The VirS/VirR Two-Component System Regulates the Anaerobic Cytotoxicity, Intestinal Pathogenicity, and Enterotoxemic Lethality of *Clostridium perfringens* Type C Isolate CN3685

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ABSTRACT ***Clostridium perfringens*** **vegetative cells cause both histotoxic infections (e.g., gas gangrene) and diseases originating in the intestines (e.g., hemorrhagic necrotizing enteritis or lethal enterotoxemia). Despite their medical and veterinary importance, the molecular pathogenicity of *C. perfringens* vegetative cells causing diseases of intestinal origin remains poorly understood. However, *C. perfringens* beta toxin (CPB) was recently shown to be important when vegetative cells of *C. perfringens* type C strain CN3685 induce hemorrhagic necrotizing enteritis and lethal enterotoxemia. Additionally, the VirS/VirR two-component regulatory system was found to control CPB production by CN3685 vegetative cells during aerobic infection of cultured enterocyte-like Caco-2 cells. Using an isogenic *virR* null mutant, the current study now reports that the VirS/VirR system also regulates CN3685 cytotoxicity during infection of Caco-2 cells under anaerobic conditions, as found in the intestines. More importantly, the *virR* mutant lost the ability to cause hemorrhagic necrotic enteritis in rabbit small intestinal loops. Western blot analyses demonstrated that the VirS/VirR system mediates necrotizing enteritis, at least in part, by controlling *in vivo* CPB production. In addition, vegetative cells of the isogenic *virR* null mutant were, relative to wild-type vegetative cells, strongly attenuated in their lethality in a mouse enterotoxemia model. Collectively, these results identify the first regulator of *in vivo* pathogenicity for *C. perfringens* vegetative cells causing disease originating in the complex intestinal environment. Since VirS/VirR also mediates histotoxic infections, this two-component regulatory system now assumes a global role in regulating a spectrum of infections caused by *C. perfringens* vegetative cells.**

IMPORTANCE ***Clostridium perfringens*** **is an important human and veterinary pathogen. *C. perfringens* vegetative cells cause both histotoxic infections, e.g., traumatic gas gangrene, and infections originating when this bacterium grows in the intestines. The VirS/VirR two-component regulatory system has been shown to control the pathogenicity of *C. perfringens* type A strains in a mouse gas gangrene model, but there is no understanding of pathogenicity regulation when *C. perfringens* vegetative cells cause disease originating in the complex intestinal environment. The current study establishes that VirS/VirR controls vegetative cell pathogenicity when *C. perfringens* type C isolates cause hemorrhagic necrotic enteritis and lethal enterotoxemia (i.e., toxin absorption from the intestines into the circulation, allowing targeting of internal organs). This effect involves VirS/VirR-mediated regulation of beta toxin production *in vivo*. Therefore, VirS/VirR is the first identified global *in vivo* regulator controlling the ability of *C. perfringens* vegetative cells to cause gas gangrene and, at least some, intestinal infections.**

Received 21 December 2010 **Accepted** 29 December 2010 **Published** 25 January 2011

Citation Ma, M., J. Vidal, J. Saputo, B. A. McClane, and F. Uzal. 2011. The VirS/VirR two-component system regulates the anaerobic cytotoxicity, intestinal pathogenicity, and enterotoxemic lethality of *Clostridium perfringens* type C isolate CN3685. mBio 2(1):e00338-10. doi:10.1128/mBio.00338-10.

Editor R. John Collier, Harvard Medical School

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Clostridium perfringens ranks among the most important bacterial pathogens affecting humans and domestic animals (1). The pathogenicity of this Gram-positive anaerobe is largely attributable to its prolific toxin-producing capacity. However, individual strains never produce all 17 identified *C. perfringens* toxins, providing the basis for a toxinotyping classification system that assigns individual isolates to types (A to E) based upon their production of alpha, beta, epsilon, and iota toxins (1–4). Besides producing one or more typing toxins, *C. perfringens* isolates commonly produce toxins such as perfringolysin O (PFO) (5–7).

Different *C. perfringens* types are associated with specific diseases (1, 3). Vegetative cells of *C. perfringens* type C strains, which by definition must produce (at minimum) alpha toxin (CPA) and beta toxin (CPB), cause human enteritis necroticans (8–10). Enteritis necroticans is currently endemic throughout the developing world but is historically most associated with the Papua New Guinea (PNG) highlands (8–10). In the 1960s to 1970s, enteritis necroticans (locally named pigbel) was the leading cause of mortality in children >1 year of age living in the PNG highlands. Controlled by vaccination during the 1980s, pigbel is now re-

emerging in the PNG highlands. Enteritis necroticans from type C infections also occasionally occurs in developed countries, predominantly affecting diabetics (11, 12).

Enteritis necroticans involves abdominal pain, bloody stool, vomiting, and, in severe (often rapidly fatal) cases, toxemia and shock (8–10). Most commonly the jejunum is affected, although the ileum or the entire small intestine can be involved. Histologically, blunted villi are observed, along with numerous *C. perfringens* vegetative cells present on the mucosal surface of necrotic intestinal tissue (13). Enteritis necroticans typically occurs in people with low intestinal trypsin levels due to malnutrition, coinfection with *Ascaris* strains producing trypsin inhibitor, or underlying pancreatic disease. Those associations suggested that trypsin is an important host intestinal defense factor against type C infection (9), as supported by the need to add trypsin inhibitor (TI) for type C cultures to produce disease in animal infection models (4, 14).

Type C isolates also cause fatal disease in most livestock species, which economically impacts the agricultural industry (1, 2). As with human disease, veterinary diseases caused by type C strains typically involve hemorrhagic necrotic enteritis and enterotoxemias, i.e., absorption of toxins from the intestines into the circulation, leading to damage of internal organs distant from the gastrointestinal tract. While adult animals can be sickened or killed by type C infection, this illness most commonly affects neonatal animals, particularly lambs, calves, piglets, and foals. This association likely reflects a greater toxin sensitivity of neonatal animals due to the low trypsin levels present in their intestines and trypsin inhibitor effects of the colostrum.

Despite their medical and veterinary importance, the molecular pathogenicity of *C. perfringens* type C vegetative cells only recently came under study. By exploiting advances in *C. perfringens* genetics (15), isogenic CN3685 toxin null mutants were constructed and their pathogenicity was tested in rabbit intestinal loops (4), which showed that CPB (but not CPA or PFO) is necessary for hemorrhagic necrotic enteritis. Furthermore, administration of purified CPB (in the presence of TI) into rabbit small intestinal loops produced pathology similar to that observed with wild-type CN3685 infection. Follow-up rabbit small intestinal loop studies then demonstrated that purified CPB (plus TI) extensively damages the jejunum and ileum and somewhat damages the duodenum but has little or no effect on the colon (16). Since rabbit small intestinal loop assays of type C isolate-induced necrotic enteritis terminate before death, a mouse intraduodenal (ID)-challenge model was developed to reproduce type C isolate-induced lethal enterotoxemia (14). When the CN3685 toxin null mutants were tested in this mouse model, CPB was identified as the major contributing toxin to fatal enterotoxemia caused by CN3685 vegetative cells (14).

Similarly, toxin production regulation by type C vegetative cells only recently came under study. Since the VirS/VirR two-component regulatory system controls CPA and PFO production by *C. perfringens* type A isolates (17–20), a *virR* null mutant of CN3685 was constructed to test whether *in vitro* CPB production might also involve VirS/VirR-mediated regulation (21). When enterocyte-like Caco-2 cell cultures were infected under aerobic conditions, the isogenic *virR* null mutant exhibited substantially less transcription of genes encoding CPB and PFO than wild-type CN3685 or a VirS/VirR complementing strain (21).

It is currently unclear how *C. perfringens* type C vegetative cells regulate their *in vivo* pathogenicity when causing diseases originating in the intestines. The *in vitro* results (21) indicating that VirS/VirR regulates toxin expression by type C vegetative cells during aerobic infection of Caco-2 cell cultures might suggest that this two-component regulatory system also mediates type C pathogenicity *in vivo*. However, *in vitro* findings cannot always be extrapolated to *in vivo* virulence, since virulence gene expression is often environmentally influenced. Virulence gene expression by type C isolates is clearly environmentally sensitive, since under aerobic conditions, CPB expression is strongly influenced by the presence of host cells (21). Thus, the regulation of virulence gene expression by type C isolates may differ *in vitro* from that in the complex intestinal environment.

Therefore, the current study directly tested whether the VirS/VirR system is important for type C isolate virulence by comparing the abilities of washed vegetative cells of wild-type CN3685, the isogenic CPJV47 *virR* null mutant of CN3685, and the VirS/VirR complementing strain CPJV47(pTS405) to cause (i) cytotoxicity in Caco-2 cell cultures infected under anaerobic conditions, (ii) necrotizing enteritis in rabbit small intestinal loops, and (iii) enterotoxemic lethality in mice. Results from these studies provide the first insights into the *in vivo* regulation of pathogenicity for *C. perfringens* vegetative cells causing enteritis and enterotoxemia.

RESULTS

CPB production by vegetative cells of type C wild-type strain CN3685, the isogenic CPJV47 *virR* null mutant, and the CPJV47(pTS405) complementing strain when grown in TGY broth containing thioglycolate. Previous studies (21) reported that, when incubated under aerobic conditions in the presence of Caco-2 cells, vegetative cells of CPJV47 do not produce CPB, in contrast to the strong CPB production observed under those conditions for vegetative cultures of CN3685 or CPJV47(pTS405). Since earlier studies have also established the importance of CPB for CN3685 pathogenicity (4), the VirS/VirR two-component system (encoded by the *virS/virR* operon) might regulate CN3685 vegetative cell pathogenicity *in vivo*. However, virulence gene expression (including CPB expression) is often influenced by environmental factors, potentially including oxygen. Therefore, before animal testing of VirS/VirR's role in type C isolate pathogenicity became justifiable, two *in vitro* studies were performed to evaluate whether the VirS/VirR system regulates CPB production by CN3685 vegetative cells under anaerobic conditions, as occurs in the intestines during disease.

VirS/VirR regulation of CPB production under anaerobic conditions was first evaluated using cultures of CN3685 or its derivatives grown in TGY (3% tryptic soy broth, 2% glucose, 1% yeast extract) broth containing thioglycolate, which removes oxygen to allow the vegetative growth of *C. perfringens*. Under those growth conditions, Western blot analyses detected CPB production by wild-type CN3685, starting within 4 h postinoculation (data not shown); the presence of CPB remained detectable in overnight cultures (Fig. 1). However, under similar growth conditions, CPB was not detected in 4-h (data not shown) or overnight (Fig. 1) CPJV47 cultures. This loss of CPB production resulted from *virS/virR* operon disruption since CPJV47(pTS405) made CPB in TGY containing thioglycolate, where this toxin production started within 4 h (not shown) and CPB remained detectable in overnight cultures (Fig. 1).

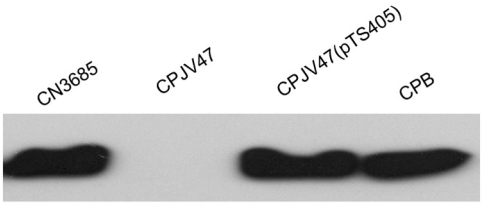

FIG 1 CPB production by CN3685 and its isogenic derivatives grown in TGY broth containing thioglycolate. Wild-type CN3685, the CPJV47 *virR* null mutant, and the CPJV47(pTS405) complementing strain were grown overnight at 37°C in TGY broth containing thioglycolate. Culture supernatants were then subjected to Western blotting using a CPB-specific monoclonal antibody. The migration of purified CPB (35 kDa) is shown in the right lane of the blot.

These CPB production differences were not due to the slower growth of CPJV47 vegetative cells, since the wild-type, *virR* null mutant, and complementing strains all grew similarly in TGY broth containing thioglycolate (data not shown).

Caco-2 cell cytotoxicity after anaerobic infection with CN3685, CPJV47, and CPJV47(pTS405). A second *in vitro* study evaluated whether the VirS/VirR system regulates the development of host cell cytotoxicity when washed vegetative cells of wild-type CN3685, the CPJV47 *virR* null mutant, or the CPJV47(pTS405) complementing strain was suspended in fresh minimal essential medium (MEM) and then used to infect enterocyte-like Caco-2 cell cultures under anaerobic conditions. This experiment represents a simplified *in vitro* model of natural disease, where type C strains encounter enterocytes in the anaerobic intestinal environment, and permitted later comparisons of VirS/VirR regulation of CN3685-induced *in vitro* cytotoxicity versus *in vivo* pathogenicity.

As shown in Fig. 2A, a 1-h anaerobic infection with washed CN3685 vegetative cells caused extensive Caco-2 cell rounding. However, under the same conditions, washed vegetative cells of CPJV47 produced little or no morphological damage to Caco-2 cells at 1 h. This cytotoxicity attenuation was specifically due to inactivation of the *virS/virR* operon in CPJV47, since a 1-h anaerobic infection with washed vegetative cells of CPJV47(pTS405) produced morphological damage similar to that caused by wild-type CN3685. The attenuated Caco-2 cell cytotoxicity observed for the CPJV47 infection did not result from reduced bacterial numbers compared to those for the wild-type and complementing strains (data not shown).

To quantify differences in Caco-2 cell cytotoxicity following anaerobic infection with each CN3685-derived strain, lactate dehydrogenase (LDH) release was measured in supernatants removed from Caco-2 cell cultures after a 1-h anaerobic infection. This analysis revealed that anaerobic infection with washed CN3685 cells caused substantial Caco-2 cell cytotoxicity within 1 h (Fig. 2B). At that same time point, considerably less LDH release occurred in Caco-2 cell cultures anaerobically infected with washed CPJV47 cells (Fig. 2B). However, washed cells of CPJV47(pTS405) caused wild-type-infection-like cytotoxicity levels after a 1-h anaerobic infection of Caco-2 cell cultures, demonstrating that the attenuated cytotoxic properties of CPJV47 for Caco-2 cells was specifically due to inactivation of the *virS/virR* operon (Fig. 2B). When these studies were extended to a 2-h anaerobic infection, extensive cell rounding and detachment became visible in all Caco-2 cultures, whether the infection involved washed CN3685, CPJV47, or CPJV47(pTS405) vegetative cells (not shown).

Western blot analyses of supernatants from anaerobically infected Caco-2 cultures did not detect CPB production after 1 h of infection with washed cells of CN3685 or its derivatives (data not shown). However, CPB production was demonstrable after a 2-h anaerobic infection with washed cells of wild-type

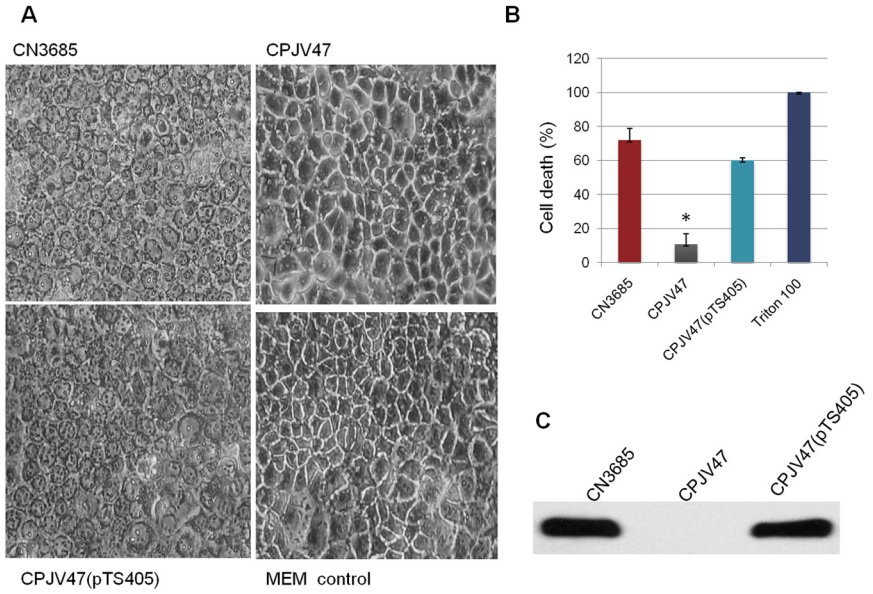

FIG 2 Anaerobic infection of Caco-2 cells by CN3685 or its isogenic derivatives. (A) Morphological damage to Caco-2 cells. Confluent Caco-2 cell cultures were infected at an MOI of 20:1 for 1 h at 37°C under anaerobic conditions. Inocula used for these infections included fresh MEM containing washed vegetative cells of wild-type CN3685, the CPJV47 *virR* null mutant, or the CPJV47(pTS405) complementing strain. Morphological damage to Caco-2 cells observed after a 1-h anaerobic infection with CN3685 or CPJV47(pTS405) included cell rounding and the beginning of cell detachment from the culture dish surface. Note that Caco-2 cell cultures infected with CPJV47 appeared similar to control cells at this time point. Magnification, ×100. (B) Lactate dehydrogenase (LDH) release by Caco-2 cells. LDH release was measured as an indicator of Caco-2 cell cytotoxicity induced by a 1-h anaerobic infection at 37°C with an MOI of 20:1 of washed cells of CN3685 or its isogenic derivatives, as specified. Culture supernatants were removed from the infected Caco-2 cell cultures and analyzed with an LDH cytotoxicity detection kit. Shown are mean results from three independent repetitions. Error bars depict standard deviations (SD). Note that no LDH activity was detectable when CN3685 (or its derivatives) was suspended in MEM in the absence of Caco-2 cells (data not shown). *, samples showing a statistically significant ($P < 0.01$) decrease in LDH cytotoxicity compared with that of Caco-2 cultures infected with washed CN3685 cells. (C) CPB production during Caco-2 cell infection. Western blot analysis was performed to evaluate *in vitro* CPB production by CN3685 and its derivatives after an anaerobic infection of Caco-2 cell cultures. After a 2-h infection at an MOI of 20:1 with washed cells of CN3685, CPJV47, or CPJV47(pTS405) suspended in fresh MEM, culture supernatants were removed from each cell culture well. An aliquot of each supernatant was then subjected to Western blotting using a CPB-specific monoclonal antibody (4).

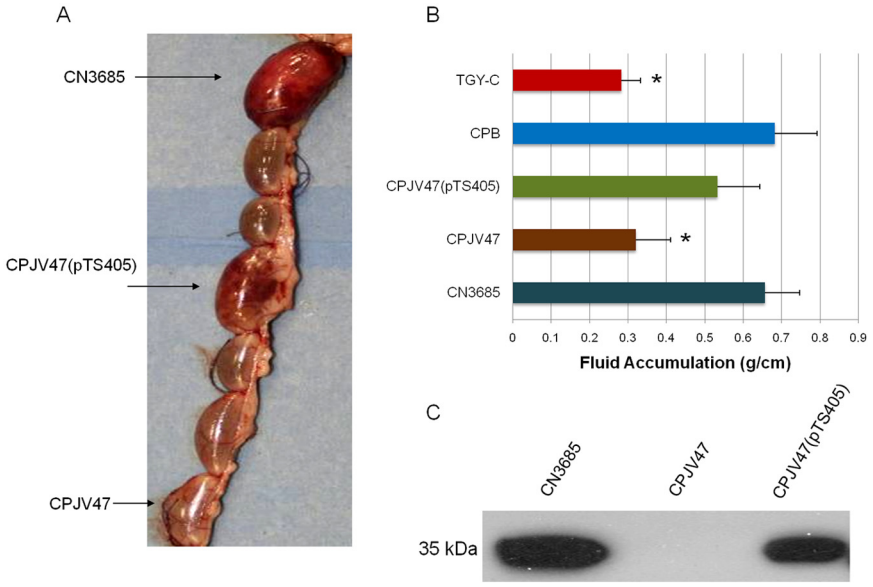

FIG 3 Ligated rabbit small intestinal loop responses after inoculation with CN3685, the CPJV47 *virR* null mutant, or the CPJV47(pTS405) complementing strain. (A) Gross pathology. Shown is the gross pathology developing in loops after a 6-h infection with TGY-C containing 10^8 washed cells of CN3685 or its derivatives. Note that loops inoculated with CN3685 or CPJV47(pTS405) were hemorrhagic and distended with fluid. No gross abnormalities were observed in loops inoculated with CPJV47 or sterile medium. Interloops, which received no inoculum, were constructed between sample loops to eliminate possible sample leakage or cross contamination. (B) Fluid accumulation. Shown are fluid accumulation levels measured in rabbit small intestinal loops 6 h after receiving an inoculum of TGY-C containing 10^8 washed cells of CN3685, CPJV47, or CPJV47(pTS405). Also shown are fluid accumulation levels measured 6 h after some loops received 10 μg of purified CPB, as a positive control, or sterile TGY-C. *, loops showing a statistically significant ($P < 0.05$) decrease in fluid levels compared to loops infected with washed CN3685 cells. Error bars show standard errors of the means (SEM). (C) Western blot analysis of *in vivo* CPB production in luminal fluids recovered from rabbit small intestinal loops after infection with CN3685, CPJV47, or CPJV47(pTS405). After a 6-h infection with fresh TGY-C containing 10^8 washed cells of CN3685 or its derivatives, luminal fluids were removed from each loop and then analyzed by Western blotting, using a CPB-specific monoclonal antibody (4).

CN3685 or the complementing strain (Fig. 2C). In contrast, no CPB production was evident after washed cells of the *virR* null mutant were used for either a 2-h (Fig. 2C) or a 3-h (data not shown) anaerobic infection of Caco-2 cultures.

Finally, Western blotting of culture supernatants from CN3685 grown anaerobically in MEM, which did or did not contain Caco-2 cells, revealed host cell-mediated upregulation of CPB expression (data not shown), similar to the results of previous reports using aerobic incubation (21).

Pathogenicity of CN3685, CPJV47, and CPJV47(pTS405) vegetative cells in the rabbit small intestinal loop model of type C hemorrhagic necrotizing enteritis. The Fig. 2 results indicating the importance of VirS/VirR for the rapid onset of CN3685 vegetative cell-induced cytotoxicity in Caco-2 cells under anaerobic infection conditions, as is present in the intestines, suggested that this two-component system could also regulate the *in vivo* pathogenicity of CN3685 in the complex intestinal environment. Since type C isolates cause hemorrhagic necrotizing enteritis in humans and livestock, the vegetative cell pathogenicities of wild-type CN3685, the isogenic CPJV47 *virR* null mutant, and the CPJV47(pTS405) VirS/VirR complementing strain were compared in a previously described rabbit small intestinal loop model of type C hemorrhagic necrotizing enteritis (4). Bacterial inocula for these experiments used washed vegetative cells suspended in fresh TGY medium containing cysteine hydrochloride (TGY-C) and TI in order to remove virulence contributions from toxins produced during the *in vitro* growth necessary to prepare the inoculating culture.

(i) Gross pathology of small intestinal loops. As in a previous report (4), mucosal and luminal hemorrhages were observed 6 h after introduction of the washed CN3685 vegetative cell inoculum into rabbit small intestinal loops (Fig. 3A). These effects were visible from both the mucosal and the serosal surfaces. The intestinal wall was thin and lacked natural tone. For comparison, 6 h of treatment of rabbit small intestinal loops with an inoculum containing purified CPB plus TI caused similar gross pathology, consistent with previous conclusions that CPB is sufficient to cause the hemorrhagic necrotizing enteritis associated with CN3685 infection of rabbit small intestinal loops (4).

A 6-h incubation of rabbit small intestinal loops with washed vegetative cells of the isogenic CPJV47 *virR* null mutant or with sterile TGY-C (negative control) caused no significant gross pathological abnormalities, in contrast to the wild-type-infection results. However, rabbit small intestinal loops incubated for 6 h with a washed vegetative cell inoculum of the CPJV47(pTS405) VirS/VirR complementing strain showed gross lesions indistinguishable from those produced by CN3685 infection, confirming that the lack of gross pathological damage associated with CPJV47 infection was attributable to inactivation of the *virS/virR* operon.

(ii) Luminal fluid accumulation in rabbit small intestinal loops. More luminal fluid accumulated in loops receiving an inoculum containing either washed CN3685 vegetative cells or purified CPB (plus TI) than in loops receiving an inoculum of sterile TGY-C medium plus TI or washed vegetative cells of CPJV47 (Fig. 3B). This attenuation of luminal fluid accumulation was specifically attributable to mutation of the *virS/virR* operon in CPJV47, since an inoculum containing washed vegetative cells of CPJV47(pTS405) caused more luminal fluid accumulation than was measured in loops receiving an inoculum containing washed vegetative cells of the CPJV47 *virR* null mutant (Fig. 3B).

(iii) Histology of rabbit small intestinal loops. When treated for 6 h with an inoculum containing washed CN3685 vegetative cells or purified CPB plus TI, small intestinal loops developed severe diffuse necrotizing and hemorrhagic enteritis, as in a previous report (4). These effects included a diffuse, nearly complete, loss of the superficial epithelium, villus blunting, pseudomembrane formation, and mucosal and submucosal thrombosis (Fig. 4; Table 1). In contrast, loops treated for 6 h with an inoculum containing either washed vegetative cells of the CPJV47 *virR* null mutant or sterile TGY-C plus TI (as a negative control) showed no significant histological abnormalities, except for mild

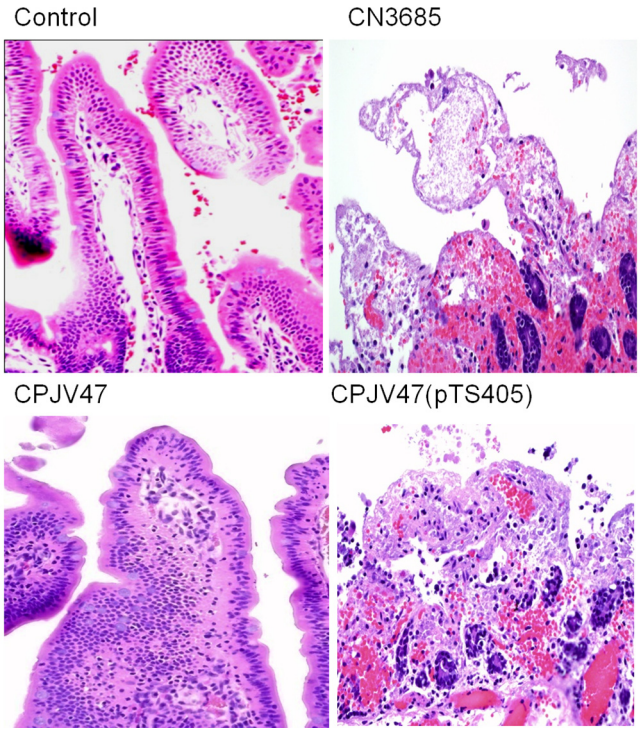

FIG 4 Histologic damage in rabbit ileal loops treated for 6 h with washed cells of wild-type CN3685, the CPJV47 *virR* null mutant, or the CPJV47(pTS405) complementing strain. Control loops, inoculated with sterile TGY-C, or loops inoculated with fresh TGY-C containing 10^8 washed cells of CPJV47 showed normal, full-length intestinal villi with a well-preserved epithelium and lamina propria. In contrast, loops inoculated with fresh TGY-C containing 10^8 washed cells of CN3685 or CPJV47(pTS405) exhibited substantial histologic damage, including necrosis and desquamation of the epithelium, necrosis of the lamina propria, blunting of villi, hemorrhaging of the mucosa, and a diffuse neutrophilic infiltration of the mucosa and submucosa. Sections were stained with hematoxylin and eosin and photographed at a ×200 magnification.

submucosal edema and dilation of submucosal lymphatic vessels. The lack of histologic damage observed in CPJV47-treated loops specifically involved inactivation of the *virS/virR* operon, since an inoculum containing washed cells of the CPJV47(pTS405) complementing strain caused lesions similar to those observed in loops receiving an inoculum of wild-type CN3685 vegetative cells (Fig. 4; Table 1).

Mechanistic basis for the differences between CN3685, CPJV47, and CPJV47(pTS405) vegetative cell pathogenicity in rabbit small intestinal loops. With analyses indicating that the VirS/VirR two-component system is necessary for CN3685 to cause hemorrhagic necrotizing enteritis in rabbit small intestinal loops, the mechanistic basis for the differences between CN3685, CPJV47, and CPJV47(pTS405) vegetative cell pathogenicity in rabbit small intestinal loops was explored (Fig. 3 and 4). A first experiment examined whether vegetative cells of the CPJV47 *virR* null mutant might be less pathogenic in rabbit loops because those mutant vegetative cells survive more poorly *in vivo* than in wild-type CN3685 vegetative cells. However, similar numbers of vegetative cells of wild-type CN3685, CPJV47, and CPJV47(pTS405) were recovered from small intestinal loops after a 6-h incubation (data not shown).

Since CPB was previously shown to be necessary for CN3685 pathogenicity in rabbit small intestinal loops (4) and VirS/VirR can regulate CPB production *in vitro* (21; this study), a second explanation for the attenuated pathogenicity displayed by vegetative cells of the CPJV47 *virR* null mutant in rabbit small intestinal loops may involve VirS/VirR regulating *in vivo* CPB production. When this hypothesis was tested by Western blot analyses of CPB levels in luminal fluid recovered from infected rabbit small intestinal loops (Fig. 3C), the presence of significant amounts (~30 μg/ml) of CPB was detected in luminal fluids recovered from loops incubated for 6 h with an inoculum containing washed wild-type CN3685 vegetative cells. In contrast, no CPB was detected in loops after a 6-h infection using an inoculum containing washed CPJV47 vegetative cells. The absence of detectable CPB in loops receiving an inoculum containing washed CPJV47 vegetative cells was reversible by complementation, with ~20 μg/ml of CPB detected in luminal fluids from loops receiving inocula containing CPJV47(pTS405) washed cells and then incubated for 6 h. Since the inocula for these experiments used washed vegetative cells, the Fig. 3C results demonstrate that the VirS/VirR two-component regulatory system governs *in vivo* CPB production.

Comparison of the lethal effects induced by CN3685, CPJV47, and CPJV47(pTS405) vegetative cells in the mouse ID-challenge model of type C enterotoxemia. Besides causing necrotizing enteritis, type C vegetative cells cause enterotoxemia, where toxins produced in the intestines are absorbed into the circulation to damage internal organs and induce lethality. Therefore, the current study applied a recently developed mouse ID-challenge model of lethal enterotoxemia caused by type C isolates (14) to compare the lethal effects of inocula containing washed vegetative cells of the wild-type strain, the isogenic *virR* null mutant, and the complementing strain resuspended in TGY-C plus TI.

(i) Mouse lethality. As reported previously (14), an ID inoculum containing washed CN3685 vegetative cells produced lethality in nearly all challenged mice (Fig. 5). Clinical signs, in most cases followed by spontaneous death, developed at 8 to 24 h post-

TABLE 1 Rabbit small intestinal loop histological changes[a]

Sample	Pathology score for:					
	Desquamation	Necrosis of epithelium	Necrosis of lamina propria	Inflammation	Villus blunting	Overall
CN3685	4.2 ± 0.8	4.2 ± 0.9	4.3 ± 0.8	3.8 ± 1.0	3.4 ± 1.1	4.28 ± 0.9
CPJV47	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.8 ± 0.5[b]	0.0 ± 0.0[b]	0.37 ± 0.2[b]
CPJV47(pTS405)	3.6 ± 0.7	3.5 ± 0.6	3.5 ± 0.5	2.9 ± 0.9	2.7 ± 0.8	3.56 ± 0.6
CPB	4.8 ± 0.4	4.8 ± 0.4	4.8 ± 0.4	4.4 ± 0.9	3.8 ± 1.4	4.84 ± 0.4
TGY	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	1.0 ± 0.0[b]	0.0 ± 0.0[b]	0.5 ± 0.0[b]

[a] Pathology was scored by a pathologist in a blind manner on a 0-to-5 point scale (with 0.5-point increments); a score of 5 represents maximal effect, and a score of 0 represents no effect. Results shown are for eight rabbits.

[b] This value indicates a statistically significant ($P < 0.05$) difference from the value for wild-type infection.

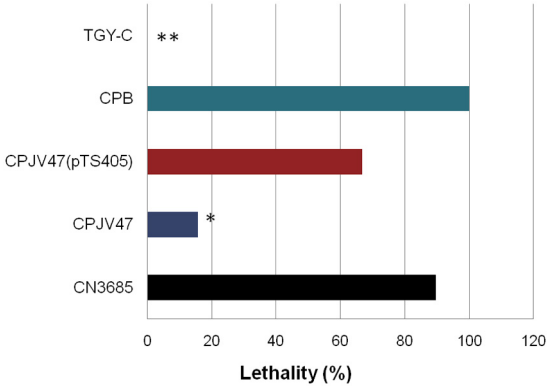

FIG 5 Lethality in mice inoculated with wild-type CN3685, the CPJV47 *virR* null mutant, or the CPJV47(pTS405) complementing strain. Mice were ID challenged with TGY-C containing washed cells of CN3685 or its derivatives. Lethality was recorded over a 48-h period postinoculation. Also shown for comparison is the lethality caused by ID injection of CPB (25 μg). Note the absence of any lethality following ID challenge with TGY-C. Each challenge group included 18 or 19 mice. The only statistically significant ($P < 0.05$) difference from wild-type-CN3685-induced lethality was observed for CPJV47. The complementing strain was also significantly ($P < 0.05$) more lethal that CPJV47.

challenge. Those signs consisted of a swollen abdomen, depression, inappetence, tachypnea with superficial abdominal breathing, and neurological signs, including circling and rolling.

Lethality in mice receiving an ID inoculum containing washed vegetative cells of CPJV47 was significantly lower (Fig. 5) than in mice receiving CN3685. This effect was specifically due to inactivation of the *virS*/*virR* operon in CPJV47, since complementation to restore VirS/VirR expression also significantly enhanced lethality (Fig. 5).

No clinical disease or lethality was observed in negative-control mice receiving an ID inoculum of sterile TGY-C broth plus TI (Fig. 5). Lethality in mice receiving an ID injection of 25 μg of purified CPB plus TI, as a positive control, was 100%.

(ii) Mouse pathology. As in previous reports (14), an ID inoculum containing washed wild-type CN3685 vegetative cells produced gross changes consisting only of a distended abdomen with large amounts of gas, predominantly in the small bowel but affecting also the cecum and colon in a few animals. Histologically, in all mice inoculated with vegetative cells of CN3685, the mucosa was severely attenuated, with villus blunting (data not shown). Mice receiving an ID-challenge inoculum containing washed vegetative cells of the CPJV47 isogenic *virR* null mutant exhibited only mild gas distention of the small intestine, with mild mucosal attenuation. In contrast, mice receiving an ID inoculation of washed CPJV47(pTS405) vegetative cells showed gross and histological changes similar to those produced by the wild type (data not shown). No other significant histological abnormalities were observed in any mice in this study.

Mouse colonization and CPB production. No differences were noted (data not shown) in the numbers of type C cells recovered from the colon/rectum or small intestine of mice challenged with CN3685, CPJV47, or CPJV47(pTS405). Attempts to detect by Western blotting the presence of CPB in the colon/rectum or small intestine of these mice were unsuccessful.

DISCUSSION

Vegetative cells of *C. perfringens* type B, C, and D strains are very important causes of disease originating in the mammalian intestines (1), including potentially fatal necrotic enteritis and/or enterotoxemia in humans (type C strains) or nonavian livestock (type B, C, and D strains). These non-type A isolates always produce numerous toxins, which once made it quite challenging to study their molecular pathogenicity. However, the recent development of improved genetic tools for *C. perfringens* has remedied this situation (15). With our exploitation of these advances, type C isolates have emerged as the initial paradigm for studying mammalian infections of intestinal origin caused by *C. perfringens* vegetative cells. For example, molecular Koch's postulate studies demonstrated that, although important for type A strains to cause gas gangrene (22–24), CPA and PFO are apparently not major contributors to hemorrhagic necrotic enteritis or lethal enterotoxemias caused by type C disease strain CN3685 (4). Instead, CPB (not produced by type A strains) was found to be important when CN3685 vegetative cells cause hemorrhagic necrotic enteritis and fatal enterotoxemia in animal models (4, 14).

Pathogens sense their environment to regulate the production of virulence factors, including toxins (25). Consistent with that theme, CN3685 vegetative cells were previously shown (21) to sense, *in vitro*, the presence of host cells and upregulate their toxin production under aerobic conditions. Furthermore, the VirS/VirR two-component system is emerging as a key controller of *in vitro C. perfringens* toxin production. Initial studies had established that VirS/VirR regulates the *in vitro* production of CPA and PFO by type A vegetative cells (17, 18), and it was later determined that this two-component system also controls the *in vitro* expression of beta2 toxin by type A strains (26). More recently, VirS/VirR was directly linked to the upregulation of *in vitro* CPB production occurring when type C CN3685 vegetative cells contact Caco-2 cells under aerobic conditions (21). The current study now demonstrates that VirS/VirR is also essential for CN3685 vegetative cells to produce CPB under *in vitro* anaerobic conditions, whether or not host cells are present. Finally, the VirS/VirR system was recently shown to regulate the *in vitro* production of NetB (27), a toxin important when type A strains cause avian necrotic enteritis (28). Collectively, these results indicate that VirR/VirS helps *C. perfringens* vegetative cells sense their environment so that they can regulate the expression of both chromosomally encoded toxins (CPA and PFO) and plasmid-encoded toxins (CPB, NetB, and beta2 toxin).

However, VirS/VirR involvement in regulating *C. perfringens* toxin production has not yet been evaluated in the complex mammalian intestinal environment. The current work now clearly demonstrates that this two-component regulatory system is essential for CPB production by type C strain CN3685 in the rabbit small intestinal loop model. The importance of VirS/VirR for *in vivo* production of other toxins produced by *C. perfringens* vegetative cells is under investigation.

The single most important finding of the current study is that VirS/VirR mediates the vegetative cell pathogenicity of *C. perfringens* strains that cause disease originating in the mammalian intestines. Specifically, our results clearly establish the importance of the VirS/VirR system when vegetative cells of type C strain CN3685 cause hemorrhagic necrotizing enteritis in rabbit small intestinal loops or lethal enterotoxemia in mice. The extent to which VirS/VirR mediates the pathogenicity of other *C. perfringens* strains causing disease that originates in animal intestines requires further study. However, since this two-component regulatory system can regulate the *in vitro* production of NetB toxin

(27), which appears important for avian necrotic enteritis (28), VirS/VirR also likely mediates avian necrotic enteritis caused by NetB-positive type A strains. Since previous studies showed that the VirS/VirR system is necessary for type A vegetative cells to cause gas gangrene in the mouse model (17), this two-component system is emerging as a key global pathogenicity regulator for many *C. perfringens* vegetative cells.

As briefly mentioned, the current study also identified a mechanism by which VirS/VirR regulates the ability of CN3685 vegetative cells to cause hemorrhagic necrotizing enteritis in rabbit small intestinal loops. Specifically, this two-component system was shown to regulate *in vivo* CPB production by washed CN3685 vegetative cells after inoculation into rabbit small intestinal loops. This finding is important for understanding type C disease pathogenesis since type C isolates cause infections, not intoxications, and previous studies established that CPB production is necessary for CN3685 vegetative cells to cause hemorrhagic necrotic enteritis in the rabbit small intestinal loop model (4).

Besides having an essential role in hemorrhagic necrotic enteritis, VirS/VirR was found to be important when CN3685 causes lethal enterotoxemias in the mouse ID-challenge model. The mechanism behind this effect could not be formally proven since Western blot analyses could not demonstrate *in vivo* CPB production even by wild-type CN3685 in the mouse ID-challenge model. The inability to detect *in vivo* CPB production in the intestines of these mice may reflect (at least in part) (i) the open nature of the mouse ID model, where TI or unbound, nonadsorbed toxin could be flushed from the gastrointestinal system, or (ii) the continual supply of fresh trypsin into the intestines of these mice, which may overwhelm the TI initially present in the intestines, leading to substantial degradation of trypsin-sensitive CPB (14).

Nevertheless, control of CPB production is probably an important mechanism behind the observed VirS/VirR regulation of lethal enterotoxemia in this mouse model, considering (i) the current findings demonstrating VirS/VirR regulation of CPB production by CN3685 during infection of rabbit small intestinal loops and (ii) the established importance of CPB production for CN3685-induced lethality in the mouse enterotoxemia model (16). In addition, the hypothesis that VirS/VirR controls, at least in part, CN3685-induced enterotoxemic lethality by regulating CPB production in ID-challenged mice is indirectly supported by the similar reductions from wild-type CN3685 lethality levels observed using either an isogenic *cpb* null mutant (4) or an isogenic *virR* null mutant (this study). Specifically, relative to the 90 to 100% lethality level induced by wild-type CN3685, those two mutants cause 23% and 15% lethality (respectively) in the mouse ID-challenge model.

The low levels of lethality observed in mice ID challenged with the *virR* mutant could reflect effects of a toxin, such as CPA, whose production is only partially regulated by this two-component regulatory system (19). It also might involve the activity of an unknown toxin that is not VirS/VirR regulated. This second possibility is indirectly supported by previous reports (14) that a CN3685 mutant deficient in the production of CPB, CPA, and PFO (all completely or partially VirS/VirR regulated) still caused a low level (9%) of lethality in the mouse ID-challenge model.

The current study also found that disrupting the VirS/VirR system delays the rapid onset of *in vitro* cytotoxicity when CN3685 infects Caco-2 cell cultures under anaerobic conditions. A 1-h anaerobic infection with either wild-type CN3685 or the CPJV47(pTS405) complementing strain caused substantially more morphological damage and cell death in Caco-2 cells than did a similar CPJV47 infection. However, by 2 h of anaerobic infection, CPJV47 also severely damaged Caco-2 cells. Interestingly, Western blots never detected CPB production by CPJV47 and detected CPB production by CN3685 and CPJV47(pTS405) only after a 2-h anaerobic infection of Caco-2 cells. The cytotoxicity observed after a 1-h wild-type CN3685 anaerobic infection was not caused by production of small amounts of CPB that had rapidly bound to cells, so it could not be detected by Western blotting culture supernatants, since (data not shown) (i) Western blots did not detect CPB bound to Caco-2 cells after a 1-h CN3685 anaerobic infection and (ii) ~10 to 20 μg of CPB/ml is needed to induce Caco-2 cell cytotoxicity within 1 to 2 h, which is well below the <200-ng CPB detection limit of the Western blot. Therefore, a nonregulated or partially VirS/VirR-regulated toxin apparently contributes to CN3685-induced cytotoxicity.

Since CPB is VirS/VirR regulated (21; this study), the ability of CPJV47 to cause cytotoxicity is consistent with previous reports that a CN3685 *cpb* null mutant still caused cytotoxicity in Caco-2 cells (14). Thus, clear differences exist between *in vivo* and *in vitro* models of CN3685 infection; i.e., while an isogenic *virR* null mutant caused extensive cell culture cytotoxicity within 2 h under anaerobic conditions, that mutant was completely or strongly, respectively, attenuated in 6-h rabbit small intestinal loop assays or 48-h mouse lethality assays. These findings emphasize the importance of animal model studies to confirm *C. perfringens* pathogenicity relationships suggested by *in vitro* cell culture infection studies.

The established or predicted vegetative cell importance of VirS/VirR when (i) type C strains cause necrotic enteritis or enterotoxemias (this study), (ii) type A strains cause gas gangrene (17), and (iii) NetB-positive type A strains cause avian necrotic enteritis (see above) is interesting since *C. perfringens* strains possess 23 sensor kinases and 17 response regulators (29, 30). Whether those other two-component regulatory systems also contribute to pathogenicity is unknown. Another open question is the identities of the environmental cues that activate VirS/VirR signaling. *C. perfringens* possesses an Agr system (31, 32), and it was suggested (31) that the Agr quorum-sensing system might interact with VirS/VirR, but this relationship remains unproven. Thus, considerable work remains for us to understand the signaling used by *C. perfringens* vegetative cells to regulate their *in vivo* toxin production and pathogenicity.

Finally, the current work suggests some potential applications. While both medical and veterinary vaccines have been used against *C. perfringens*, vaccination is complicated by the variety of toxins produced by different *C. perfringens* strains. Similarly, toxin production diversity complicates the use of antitoxins as therapeutic adjuvants against *C. perfringens* infection. However, the emerging evidence implicating VirS/VirR in controlling *C. perfringens* toxin production during both histotoxic infections and, at least some, infections originating in the intestines may suggest an alternative therapeutic approach. If identified, VirS/VirR inhibitors could be useful therapeutics. For example, since *C. perfringens* diseases are not intoxications but rather infections involving *in vivo* toxin production, treating patients with an inhibitor of the VirS membrane sensor might reduce *in vivo* toxin production by *C. perfringens* vegetative cells and thus ameliorate some, if not many, diseases caused by this bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. perfringens* type C strain CN3685, isolated from peritoneal fluid of a sheep with struck (a rapidly fatal type C enterotoxemia), was the wild-type strain used for *in vivo* and *in vitro* infections and for purifying CPB (see below). The isogenic CPJV47 *virR* null mutant and the CPJV47(pTS405) VirR/VirS complementing strain used in the current study were constructed and genotyped previously (21). Reverse transcription (RT)-PCR analyses and Western blot analyses performed in that earlier study confirmed the phenotypes of these parent, mutant, and complementing strains. Strains were grown in TGY (3% tryptic soy broth [Becton Dickinson], 2% glucose [Sigma Aldrich], 1% yeast extract [Becton Dickinson], supplemented with 0.1% sodium thioglycolate [Sigma Aldrich]). Tetracycline (2.5 μg/ml) or chloramphenicol (15 μg/ml) was added, respectively, for growth of CPJV7 or CPJV47(pTS405).

Preparation of inocula for *in vivo* pathogenicity testing. Inocula used for injecting into rabbit intestinal loops and for ID inoculation into mice were prepared by overnight growth at 37°C of CN3685, CPJV47, and CPJV47(pTS405) in TGY-C (TGY broth containing 0.1% cysteine hydrochloride, a reducing agent), with the addition of 2.5 μg/ml of tetracycline (for CPJV47) or 15 μg/ml of chloramphenicol [for CPJV47(pTS405)]. After centrifugation of the overnight cultures, the vegetative cells were resuspended in fresh TGY-C (to remove preformed toxins) at a concentration of 10^8 CFU/ml. A 1-ml aliquot of each vegetative cell suspension (mixed with 1 mg/ml of TI) was inoculated into a rabbit small intestinal loop or a mouse. Inocula for control loops and mice included sterile TGY-C plus TI or sterile TGY-C plus TI containing 10 μg (for rabbit loops) or 25 μg (for mice) of purified CPB.

Beta toxin purification. An isolated CN3685 colony was cultured for 8 h in TGY broth with thioglycolate to produce CPB. The toxin was purified from supernatants of those cultures by anion-exchange chromatography, as described previously (4, 21), and then evaluated for purity by SDS-PAGE and Western blotting, followed by densitometric analysis. The final preparation was ~95% homogeneous and showed no apparent contamination with CPA or PFO.

Culture of Caco-2 cells. Human-derived, enterocyte-like Caco-2 cells were maintained in Eagle's MEM (Sigma) supplemented with 10% fetal bovine serum (Mediatech Inc.); 1% L-glutamine (Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml); and 1% nonessential amino acids (Sigma). Cells were incubated at 37°C in 5% of CO_2 humidified atmosphere.

Microscopic assessment of Caco-2 cell culture morphological damage caused by anaerobic infection using vegetative cells of CN3685, CPJV47, or CPJV47(pTS405). To compare their abilities to morphologically damage Caco-2 cells, CN3685, CPJV47, and CPJV47(pTS405) vegetative cells were grown overnight in TGY broth containing thioglycolate. A 5-ml aliquot of each overnight culture was then centrifuged at 4°C. After two washes with phosphate-buffered saline (PBS; pH 7.4), the bacterial cells were suspended in 5 ml of fresh MEM without serum or antibiotics. Confluent Caco-2 cell monolayers in six-well plates were washed three times with prewarmed PBS (pH 7.4) and then infected, in an anaerobic bag (Mitsubishi Gas Chemical Co. Inc.), with the washed bacterial cell suspension (multiplicity of infection [MOI] = 20) for 1 or 2 h at 37°C. Photographs of morphological damage were taken using a Canon PowerShot G5 camera fitted to a Zeiss Axiovert microscope.

LDH cytotoxicity assay. To quantify their cytotoxic effects, washed vegetative cells of CN3685 and of its isogenic derivatives, prepared as described above, were suspended in 5 ml of fresh MEM without serum or antibiotics. Confluent Caco-2 cell monolayers in a 6-well plate were washed three times with warm PBS and then infected with the washed bacterial cell suspension (MOI = 20) for 1 h at 37°C under anaerobic conditions. Supernatants of the infected cultures were carefully removed and centrifuged for 5 min. Each supernatant was then analyzed using the LDH cytotoxicity detection kit (Roche), with the absorbance of each sample measured at 490 nm with an iMark microplate reader (Bio-Rad). As described in the kit instructions, percent cytotoxicity was calculated for infected cultures after subtraction of background LDH release values from uninfected Caco-2 cells. Triton X-100 was used to determine maximal LDH release.

Comparison of levels of virulence of CN3685, CPJV47, and CPJV47(pTS405) vegetative cells in rabbit small intestinal loops. Inocula containing 10^8 washed vegetative cells of each *C. perfringens* strain resuspended in TGY-C plus TI were injected into small intestinal loops prepared, as described previously (4), in young adult New Zealand White rabbits. After closing of the abdominal incision by separate muscle and skin sutures, the animals were kept deeply anesthetized throughout the experiment. After 6 h, the rabbits were euthanized by an overdose of sodium barbiturate (Beuthanasia; Schering-Plough Animal Health, Kenilworth, NJ) and the small intestinal loops were excised and weighed, before and after the fluid was removed. They were also examined grossly, and length was measured. Fluid secretion was expressed as the loop weight-to-length ratio (g/cm). For histological analysis, all tissues were fixed and stained with hematoxylin and eosin as described previously (4). Tissue sections were examined by a pathologist in a blind fashion, using a quantitative scoring system as described previously (4). Briefly, the degree of damage for each parameter (mucosal necrosis, desquamation of the epithelium, inflammation, villous blunting, edema, and hemorrhage) was scored using a scale of 1 to 5, with 1 indicating no histologic damage and 2, 3, 4, and 5 indicating increasingly severe damage.

Ethics statement. The animal research in this project was approved by the Animal Care and Use Committee of the University of California—Davis (permit number 13222). All rabbit surgery was performed under inhalatory isoflurane anesthesia, and all mouse surgery was performed under Avertin anesthesia. All efforts were made to minimize suffering.

Comparison of levels of lethality induced by CN3685, CPJV47, and CPJV47(pTS405) vegetative cells in the mouse ID enterotoxemia model. To determine their ability to cause enterotoxemic lethality, washed vegetative cells of wild-type CN3685, the CPJV47 *virR* null mutant, and the CPJV47(pTS405) complementing strain were each tested in our previously described mice ID-challenge inoculation model for type C enterotoxemias (14). Inoculum preparations contained 10^8 CFU washed vegetative cells of CN3685, CPJV47, or CPJV47(pTS405) suspended in fresh TGY-C; those samples were then inoculated ID into 4 to 5 groups of 4 mice. The animals were then monitored until the assay endpoint (see below).

Mouse lethality assay endpoint and interpretation of results. Assay endpoints for the mouse ID-challenge experiments included spontaneous death, development of severe neurological or respiratory signs necessitating euthanasia, or survival without clinical alterations after 48 h. Because our experience indicates that mice developing severe neurological or respiratory signs after inoculation with *C. perfringens* type C vegetative cells never recover, animals were euthanized after developing such signs and included in the calculation of lethality. Clinical signs necessitating euthanasia included severe respiratory distress, depression, incoordination, ataxia, and circling. Euthanasia was performed by inhalation of carbon dioxide.

Western blot analysis of CPB levels in infected rabbit small intestinal loops. To evaluate *in vivo* CPB production by vegetative cells of wild-type CN3685, CPJV47, or CPJV47(pTS405), intestinal fluids were collected from loops from experimental rabbits. A 10-μl aliquot of fluid was Western blotted using a CPB-specific monoclonal antibody, as described previously (4).

To quantify *in vivo* CPB production levels, serial dilutions of purified beta toxin (2 to 68 μg) were electrophoresed on a 12% polyacrylamide gel containing SDS, together with intestinal fluid samples collected from experimental rabbit loops. Separated proteins were transferred onto a nitrocellulose membrane, which was blocked with Odyssey blocking buffer for 1 h at room temperature and then probed with mouse anti-CPB primary antibody diluted with Odyssey blocking buffer plus 0.1% Tween 20 overnight at 4°C. IRDye-labeled secondary antibody (Li Cor) diluted in Odyssey blocking buffer was added, and reaction mixtures were kept for 1 h at room temperature with protection from light. CPB amounts in each sample were calculated by densitometric scanning (Li Cor Odyssey) against a standard curve generated using diluted purified CPB.

Comparison of *in vivo* colonization levels for CN3685, CPJV47, and CPJV47(pTS405). To determine possible *in vivo* colonization differences between the wild-type strain, CPJV47, and CPJV47(pTS405), the contents of the small intestine and colon/rectum of groups of mice, or the small intestinal contents of rabbit loops inoculated with these three strains were aseptically collected. Contents were diluted in TGY-C, and the number of CFU/ml of each strain was determined using a standard spread technique on blood agar. Rabbit intestinal loops or mice receiving only TGY-C were used as negative controls.

Statistical analyses. Each rabbit experiment was performed with two repetitions in each of eight different rabbits. For mice, each experiment was repeated 4 or 5 times in groups of at least 4 mice. All statistical analyses were done using the Minitab 15 software and SAS 9.2 software. The fluid accumulation data were analyzed using the two-way analysis of variance (ANOVA) with *post hoc* test. The Table 1 data were analyzed using the Friedman test, and the LDH cytotoxicity data were analyzed using the two-tailed, unpaired Student *t* test.

ACKNOWLEDGMENTS

This research was generously supported by grant AI056177-08 from the National Institute of Allergy and Infectious Diseases.

We thank Paul Hauer for supplying a CPB-specific monoclonal antibody.

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