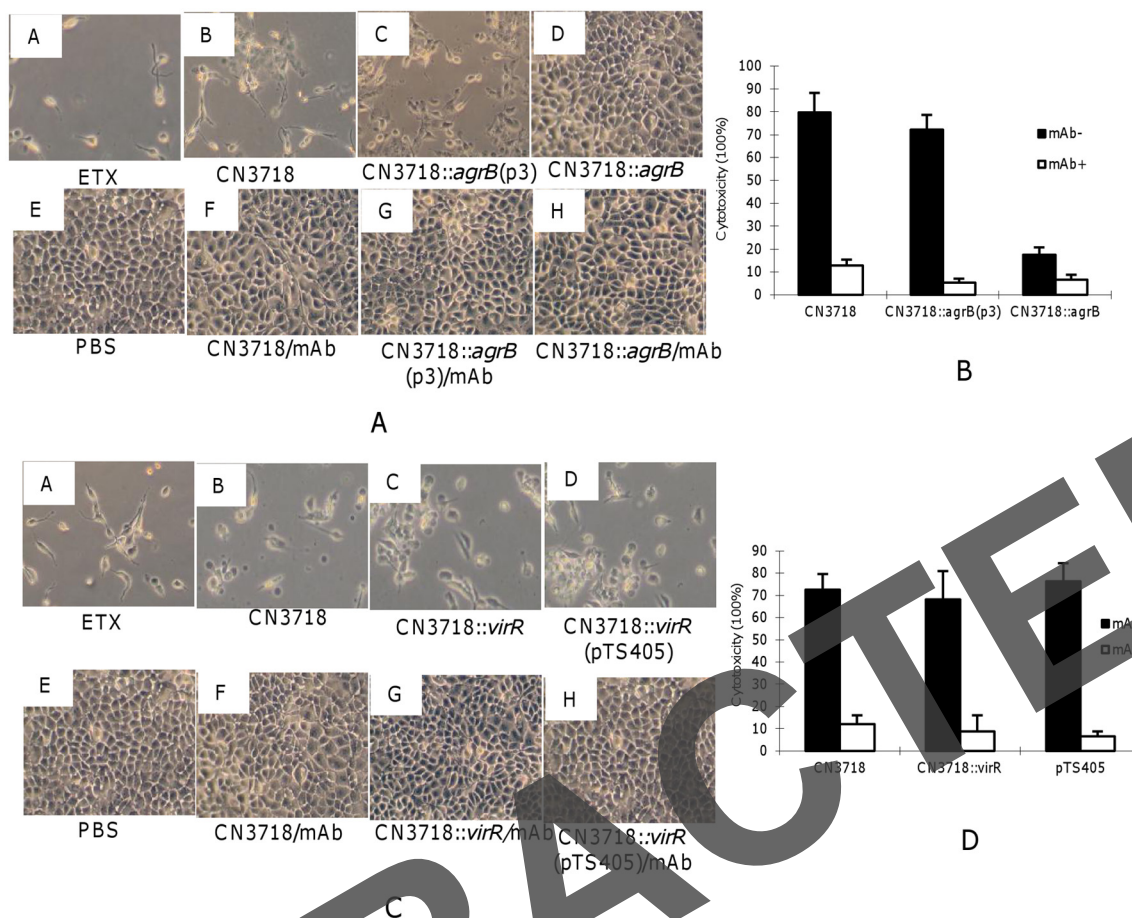
Transwell plates

**FIG 4** The agr quorum-sensing system uses a diffusible signaling molecule to control CN3718 toxin production. At two time points (3.5 h and 5 h), samples were collected from the indicated Transwell chambers for ETX Western blot detection. The bottom panel shows a schematic diagram for the different *C. perfringens* strains inoculated into each chamber of the Transwell plate.

toxin production by CN3718 has cytotoxic consequences. For this experiment, an *in vitro* cell culture model was developed to mimic type D enteric disease, where *C. perfringens* contacts enterocytes and produces toxins that are then absorbed into the circulation to damage nonintestinal internal organs, such as the kidneys or brain. In our *in vitro* model of type D disease, enterocyte-like Caco-2 cells (which are naturally ETX insensitive) were anaerobically infected with wild-type CN3718, the isogenic CN3718::agrB null mutant, or the CN3718::agrB(p3) complemented strain for 2 h. Supernatants from those infected cultures were collected and filter-sterilized, and the sterile supernatants were then treated with trypsin to activate the ETX prototoxin, as would occur in the intestines during natural disease. The trypsin-treated ETX in these samples produced a slightly lower-molecular-mass (~30-kDa) ETX species on Western blots than did non-trypsin-treated ETX (data not shown), confirming that trypsin-induced cleavage of ETX had occurred.

The trypsin-activated supernatants were then applied to MDCK cells for 2 h, mimicking enterotoxemic effects of type D infection on the kidney (Fig. 5A). Under these conditions, the activated supernatants from the wild-type strain caused morphological changes, including rounding and swelling of the MDCK cells. On prolonged incubation, detachment of cells from the cell culture dish was also observed. This damage could be inhibited by pretreating these supernatants with an ETX-neutralizing monoclonal antibody (MAb), confirming the involvement of ETX in this cytotoxicity. In contrast, the activated supernatants from the agrB null mutant caused little or no detectable damage; this loss of cytotoxicity was reversible by complementation of the mutant with a wild-type agr operon.

Lactate dehydrogenase (LDH) release from damaged or destroyed cells was then measured to quantify cytotoxicity with this model (Fig. 5B). Rates of cell death caused by the wild-type and CN3718::agrB(p3) complemented strain were similarly high. In contrast, there was significantly less cytotoxicity caused by the agrB null mutant. When the sterile supernatants were preincubated with an ETX-neutralizing MAb, cytotoxicity caused by the wild-type or complemented strains decreased significantly, confirming a substantial role for ETX in the cytotoxic activity of these supernatants.



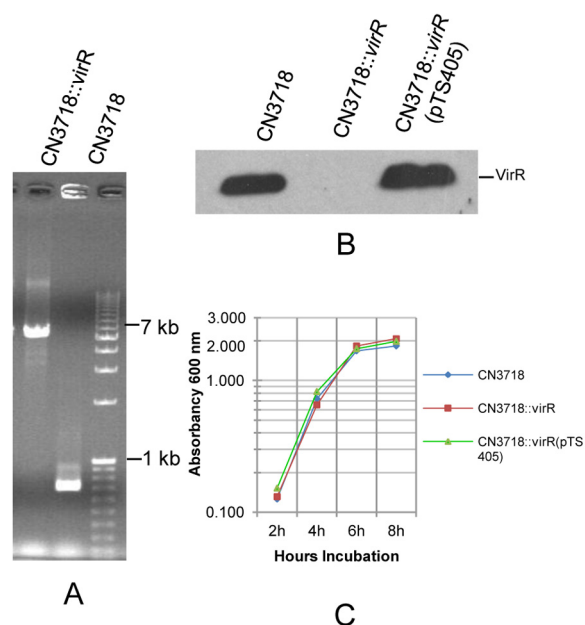
**FIG 5** Cytotoxic activities of trypsin-activated supernatants from *agrB* and *virR* null mutants. (A) Morphological damage to MDCK cells induced by infection with  $1.5 \times 10^7$  CFU of wild-type CN3718, the *agrB* mutant, or the complemented strain. Each strain was cocultured with Caco-2 cells for 2 h. The supernatants were collected and concentrated  $10\times$  before activation by trypsin. After blockage of trypsin activity with trypsin inhibitor, the activated supernatants were applied for 2 h to MDCK cells. Cultures were then visualized by microscopy and photographed. The samples shown include MDCK cells treated with activated, purified ETX as a positive control (panel A), MDCK cells treated with activated Caco-2 culture supernatants after the indicated infection (panels B to D), MDCK cells treated with PBS as a negative control (panel E), and MDCK cells treated with the specified activated Caco-2 culture supernatants after those supernatants had been preincubated with ETX MAb for 15 min before their addition to MDCK cells (panels F to H). (B) Quantitative MDCK cytotoxicity (measured by LDH release) of the activated Caco-2 cell supernatants after the specified infections with  $1.5 \times 10^7$  CFU. The error bars shown represent the standard error of the mean calculated from three independent experiments. (C) Each *C. perfringens* strain indicated ( $1.5 \times 10^7$  CFU) was cocultured with Caco-2 cells for 2 h. The supernatants were concentrated  $10\times$  before activation by trypsin. After blockage of trypsin activity with trypsin inhibitor, the activated supernatants were applied for 2 h to MDCK cells. Cultures were then visualized by microscopy and photographed. The samples shown include MDCK cells treated with activated, purified ETX as a positive control (panel A), MDCK cells treated with the specified activated Caco-2 culture supernatants obtained after the indicated infection (panels B to D), MDCK cells treated with PBS as a negative control (panel E), and MDCK cells treated with the specified activated Caco-2 culture supernatants after those supernatants had been preincubated with ETX MAb for 15 min before being added to MDCK cells (panels F to H). (D) Quantitative MDCK cytotoxicity (measured by LDH release) of the activated Caco-2 cell supernatants after the specified infections with  $1.5 \times 10^7$  CFU. The error bars represent the standard error of the mean calculated from three independent experiments.

**The VirS/VirR two-component regulatory system positively regulates the ability of CN3718 to produce PFO and CPA, but not ETX.** The VirS/VirR two-component regulatory system positively controls production of several toxins by *C. perfringens* type A strains (17, 18, 25). To address whether the VirS/VirR regulatory system might also be involved in regulating toxin production by a type D isolate, a CN3718 *virR* mutant (named CN3718::*virR*) was constructed using the *Escherichia coli*-based suicide plasmid pKOR (17) and confirmed by PCR (Fig. 6A). As shown by Western blotting using an anti-VirR antibody, the VirR protein was not produced by CN3718::*virR*. In contrast, this analysis detected VirR production by wild-type CN3718 (Fig. 6B). To complement CN3718::*virR*, pTS405 (containing the whole *virR*-S operon [26])

was transformed into the CN3718 *virR* null mutant by electroporation to create CN3718::*virR*(pTS405). Western blotting confirmed VirR protein expression by this complemented strain (Fig. 6B). Growth rates of the wild-type parent, *virR* null mutant, and CN3718::*virR*(pTS405) complemented strain were compared in TGY medium and were found to be nearly identical (Fig. 6C).

To begin evaluating whether VirS/VirR can regulate toxin production in CN3718, the wild-type, CN3718::*virR* mutant, and VirS/VirR complemented strain were each streaked onto sheep blood agar plates. Colonies of wild-type CN3718 and the CN3718::*virR*(pTS405) complemented strain, but not the CN3718::*virR* mutant, were surrounded by an inner zone of beta-hemolysis indicative of PFO production (Fig. 7A). Western blot





**FIG 6** Construction of a CN3718 *virR* null mutant. (A) PCR was performed using *virR* gene primers and genomic DNA. Using DNA from the wild-type CN3718, a 600-bp *virR* gene product was amplified. However, larger bands were amplified from the CN3718::*virR* null mutant because the pKOR mutagenesis vector (6 kb) had inserted into the *virR* ORF by homologous recombination. (B) Western blot analyses of VirR expression by wild-type CN3718, the *virR* null mutant, or CN3718::*virR*(pTS405), the VirS/VirR complemented strain. (C) Growth comparison of wild-type CN3718, the *virR* null mutant, and the complemented strain. The optical density (OD<sub>600</sub>) of each strain growing in TGY medium was measured using a Bio-Rad SmartSpec spectrophotometer. Shown are representative results from two similar repetitions.

analysis then directly demonstrated that PFO expression by this mutant was absent, while wild-type CN3718 produced PFO (Fig. 7B). When VirR expression was restored by complementation, PFO production was also restored (Fig. 7E).

When grown on egg yolk agar plates, colonies of both the wild-type parent and the CN3718::*virR*(pTS405) complemented strain were surrounded by the precipitation zone, indicative of lecithin breakdown due to the phospholipase C activity of CPA (21, 22). In contrast, the CN3718::*virR* mutant produced a much smaller halo zone around its colonies when growing on this medium (Fig. 7C). This result demonstrated that the VirS/VirR two-component regulatory system only partially regulates CPA production by CN3718. When TGY culture samples were subjected to Western blot analysis, the results directly demonstrated that CPA production was decreased compared to that of wild-type CN3718 or the CN3718::*virR*(pTS405) complemented strain (Fig. 7D).

To evaluate whether the VirS/VirR two-component regulatory system can positively regulate ETX production, Western blot analyses were performed to evaluate ETX production by wild-type CN3718, the CN3718::*virR* mutant, and the CN3718::*virR*(pTS405) complemented strain. Surprisingly, silencing of VirR protein production in the CN3718::*virR* mutant had no effect on ETX expression compared to the wild-type and VirR complemented strains (Fig. 7E).

Previous studies (24, 27) had shown that the positive regulation of PFO and NetB production, as well as all directly VirR-regulated genes (28), by the VirS/VirR two-component system in

*C. perfringens* type A strains involves *virR* boxes, which are two imperfect 12-bp directly repeated sequences located upstream of such genes. However, when the region upstream of the *etx* gene in CN3718 was sequenced and subjected to bioinformatics analysis, no obvious *virR* box sequence was identified.

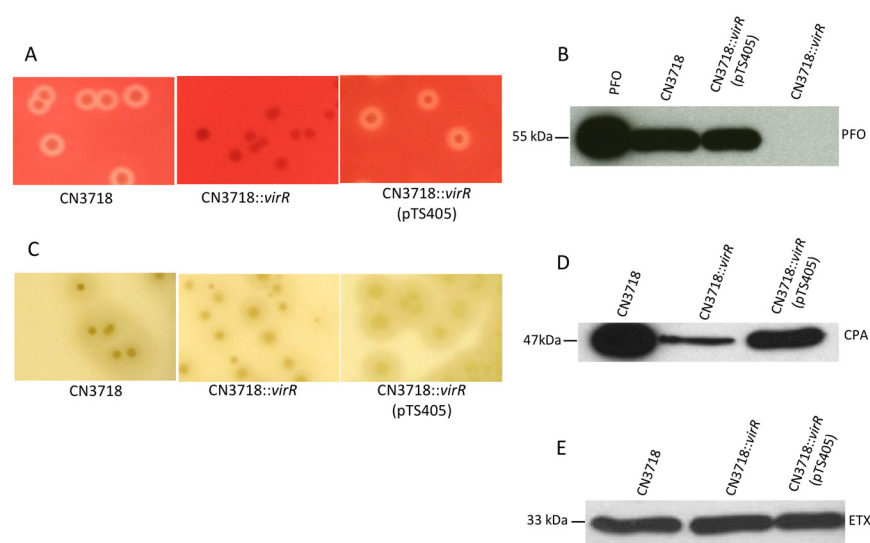
**MDCK cell cytotoxicity of the *virR* mutant.** As described earlier in this study using the *agrB* mutant, sterile supernatants from Caco-2 cell cultures infected with wild-type CN3718, the CN3718::*virR* null mutant, or the CN3718::*virR*(pTS405) complemented strain were collected and then treated with trypsin to activate ETX. After inactivation of trypsin, these supernatants were tested in our MDCK cytotoxicity assay to evaluate whether trypsin-activated supernatants from the *virR* null mutant have similar cytopathic effects to the activated wild-type supernatants. Observation of morphological changes by microscopic examination revealed that the activated supernatants from the *virR* null mutant can still clearly damage MDCK cells, similar to the effects caused by activated supernatants from the wild-type CN3718 and the *virS/virR* complemented strain (Fig. 5C). This damage involves ETX production since preincubating any of these activated supernatants with an ETX-neutralizing MAb blocked the development of cytotoxicity (Fig. 5C).

An LDH release assay then confirmed that there were no quantitative differences in MDCK cytotoxicity between the activated supernatants of CN3718, the isogenic *virR* mutant, or the complemented strain (Fig. 5D). The LDH release induced from MDCK cells by these activated supernatants involves ETX since this effect was blocked by preincubation of any of these activated supernatants with an ETX-neutralizing MAb (Fig. 5D).

## DISCUSSION

*C. perfringens* type B or D strains cause enteritis or enterotoxemias, both of which start with the presence of vegetative bacteria in the intestines, where they contact host enterocytes (1). Recent *in vitro* studies showed that *C. perfringens* type C vegetative cells, which also cause disease when present in the intestines, upregulate their production of beta-toxin (CPB) when those bacteria encounter cultured enterocyte-like Caco-2 cells (25). Since type C strains do not produce ETX, the present study performed a similar analysis using a type D strain and found that the ETX-producing strain CN3718 substantially upregulated its ETX production upon encountering Caco-2 cells. These results suggest that, during *in vivo* disease, ETX-producing strains like CN3718 could sense the presence of host cells and then respond by upregulating their ETX production to facilitate the pathogenesis of infections such as enterotoxemia and enteritis, which involves bacterial production of ETX in the intestines.

The present work has identified the *agr* locus as the first regulator that can control ETX production. When the *agrB* gene in CN3718 was disrupted, the resultant isogenic null mutant failed to produce ETX in broth culture due to impaired *etx* transcription. Consistent with previous studies analyzing *C. perfringens* type A strain 13 (13, 14), the CN3718 *agrB* mutant also produced sharply reduced amounts of PFO and CPA during growth in broth culture. Complementation of the CN3718 *agrB* mutant restored nearly wild-type levels of toxin production, including ETX, during growth in broth cultures. The *agrB* null mutant was also unable to upregulate its ETX production upon close contact with Caco-2 cells, and this defect was completely reversible by complementation with the wild-type *agr* operon, indicating it was not attribut-



**FIG 7** *VirR* positively regulates the *in vitro* production of CPA and PFO, but not ETX, by CN3718. (A) PFO activity detection on sheep blood agar plates. Colonies of the wild-type and CN3718::virR(pTS405) complemented strain, but not the CN3718::virR null mutant, are surrounded by an inner zone of PFO-induced beta-hemolysis. (B) Western blot analysis of PFO production by the wild-type, *virR* null mutant, or complemented strain grown overnight in TGY medium. (C) CPA activity detection on egg yolk agar plate. The wild-type or complementing strain, but not the *virR* null mutant, showed characteristic CPA-induced precipitation surrounding their colonies when growing on egg yolk agar plates. (D) Western blot analysis of CPA production by the wild-type, *virR* null mutant, and complemented strain grown overnight in TGY medium. (E) Western blot analyses of ETX production by wild-type CN3718, the *virR* null mutant, and the complementing strain after 4 h of growth in TGY.

able to a secondary mutation. Furthermore, the ability of the *agr* operon to control ETX production by CN3718 was shown to involve a diffusible signal, consistent with a QS effect. Collectively, these *in vitro* results suggest the *agr* operon, apparently acting via a QS effect, might function as an important regulator of ETX production during natural disease caused by isolates like CN3718, which are in contact with host enterocytes.

It has now been shown that the *agr* operon, apparently acting by a QS effect since it involves a secreted signal, can positively regulate the production of many *C. perfringens* toxins, including ETX, PFO, CPA, beta2-toxin, and CPE (13, 14, 16). Thus, there is increasing evidence that the Agr-like QS system is a global regulator of *C. perfringens* toxin production. Agr-mediated control of clostridial toxin production is not restricted to *C. perfringens* since this QS system has also been implicated in positive control of botulinum toxin production by *C. botulinum* (29). Since toxins play a critical role in numerous clostridial infections, the apparent ability of the Agr-like QS system to regulate production of such different toxins suggests this QS system could be a master virulence regulator for several pathogenic clostridial species. The ability of the Agr-like QS system to regulate production of many clostridial toxins also suggests that this QS system could represent a potential target for therapeutic development.

There appear to be important differences between the *agr* operons in *S. aureus* and *C. perfringens*. Most notably, due to the absence of orthologs of the AgrA/AgrC two-component regulatory system in the *agr* operon of *C. perfringens*, it has been suggested that the Agr-like QS system of *C. perfringens* works through another two-component regulatory system, namely, the VirS/VirR system (13). Consistent with this possibility, other studies have shown that production of PFO, CPA, and beta2-toxin is under the

complete or partial positive control of both the Agr-like QS system and the VirS/VirR two-component regulatory system (14, 16, 17, 19, 24, 30–32).

In contrast, the present study found that vegetative cells of type D strain CN3718 still made wild-type levels of ETX after inactivation of the *virS-R* operon. This continued ETX production was not explainable by a failure to inactivate *virS* and *virR* genes during insertional mutagenesis since Western blotting confirmed the loss of VirR expression by the *virR* null mutant and this regulatory effect was reversible by complementation with the wild-type *virS-R* operon. Also supporting successful inactivation of the *virS-R* operon in the CN3718 *virR* null mutant are this strain's inability to produce PFO and its reduced production of CPA, which is fully consistent with previous reports that PFO production and CPA production are completely or partially (respectively) controlled by the VirS/VirR two-component system (17, 24). Nor was the continued production of wild-type ETX levels by the *virR* mutant due to reversion of the *virR* mutation based upon Western blotting

results (data not shown), which using the same overnight culture supernatant, detected virtually no PFO production, but wild-type levels of ETX production, by the *virR* mutant.

Thus, the finding that CN3718 still produces *agr* locus-regulated ETX in the absence of a functional VirS/VirR two-component system appears to conflict with the recent hypothesis (13) proposing that Agr QS-mediated regulation of toxin production involves the VirS/VirR two-component regulatory system. One possibility is that another two-component regulatory system can also regulate ETX production by CN3718 in a redundant manner. Alternatively, ETX production by this strain may be completely VirS/VirR independent, possibly involving instead another of the ~20 two-component regulatory systems present in *C. perfringens* (33, 34). Consistent with that second possibility, no *virR* boxes were detected upstream of the *etx* gene in CN3718. However, some *C. perfringens* genes lacking *virR* boxes are still indirectly regulated by VirS/VirR via a regulatory RNA named VR-RNA (31), so the absence of detectable *virR* boxes upstream of the *etx* gene in CN3718 could still be compatible with the redundant regulation of ETX expression by both VirS/VirR and another two-component regulatory system. The current demonstration of wild-type levels of ETX production by the isogenic *virR* null mutant of CN3718 may suggest that future studies examining the involvement of other *C. perfringens* two-component systems in ETX regulation are warranted. In addition, it will be important to explore toxin regulation in other ETX-producing strains, including type B strains.

## MATERIALS AND METHODS

**Strains, media, and culture conditions.** *C. perfringens* type D strain CN3718, which can produce CPA, PFO, and ETX, is an animal disease



isolate that was originally part of the Burroughs-Wellcome Collection and had been provided by Russell Wilkinson (University of Melbourne). Construction and characterization of the CN3718 *etx* null mutant (JIR4982) were performed as reported previously (35). For use, a cooked-meat-stock culture of these strains was first streaked onto a Shahidi-Ferguson *perfringens* (SFP) agar plate and then grown overnight at 37°C under anaerobic conditions. Bacterial culture media used in this study included fluid thioglycolate medium (FTG) (Difco Laboratories), TGY (3% tryptic soy broth [Becton-Dickinson], 2% glucose [Sigma Aldrich], 1% yeast extract [Becton-Dickinson], and 0.1% sodium thioglycolate [Sigma Aldrich]), tryptose-sulfite-cycloserine (TSC) agar medium (SFP agar [Difco Laboratories], supplemented with 0.04% of D-cycloserine [Sigma Aldrich]), and brain heart infusion (BHI) agar (Becton-Dickinson). When indicated, tetracycline (Tet; 2.5 µg/ml) or chloramphenicol (Cm; 15 µg/ml) was added to the culture medium.

**Sequencing of the CN3718 *agr* locus and the region upstream of the *etx* gene.** DNA was purified from CN3718 using the MasterPure Gram-positive DNA purification kit (Epicentre Biotechnologies, Madison, WI). PCR was then performed on this DNA using previously described amplification conditions with primers designed using the strain 13 *agr* locus sequences. The ~2.9-kb PCR product amplified from CN3718 DNA was then sequenced by Genewiz, Inc. (South Plainfield, NJ).

To evaluate whether *virR* boxes are present, DNA sequences located 0.8 kb upstream of the *etx* gene were amplified by PCR under the same conditions used for amplifying the *agr* locus, except with two different primers (VirSeq1F [5'-CCTTATTCACCTGTAATGCGTGTCCC-3'] and VirSeq1R [5'-ATATCACGCTGATGCGAT-TGCTAAA-3']). The PCR product was then sequenced by Genewiz, Inc., and the resultant CN3718 *etx* upstream sequence was deposited in GenBank.

**Construction of a CN3718 *agrB* null mutant.** The previously constructed, *agrB*-targeted intron donor plasmid pJIR750agrBi (16), which specifies insertion (in a sense orientation) of a group II intron between *agrB* nucleotides 566 and 567, was used to inactivate the chromosomal *agrB* gene of CN3718. After pJIR750agrBi was electroporated into wild-type CN3718, transformants were selected on BHI agar plates containing 15 µg/ml of chloramphenicol.

Putative *agrB* null mutants were then screened by PCR using the primers AgrBKO-F and AgrBKO-R (16). PCRs were performed in a Techne (Burkhardtendorf, Germany) thermocycler using the following PCR amplification conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2 min, followed by a 10-min extension at 68°C. PCR products were electrophoresed on a 1% gel and stained with ethidium bromide for visualization. The confirmed mutant carrying an intron insertion in the *agrB* gene was then grown in FTG medium without antibiotics and subcultured daily for ~10 days to cure the intron-carrying donor plasmid pJIR750agrBi. Curing was initially shown by susceptibility to chloramphenicol and then confirmed by Southern blotting, which demonstrated the presence of a single intron in the mutant, named CN3718::*agrB*.

To complement the CN3718::*agrB* null mutant strain, a previously constructed *E. coli*-*C. perfringens* shuttle plasmid, CPJVp3 (14), which contained the wild-type *agr* operon, was introduced into the CN3718::*agrB* null mutant strain by electroporation. This complemented strain was named CN3718::*agrB*(p3).

**Inactivation of the *virR* gene in CN3718.** To inactivate the *virR* gene in CN3718, the *E. coli*-based, *C. perfringens* suicide plasmid pKOR (17) was used. This vector, which contains an ~590-bp fragment of the *virR* gene upstream of a tetracycline resistance gene, was transformed into CN3718 by electroporation. Transformants were selected on BHI agar plates containing tetracycline (2.5 µg/ml). Several tetracycline-resistant colonies were observed on the plates, and their identity as *virR* mutants was confirmed by PCR and Western blotting.

To construct a VirR/VirS complementing strain, the previously constructed *E. coli*-*C. perfringens* shuttle vector pTS405 (26), encoding the

*virR*-S operon, was electroporated into the *virR* null mutant strain, and transformants were then selected on BHI-Cm plates.

**Southern blot assay.** The Southern blotting procedure used in this study was described previously (16, 21). Briefly, *C. perfringens* genomic DNA from wild-type CN3718, the *agrB* or *virR* null mutants, or the complementing strains was extracted using the MasterPure Gram-positive DNA purification kit (Epicentre Biotechnologies). The purified DNA was then digested with EcoRI (New England Biolabs) and separated by electrophoresis on a 0.8% agarose gel. The DNA in the gel was transferred to a positively charged nylon membrane (Roche) and then probed with a digoxigenin (DIG)-labeled probe specific for the intron sequence. This probe was prepared using the primer pair AgrB-IBS and AgrB-EBS1d (16) and then DIG labeled with a DIG labeling kit obtained from Roche Applied Science. CSPD substrate (Roche Applied Science) was used for detection of probe hybridization according to the manufacturer's instructions.

**Western immunoblot assay.** Each sample was mixed with 5× loading buffer and electrophoresed on an SDS-containing, 12% polyacrylamide gel. The separated proteins were then transferred onto a nitrocellulose membrane, and the blot was incubated for 1 h with blot washing buffer (20 mM Tris-HCl [pH 8.0], 0.3 M NaCl, 0.5% [vol/vol] Tween 20) containing 5% (wt/vol) nonfat dry milk before incubation with primary antibody overnight at 4°C. The toxin antibodies used in these analyses have been described previously (3, 14, 16). Blots were then washed three times with Tris buffer and incubated with goat anti-mouse-horseradish peroxidase (HRP) or anti-rabbit-HRP for 1 h at room temperature. After three more washes, the blots were treated with the ECL (enhanced chemiluminescence) Western blotting detection kit (Amersham) and exposed to X-ray film (Life Science Products) to detect the immunoreactive protein bands.

To quantify ETX secretion levels at different time points, *C. perfringens* TGY culture supernatant samples and a range of purified ETX standards (31.25, 62.5, 125, 187.5, 250, and 312.5 ng) were run on an SDS-containing, 12% polyacrylamide gel and then Western blotted. Those immunoblots were quantified using a scanning densitometer in conjunction with NIH ImageJ software.

**RT-PCR.** Total RNA was extracted from *C. perfringens* cultures according to a previously described procedure (16). Briefly, 2 ml of 3-h TGY *C. perfringens* cultures was centrifuged at 4°C. The pellet was resuspended in 200 µl of acetate solution (20 mM sodium acetate [pH 5], 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS; Bio-Rad]). That suspension then received 200 µl of saturated phenol (Fisher Scientific), and the mixture was thoroughly resuspended before incubation at 60°C in a water bath with vigorous shaking for 5 min. After centrifugation at 4°C for 5 min, the nucleic acid-containing supernatant received cold ethanol, and the sample was mixed well. The mixed sample was then centrifuged at 4°C for 5 min to obtain the RNA pellet. This pellet was washed two times with cold 70% ethanol and finally resuspended in 100 µl of DNase-free, RNase-free water. All RNA samples were additionally treated with 2 U of DNase I (Promega) at 37°C for 30 min. To stop DNase I activity, DNase I inhibitor (Promega) was added to each reaction tube. RNA was quantified by absorbance at 260 nm and stored at -80°C.

RT-PCR was performed using the AccessQuick RT-PCR system (Promega). Briefly, the RT-PCR mixture (25 µl) contained the following components: 50 ng of each RNA sample, 20 pmol each of the forward and reverse primers, and 1 U of reverse transcriptase. cDNA synthesis was conducted at 45°C for 45 min. The following cycle conditions were used for PCR: 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C for extension. A DNA polymerase III (*polC*) housekeeping gene served as an internal control to normalize the expression levels between samples.

**Cell culture.** Human-derived, enterocyte-like Caco-2 cells were routinely maintained in minimal essential Eagle's medium (MEM) (Sigma) containing 10% fetal bovine serum (FBS; Life Technologies), 1% minimal essential medium with nonessential amino acids (Sigma), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Madin-Darby canine kidney

(MDCK) cells were cultured in a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) (Sigma) and Ham's F-12 (Sigma) supplemented with heat-inactivated 3% fetal calf serum (FCS), 1% nonessential amino acids, 1% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Each cell line was normally harvested with 0.25% trypsin (Gibco), resuspended in the cell culture medium, and maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub>.

**C. perfringens infection of Caco-2 cell cultures.** For *in vitro* infection experiments, Caco-2 cells were routinely cultured (4 to 5 days) in MEM until reaching confluence in either 100-mm tissue culture plate or 12-well microplates. Caco-2 cells were then washed 3 times with prewarmed phosphate-buffered saline (PBS [pH 7.4]). The washed cells were incubated in serum-free and antibiotic-free MEM before *C. perfringens* infection. As a control, the same volume of serum-free and antibiotic-free MEM (no Caco-2 cells included) was added to the microplate wells.

For some experiments, overnight cultures of wild-type CN3718, the isogenic *agrB* null mutant, or the complemented strain were pelleted and washed three times with prewarmed PBS. Caco-2 cells were infected with the washed *C. perfringens* cells in cell culture medium at a multiplicity of infection (MOI) of 20 bacteria per cell and then incubated for the indicated times at 37°C under anaerobic conditions using an anerobic pouch with the GasPak EZ anaerobe container system (BD, Franklin Lakes, NJ). After that challenge, the infected supernatants were collected, centrifuged at 4°C for 30 min, and analyzed by Western blotting for ETX levels using a mouse monoclonal anti-ETX antibody.

To further assess whether the upregulation of ETX noted in the above experiments (see Results) was caused by secreted factors, overnight cultures of CN3718 were pelleted and washed three times with prewarmed PBS. Those washed CN3718 cells were then resuspended in one of the following: (i) filter-sterilized serum- and antibiotic-free MEM supernatants removed from Caco-2 cell cultures that had been infected with the CN3718 *agrB* mutant for 5 h, (ii) filter-sterilized serum- and antibiotic-free MEM supernatants removed from noninfected Caco-2 cells that had been grown for 5 h, or (iii) filter-sterilized serum- and antibiotic-free MEM supernatants that had been removed from the CN3718 *agrB* mutant (no Caco-2 cells) grown for 5 h. All incubations were performed under anaerobic conditions.

**Cytotoxicity of *C. perfringens* type D strains.** (i) **Visualization of morphological damage.** To evaluate the cytotoxic consequences of the toxin upregulation detected upon contact of a type D strain with Caco-2 cells (see Results), Caco-2 cell cultures were first challenged with washed cells of wild-type CN3718, the *agrB* null mutant, or the CN3718:*agrB*(p3) complemented strain. After a 2-h anaerobic infection at 37°C, the culture supernatants were removed and filter sterilized using a 0.22-µm filter (Millipore). Those sterile supernatants, containing secreted proteins, were concentrated 10× using an Amicon Ultra-4 10K device. To activate the epsilon protoxin present in the supernatants, the concentrated supernatants were treated with trypsin (Sigma Aldrich) at a concentration of 12.5 µg/ml at 37°C for 1 h. The trypsin was then inactivated by adding an equal volume of trypsin inhibitor (Sigma Aldrich) at room temperature for 30 min. After centrifugation, the activated supernatants were applied to MDCK cells.

To verify that upregulation of ETX production by the Agr-like QS system was involved in causing any observed MDCK cell damage, a seroneutralization approach was applied. Briefly, a neutralizing MAb against ETX was added to the trypsin-activated supernatants, and the supernatants were then incubated at room temperature for 15 min.

After a 2-h treatment, the cytopathic effects caused by the toxin were analyzed in terms of morphological changes. Control or damaged cells were photographed using a Canon Powershot G5 fitted to the Zeiss Axiovert 25 microscope, and images were processed using Adobe Photoshop 8.0.

(ii) **Evaluation of MDCK cytotoxic effects.** A lactate dehydrogenase (LDH) release assay kit (Invitrogen) for mammalian cell death was used to assess the involvement of the Agr-like QS system or VirR/VirS two-

component regulatory system in CN3718-induced MDCK cell cytotoxicity. The assay was performed as described by the supplier, after treatment of MDCK monolayers for 2 h at 37°C with trypsin-activated Caco-2 cell supernatants, prepared as described above. The absorbance of each sample was measured at 490 nm with an iMark microplate reader (Bio-Rad). The results are expressed as the percentage of LDH release versus the total LDH present in cells.

**Analysis of cross talk signaling.** Wild-type CN3718, a previously prepared *etx* null mutant (35), and the *agrB* null mutant were each grown in TGY broth at 37°C overnight. All strains were centrifuged at 4°C, and the pellets were washed 3 times with warmed PBS. After resuspension in fresh TGY medium, the washed bacterial cells were seeded, as specified, into the top or bottom chambers of Transwell plates (0.4 µm pore size; Corning). The ratio of *agrB* null mutant to *etx* null mutant inoculated into the chambers in this cross talk experiment was 1:3. After a 3.5- or 5-h incubation, the supernatants were collected and subjected to Western blot analysis using anti-ETX MAb.

**Nucleotide sequence accession number.** The sequence of the CN3718 *agr* locus has been deposited in GenBank under accession no. JN543538. The CN3718 *etx* upstream sequence has been deposited in GenBank under accession no. JN543539.

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