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Characterization of Toxin Plasmids in *Clostridium perfringens* Type C Isolates[▽]

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Clostridium perfringens **type C isolates cause enteritis necroticans in humans or necrotizing enteritis and enterotoxemia in domestic animals. Type C isolates always produce alpha toxin and beta toxin but often produce additional toxins, e.g., beta2 toxin or enterotoxin. Since plasmid carriage of toxin-encoding genes has not been systematically investigated for type C isolates, the current study used Southern blot hybridization of pulsed-field gels to test whether several toxin genes are plasmid borne among a collection of type C isolates. Those analyses revealed that the surveyed type C isolates carry their beta toxin-encoding gene (*cpb*) on plasmids ranging in size from ~65 to ~110 kb. When present in these type C isolates, the beta2 toxin gene localized to plasmids distinct from the *cpb* plasmid. However, some enterotoxin-positive type C isolates appeared to carry their enterotoxin-encoding *cpe* gene on a *cpb* plasmid. The *tpeL* gene encoding the large clostridial cytotoxin was localized to the *cpb* plasmids of some *cpe*-negative type C isolates. The *cpb* plasmids in most surveyed isolates were found to carry both IS*1151* sequences and the *tcp* genes, which can mediate conjugative *C. perfringens* plasmid transfer. A *dcm* gene, which is often present near *C. perfringens* plasmid-borne toxin genes, was identified upstream of the *cpb* gene in many type C isolates. Overlapping PCR analyses suggested that the toxin-encoding plasmids of the surveyed type C isolates differ from the *cpe* plasmids of type A isolates. These findings provide new insight into plasmids of proven or potential importance for type C virulence.**

Clostridium perfringens isolates are classified into five toxinotypes (A to E) based upon the production of four (α, β, ε, and ι) typing toxins (29). Each toxinotype is associated with different diseases affecting humans or animals (25). In livestock species, *C. perfringens* type C isolates cause fatal necrotizing enteritis and enterotoxemia, where toxins produced in the intestines absorb into the circulation to damage internal organs. Type C-mediated animal diseases result in serious economic losses for agriculture (25). In humans, type C isolates cause enteritis necroticans, which is also known as pigbel or Darmbrand (15, 17), an often fatal disease that involves vomiting, diarrhea, severe abdominal pain, intestinal necrosis, and bloody stools. Acute cases of pigbel, resulting in rapid death, may also involve enterotoxemia (15).

By definition, type C isolates must produce alpha and beta toxins (24, 29). Alpha toxin, a 43-kDa protein encoded by the chromosomal *plc* gene, has phospholipase C, sphingomyelinase, and lethal properties (36). Beta toxin, a 35-kDa polypeptide, forms pores that lyse susceptible cells (28, 35). Recent studies demonstrated that beta toxin is necessary for both the necrotizing enteritis and lethal enterotoxemia caused by type C isolates (33, 37). Besides alpha and beta toxins, type C isolates also commonly express beta2 toxin, perfringolysin O, or enterotoxin (11).

There is growing appreciation that naturally occurring plasmids contribute to both *C. perfringens* virulence and antibiotic resistance. For example, all typing toxins, except alpha toxin, can be encoded by genes carried on large plasmids (9, 19, 26, 30–32). Other *C. perfringens* toxins, such as the enterotoxin or beta2 toxin, can also be plasmid encoded (6, 8, 12, 34). Furthermore, conjugative transfer of several *C. perfringens* antibiotic resistance plasmids or toxin plasmids has been demonstrated, supporting a key role for plasmids in the dissemination of virulence or antibiotic resistance traits in this bacterium (2).

Despite their pathogenic importance, the toxin-encoding plasmids of *C. perfringens* only recently came under intensive study (19, 26, 27, 31, 32). The first carefully analyzed *C. perfringens* toxin plasmids were two plasmid families carrying the enterotoxin gene (*cpe*) in type A isolates (6, 8, 12, 26). One of those *cpe* plasmid families, represented by the ~75-kb prototype pCPF5603, has an IS*1151* sequence present downstream of the *cpe* gene and also carries the *cpb2* gene, encoding beta2 toxin. A second *cpe* plasmid family of type A isolates, represented by the ~70-kb prototype pCPF4969, lacks the *cpb2* gene and carries an IS*1470*-like sequence, rather than an IS*1151* sequence, downstream of the *cpe* gene. The pCPF5603 and pCPF4969 plasmid families share an ~35-kb region that includes transfer of a clostridial plasmid (*tcp*) locus (26). The presence of this *tcp* locus likely explains the demonstrated conjugative transfer of some *cpe* plasmids (5) since a similar *tcp* locus was shown to mediate conjugative transfer of the *C. perfringens* tetracycline resistance plasmid pCW3 (2).

The iota toxin-encoding plasmids of type E isolates are typically larger (up to ~135 kb) than *cpe* plasmids of type A

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TABLE 1. Summary of plasmid sizes in *C. perfringens* type C isolates

Isolate[a]	Apparent size (kb) of plasmid containing:						
	cpb (beta toxin)	*cpe* (enterotoxin)	*tpeL* (large cytotoxin)	*tcpH* (conjugative transfer)	*rep* (replication)	IS*1151* (insertion sequence)	*cpb2* (beta 2 toxin)
CN882[c]	65		65	65	65	65	
CN885[d]	65		65	65	65	65	
CN886[d]	65		65	65	65	65	
CN887[c]	65		65	65	65	65	
CN1797[d]	65		65	65	65	65	
CN2078[d]	75	75		75	75	75	
CN3711[e]	90		90	90	90	90	
CN3717[c]	90		90	90	90	90	
CN3728[e]	90		90	90	90	90	
CN3732[e]	90		90	90	90	90	
CN3753[d]	85	85		85	85/65	85/110	
CN3763[d]	75	110		75/110	75/110	75/110	
CN5383[b]	110		65	65/110	65/110	110	
CN5388[b]	110	90		90/110	90/110	90/110	90
NCTC10719[f]	95		95	95	65/95	95	65
Bar3[b]	110		65	65/110	65/110	110	75

[a] Isolates with a "CN" prefix originated from the Burroughs-Wellcome collection of *C. perfringens* isolates collected globally during the 1930s to 1960s and were provided by Russell Wilkinson (University of Melbourne), Bar3 is from Australia and was provided by Francisco Uzal, and NCTC10719 came originally from Denmark.
[b] Originated from human pigbel cases.
[c] Originated from diseased sheep.
[d] Originated from animals (species unknown).
[e] Originated from diseased cattle.
[f] Originated from diseased pigs.

isolates (19). Plasmids carrying iota toxin genes often encode other potential virulence factors, such as lambda toxin and urease, as well as a *tcp* locus (19). Many iota toxin plasmids of type E isolates share, sometimes extensively, sequences with *cpe* plasmids of type A isolates (19). It has been suggested that many iota toxin plasmids evolved from the insertion of a mobile genetic element carrying the iota toxin genes near the plasmid-borne *cpe* gene in a type A isolate, an effect that silenced the *cpe* gene in many type E isolates (3, 19).

Plasmids carrying the epsilon toxin gene (*etx*) vary from ~48 kb to ~110 kb among type D isolates (32). In part, these *etx* plasmid size variations in type D isolates reflect differences in toxin gene carriage. For example, the small ~48-kb *etx* plasmids present in some type D isolates lack both the *cpe* gene and the *cpb2* gene. In contrast, larger *etx* plasmids present in other type D isolates often carry the *cpe* gene, the *cpb2* gene, or both the *cpe* and *cpb2* genes. Thus, the virulence plasmid diversity of type D isolates spans from carriage of a single toxin plasmid, possessing from one to three distinct toxin genes, to carriage of three different toxin plasmids.

In contrast to the variety of *etx* plasmids found among type D isolates, type B isolates often or always share the same ~65-kb *etx* plasmid, which is related to pCPF5603 but lacks the *cpe* gene (27). This common *etx* plasmid of type B isolates, which carries a *cpb2* gene and the *tcp* locus, is also present in a few type D isolates. Most type B isolates surveyed to date carry their *cpb* gene, encoding beta toxin, on an ~90-kb plasmid, although a few of those type B isolates possess an ~65-kb *cpb* plasmid distinct from their ~65-kb *etx* plasmid (31).

To our knowledge, the *cpb* gene has been mapped to a plasmid (uncharacterized) in only a single type C strain (16). Furthermore, except for the recent localization of the *cpe* gene to plasmids in type C strains (20), plasmid carriage of other potential toxin genes in type C isolates has not been investigated. Considering the limited information available regarding the toxin plasmids of type C isolates, our study sought to systematically characterize the size, diversity, and toxin gene carriage of toxin plasmids in a collection of type C isolates. Also, to gain insight into possible mobilization of the *cpb* gene by insertion sequences or conjugative transfer, the presence of IS*1151* sequences or the *tcp* locus on type C toxin plasmids was investigated.

MATERIALS AND METHODS

Bacterial cells, media, and reagents. All type C isolates examined in this study are listed and described in Table 1. Carriage of *cpb*, *plc*, *cpb2*, and *cpe* genes by these isolates was determined in a previous study by multiplex PCR (11). The type C isolates were grown overnight at 37°C under anaerobic conditions on Shahidi-Ferguson perfringens (SFP) agar (Difco Laboratories) containing 0.04% D-cycloserine (Sigma-Aldrich) to maintain culture purity. For growth of broth cultures, fluid thioglycolate (FTG) medium (Difco Laboratories) and TGY medium (3% tryptic soy broth [Becton Dickinson] containing 2% glucose [Fisher Scientific], 1% yeast extract [Becton Dickinson], and 0.1% sodium thioglycolate [Sigma-Aldrich]) were used.

Pulsed-field gel electrophoresis (PFGE). For PFGE analysis, individual *C. perfringens* type C isolates were grown in FTG broth overnight at 37°C. A 0.1-ml aliquot of those starter cultures was then inoculated into 10 ml of TGY medium. After overnight growth at 37°C, the TGY cultures were used to prepare *C. perfringens* genomic DNA agarose plugs. Cells were harvested by centrifugation and washed in TES buffer (1 M Tris, 0.5 M EDTA, 5 M sucrose [pH 8.0]). The washed cells were resuspended in 0.2 ml of Tris-EDTA buffer and embedded in an equal volume of melted 2% Certified low-melt agarose (Bio-Rad Laboratories). The mixture was dispensed into plug molds, solidified at 4°C, and then cut into 2- to 3-mm slices. The agarose-embedded cells were lysed by incubation, with gentle shaking of the plugs overnight at 37°C in lysis buffer (0.5 M EDTA [pH 8.0], 0.5% [vol/vol] Sarkosyl, 0.5% lysozyme [Sigma], 0.4% deoxycholic acid). Finally, the lysed plugs were incubated for 2 days at 55°C in 0.2% proteinase K (Gene Choice)-0.5 M EDTA (pH 8.0) buffer.

PFGE was performed with 1% agarose gels by using a CHEF-DR II system (Bio-Rad Laboratories) and 0.5× Tris-borate-EDTA buffer at 14°C. For the undigested DNA plugs, the running parameters were as follows: initial pulse, 1 s; final pulse, 25 s; voltage, 6 V/cm; and time, 24 h. For isolates that showed

Southern blot signal colocalization using probes for two different open reading frames (ORFs) (as described below), DNA plugs were digested with the restriction endonucleases ApaI, AvaI, ClaI, KpnI, NcoI, NheI, SphI, and XhoI (New England Biolabs). Briefly, a plug slice (approximately one-sixth of the plug) was incubated overnight, at the proper temperature as instructed by the enzyme manufacturer, in 30 μl of 10× commercial restriction endonuclease buffer, 4 μl of a selected enzyme, and 266 μl of distilled water. The digested plugs were then electrophoresed in the CHEF-DR II system (Bio-Rad Laboratories) and 0.5× Tris-borate-EDTA buffer at 14°C. The running parameters were as follows: initial pulse, 1 s; final pulse, 12 s; voltage, 6 V/cm; and time, 15 h.

Pulsed-field gel Southern blot analyses. Digoxigenin (DIG)-labeled DNA probes were prepared using a PCR DIG probe synthesis kit (Roche). Primers used for amplifying internal *cpb*, *cpe*, IS*1151*, *tcpH*, *rep*, *tpeL*, and *cpb2* gene sequences (Table 1 lists what each of these genes encodes) have been described previously (19, 31, 32). The DIG-labeled probes were then used to perform Southern hybridization of pulsed-field gels using a previously described technique (32). CSPD substrate (Roche) was used for detection of hybridized probes according to the manufacturer's protocol.

Overlapping PCR analyses to determine whether type C isolates carry the conserved or variable regions of pCPF5603 and pCPF4969. The DNA for these overlapping, short-range PCRs was obtained from *C. perfringens* colony lysates as described previously (26). Each PCR mixture (20 μl) contained 2 μl of template DNA, 10 μl of *Taq* complete 2× mastermix (New England Biolabs), and 1 μl of each primer pair (1 μM final concentration). The primers used for investigating the presence of pCPF5603/pCPF4969 conserved regions and pCPF5603 or pCPF4969 variable regions in type C isolates have been described previously (26). The PCR amplification conditions used for these overlapping PCR analyses were 1 cycle of 95°C for 2 min and 35 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 1 min 40 s, followed by a single extension of 68°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide for visualization.

PCR analyses of *tpeL* carriage and linkage analysis of the *cpb* and *tpeL* genes in type C isolates. A PCR survey was performed to evaluate carriage among type C isolates of the *tpeL* gene, encoding the *C. perfringens* large cytotoxin (TpeL). The primers used for these PCR analyses were described previously (28). To evaluate potential linkage between the *tpeL* and *cpb* genes in type C isolates by overlapping PCR, primers were used from a former study (31) based upon the unfinished *C. perfringens* type B strain ATCC 3626 genome sequence publicly released by the J. Craig Venter Institute (JCVI). The PCR amplification conditions used for this overlapping PCR assay were 1 cycle of 94°C for 3 min and 35 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 1 min 30 s, followed by a single extension of 68°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide for visualization.

For long-range PCR to attempt connecting the *tpeL* and *cpb* genes, DNA was isolated from type C strains CN882, CN886, CN3711, CN3717, CN3732, and NCTC10719 by using a MasterPure Gram-positive DNA purification kit (Epicentre). Each PCR mixture contained 1 μl of template DNA, 25 μl of *Taq* long-range complete 2× mix (New England Biolabs), and 1 μl each of the primer pair SeqR5 and cpbF3 (1 μM final concentration) (31). The reaction mixtures, with a total volume of 50 μl, were placed in a thermocycler (Techne) and subjected to the following amplification conditions: 1 cycle of 95°C for 2 min, 35 cycles of 95°C for 30 s, 56°C for 40 s, and 65°C for 3 min, and a single extension at 65°C for 10 min. PCR products were then electrophoresed on a 1% agarose gel, which was stained with ethidium bromide for product visualization. The long-range PCR products amplified from CN886 and NCTC10719 were PCR cloned into the pCR2.1-TOPO vector (Invitrogen), and those inserts were then sequenced at the University of Pittsburgh Core sequencing facility using previously described primers (31).

PCR linkage of the *cpb* gene with IS*1151* and the *dcm* gene in type C isolates. Based upon the JCVI JGS1495 sequence, a series of primers were constructed (Table 2) to evaluate, by overlapping PCR, a potential linkage between the *cpb* gene and IS*1151* sequences or the *dcm* gene in type C isolates. PCR conditions for these amplifications were 1 cycle of 94°C for 3 min and 35 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 1 min 30 s, followed by a single extension of 68°C for 10 min. PCR products were run on a 1% gel and stained with ethidium bromide for visualization.

Reverse transcriptase PCR (RT-PCR) analysis of *tpeL* gene expression by type C isolates. RT-PCR analyses were performed to evaluate whether the *tpeL* gene is expressed by *tpeL*-positive type C isolates. Strains were grown as described above, and cells were collected by centrifugation. Total bacterial RNA was extracted from the pelleted cells using a previously described method (38). All RNA samples were treated with DNase I at 37°C for 30 min. RT-PCRs were then performed on those DNase-treated RNA samples by using an AccessQuick RT-PCR system (Promega). Briefly, 100 ng of each RNA sample was reverse transcribed to cDNA at 45°C for 1 h and then used as a template for PCR (35 cycles, each consisting of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min) with *tpeL*-specific primers (31).

TABLE 2. PCR primers used to link the *cpb* gene to the *dcm* gene

Primer	Sequence (5′ to 3′)	PCR product (size [kb])
F1 R1	TCTAGTTACCCTAGAAAGCATTACT GCTCTAAAAAAGAGCTTAAAAGCA	A (1.0)
F2 R2	ATGATAAGTGAGGACCTTCC TGGTCATATTTCATGTATAACT	B (1.2)
F3 R3	CTGCTTTTAAGCTCTTTTTTAGAGC CCTCCTTTTGTATATAGATGATCTG	C (1.1)
F4 R4	AGTTATACATGAAATATGACCA CCAGTTAACACCATTCCAATTAAGA	D (1.1)
F5 R5	CAGATCATCTATATACAAAAGGGAGAGG CCCAGCCATCTTATACATTTG	E (0.9)

Nucleotide sequence accession numbers. The *tpeL* to *cpb* sequences determined for CN886 and NCTC10719 in this study were submitted to GenBank under the accession numbers HM480840 and HM480841.

RESULTS

Characterization of *cpb* gene location in type C isolates. Studies from several laboratories have established that Southern blot assays of pulsed-field gels provide useful insight into the size, diversity, and gene carriage of *C. perfringens* toxin plasmids (3, 7, 8, 10, 12, 16, 19, 26, 27, 31, 32). Furthermore, those studies have also shown that the PFGE conditions used in the current study do not allow *C. perfringens* chromosomal DNA to enter pulsed-field gels unless first digested by restriction enzymes (8, 16, 19, 26, 27, 31, 32).

Since type C isolates must (by definition) produce beta toxin, the current study first subjected DNA from ~30 type C isolates to PFGE, followed by Southern blot hybridization with a *cpb* probe (Fig. 1A and Table 1), in order to evaluate whether the *cpb* gene is typically plasmid borne among type C isolates. As noted previously for some *C. perfringens* type A and type D isolates, DNA from certain type C isolates exhibited noticeable smearing on these Southern blots (26, 32). This smearing is indicative of high nuclease levels and prohibited further analysis of those isolates. However, *cpb* probe hybridization was discernible on Southern blots of 16 surveyed type C isolates. Each of these surveyed type C isolates was found to carry a plasmid-borne *cpb* gene. As expected for a negative control, the *cpb* probe did not hybridize with DNA from type A isolate F5603, which is *cpb* negative by PCR (Fig. 1A and data not shown).

Previous studies by several laboratories have established that probes hybridize predominantly to linearized plasmids on these pulsed-field gel Southern blots, thus providing a relatively accurate estimation of plasmid size (8, 10, 12, 16, 19, 20, 26, 27, 31). Therefore, the Southern blot results shown in Fig. 1A also revealed the existence of significant size variations among the *cpb* plasmids of the surveyed type C isolates. Specifically, the results in Fig. 1A and Table 1 indicate that, for the 16 type C isolates giving discernible *cpb* probe hybridization, *cpb* plasmid size ranged from ~65 kb to ~110 kb.

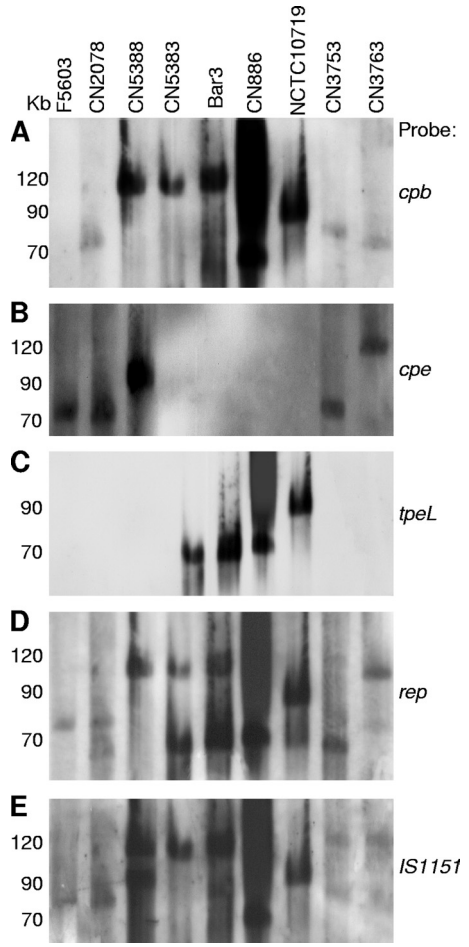

FIG. 1. Southern hybridization analyses of pulsed-field gels run with DNA extracted from representative type C isolates CN2078, CN5388, CN5383, Bar3, CN886, NCTC10719, CN3753, and CN3763. Type A isolate F5603 (*cpe* positive, *cpb* negative) is included as a control. (A) The blot was hybridized with a *cpb*-specific probe. (B) The blot from panel A was stripped and hybridized with a *cpe*-specific probe. (C) The blot from panel B was stripped and rehybridized with a *tpeL*-specific probe. (D) The blot from panel C was stripped and rehybridized with a *rep*-specific probe. (E) The blot from panel D was stripped and rehybridized with an IS*1151*-specific probe. Migration of molecular size markers (in kb) is indicated on the left of the blot.

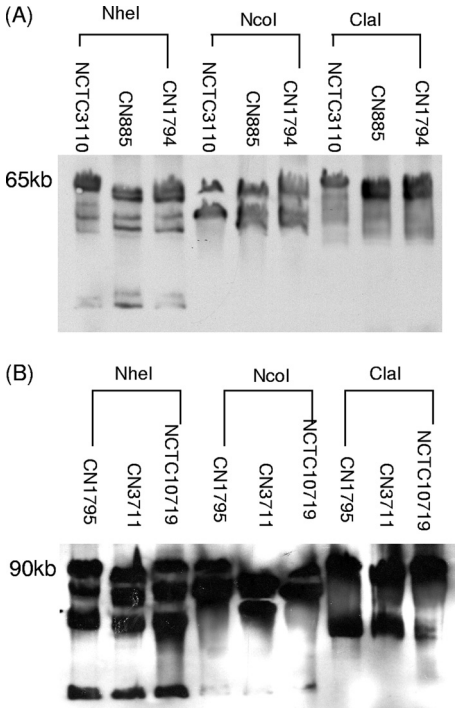

FIG. 2. Comparison of the ~65-kb (A) and ~90- to 95-kb (B) *cpb* plasmids carried by certain type B and type C isolates using Southern hybridization analyses of pulsed-field gels run with restriction enzyme-digested DNA. The type C isolates analyzed include CN885 and CN1794 (A) and CN3711 and NCTC10719 (B). The type B isolates analyzed include NCTC3110 (A) and CN1795 (B). Both blots were hybridized with a *cpb*-specific probe. Migration of molecular size markers is indicated on the left.

Comparison of the ~65-kb and ~90- to 95-kb *cpb* plasmids of type B isolates with those of type C isolates. The results in Fig. 1A reveal that the *cpb* plasmids in some type C isolates are very similar in size to the ~65-kb and ~90-kb *cpb* plasmids found in many, if not all, type B isolates (31). To help assess whether these are the same plasmid or two different but comigrating plasmids, DNA from type B isolate NCTC3110 or type C isolates CN885 and CN1794, which each carry an ~65-kb *cpb* plasmid, was digested with three different restriction enzymes (NheI, NcoI, and ClaI) and then subjected to pulsed-field Southern blot analyses with a *cpb* probe (Fig. 2A). In this experiment, migration of *cpb* genes residing on the same plasmid in different isolates should consistently exhibit the same response (sensitivity or insensitivity) to digestion using each restriction endonuclease. However, if the plasmid-borne *cpb* gene from one isolate showed no change in migration after digestion with a particular restriction endonuclease but migration of the plasmid-borne *cpb* gene in another isolate was affected by digestion with the same enzyme, that result would indicate that the two *cpb* genes are present on similar-sized, but different, plasmids (8, 19, 31, 32). The results obtained (Fig. 2A) showed similar susceptibility patterns to restriction enzyme digestion for all three isolates carrying an ~65-kb *cpb* plasmid, consistent with these isolates carrying similar *cpb* plasmids. Similar conclusions were obtained when this experiment was performed using DNA from type B isolate CN1795 and type C isolates CN3711 and NCTC10719, all of which carry *cpb* plasmids of ~90 to 95 kb (Fig. 2B).

Analysis of *cpe* gene location among the surveyed *cpe*-positive type C isolates. Four of the 16 type C isolates that did not show extensive smearing on pulsed-field Southern blots had previously been identified as *cpe* positive; each of those four type C isolates had also previously been shown to carry their *cpe* gene on plasmids (20). Those conclusions were confirmed by stripping the Southern blots hybridized with a *cpb* probe and then rehybridizing those stripped blots with a *cpe* probe. This analysis showed that these four *cpe*-positive type C isolates carry their *cpe* gene on large (ranging from ~75 kb to ~110 kb) plasmids (Fig. 1B and Table 1).

Evaluation of whether *cpb* and *cpe* are present on the same or different plasmids in type C isolates CN2078 and CN3753. Beyond confirming previous reports that the *cpe* gene is plas-

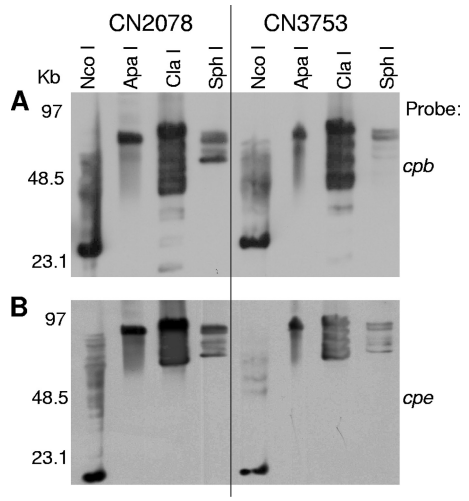

FIG. 3. Southern hybridization analyses of pulsed-field gels run with restriction enzyme-digested DNA extracted from *cpe*-positive type C isolates CN2078 and CN3753. The blot was first hybridized with a *cpb*-specific probe (A) and then stripped and reprobed with a *cpe*-specific probe (B). Restriction enzymes used for digestion are shown at the top of the blot. Migration of molecular size markers is indicated on the left.

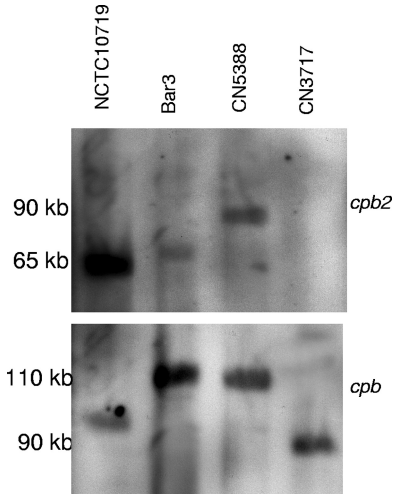

FIG. 4. Southern hybridization analyses of pulsed-field gels run with DNA extracted from *cpb2*-positive type C isolates NCTC10719, Bar3, and CN5388. *cpb2*-negative type C isolate CN3717 is included as a control. (Top) The blot was hybridized with a *cpb2*-specific probe. (Bottom) The blot was stripped and rehybridized with a *cpb*-specific probe. Migration of size markers is indicated on the left of the blot.

mid borne in most, if not all, type C isolates, the major reason for performing the *cpe* Southern blot whose results are shown in Fig. 1B was to allow the first head-to-head comparison of the relative sizes of the *cpb* and *cpe* plasmids in these four *cpe*-positive type C isolates. By overlapping the blots from Fig. 1A and B, it became readily apparent that the *cpb* and *cpe* genes are present on two distinct plasmids in type C isolates CN5388 and CN3763. However, this analysis also showed that the *cpb* and *cpe* probes hybridize to the same Southern blot location using DNA from type C isolates CN2078 and CN3753. Those results in Fig. 1A and B could indicate that both the *cpb* and *cpe* genes are present on the same ~75-kb or ~85- to 90-kb plasmid, respectively, in type C isolates CN2078 and CN3753. Alternatively, those two isolates could carry two distinct comigrating plasmids of similar sizes.

To help discriminate between those two possibilities, the same approach described for Fig. 2 was employed. DNA from CN2078 and CN3753 was digested with several different restriction endonucleases and then subjected to pulsed-field Southern blot analyses. For CN2078 DNA, migration of *cpb*- and *cpe*-containing DNA exhibited similar susceptibility patterns in response to restriction enzyme digestion (Fig. 3). These results are consistent with CN2078 carrying its *cpb* gene on the same ~75-kb plasmid that also carries the *cpe* gene. When this analysis was performed with CN3753 DNA (Fig. 3), the results obtained were also consistent with the *cpb* and *cpe* genes of that type C isolate being present together on the same ~85-kb plasmid.

Mapping of the *cpb2* gene location among *cpb2*-positive type C isolates. Three of the 16 surveyed type C isolates not showing extensive smearing on pulsed-field Southern blots had been determined by a previous study to carry the *cpb2* gene (11). Since it had not yet been determined whether the *cpb2* gene in type C isolates is plasmid borne or chromosomal, those three *cpb2*-positive type C isolates were subjected to pulsed-field Southern blot analyses using a *cpb2* probe (Fig. 4 and Table 1). Results from that experiment mapped the *cpb2* gene to plasmids of ~65 to 90 kb in these three type C isolates. Additionally, when that blot was stripped and then rehybridized with a *cpb* probe, the *cpb2*-carrying plasmid was clearly distinct from the *cpb* plasmid in each of the three *cpb2*-positive type C isolates.

PCR evaluation of *tpeL* gene carriage among type C isolates. While carriage of *cpb*, *cpe*, and *cpb2* genes among the surveyed type C isolate collection has previously been evaluated by PCR assays (11), a new toxin encoded by the *tpeL* gene has been discovered since that earlier work (1). Therefore, a PCR assay was performed to assess the presence of the *tpeL* ORF among the 16 type C isolates characterized in Table 1. This analysis determined that DNA from 12 of these 16 type C isolates supported amplification of a *tpeL* PCR product (Table 1). Interestingly, none of those *tpeL*-positive type C isolates was also *cpe* positive.

Expression of TpeL by *C. perfringens* type C isolates. The expression of alpha toxin, beta toxin, enterotoxin, and beta2 toxin by type C isolates has been evaluated in earlier studies (11), but TpeL expression by type C isolates has not yet been systematically assessed because TpeL was identified (1) only after completion of that earlier study. Since the current PCR assays detected *tpeL* ORF sequences in many surveyed type C isolates (Table 1), RT-PCR analyses were performed to evaluate whether the *tpeL* gene is expressed by five representative *tpeL*-positive type C isolates (NCTC10719, CN5383, CN1797, CN886, and Bar3). For all five surveyed *tpeL*-positive type C isolates, *tpeL* transcripts were detected after either 6 h or 24 h of growth in TGY broth (data not shown), opening the possibility that TpeL might contribute to the virulence of these isolates. No product was amplified when this RT-PCR analysis was performed with CN2078 (data not shown), consistent with

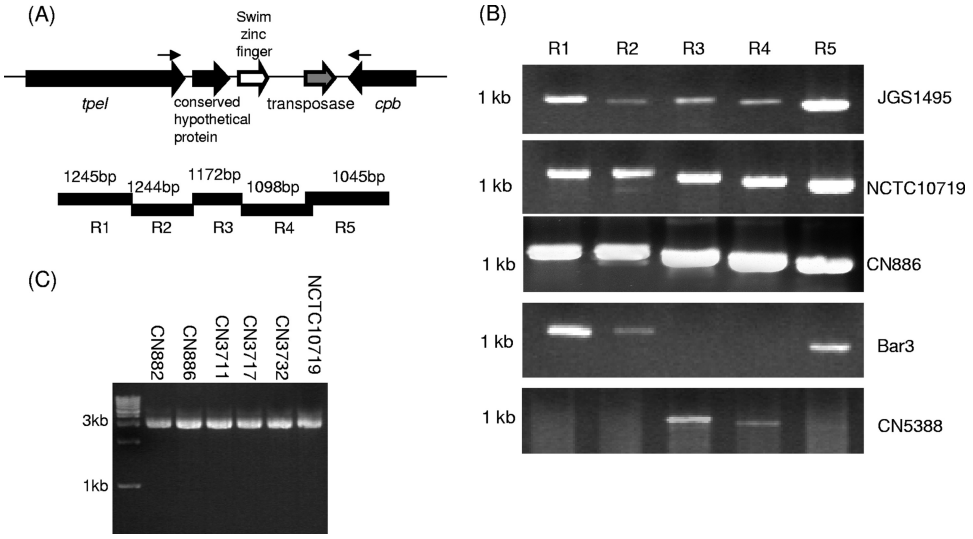

FIG. 5. Linkage of the *tpeL* and *cpb* genes in some type C isolates by long-range PCR and overlapping PCR approaches. (A) Arrangement of the *cpb* and *tpeL* gene loci in *C. perfringens* type C isolate JGS1495 (based upon sequencing results released from JCVI). Solid bars underneath the locus diagram depict the 5 overlapping PCRs used in the experiment for panel B. Small arrows indicate primers used in the long-range PCR studies whose results are shown in panel C. (B) Overlapping PCR analysis of the region extending from *cpb* to *tpeL* in type C isolates NCTC10719, JGS1495, CN886, Bar3, and CN5388 using reaction primers R1 to R5 (31). (C) Long-range PCR analyses to study the linkage of the *tpeL* and *cpb* genes in 6 type C isolates.

this isolate's testing PCR negative for the presence of *tpeL* sequences.

Analysis of *tpeL* gene location in *tpeL*-positive type C isolates. As shown in Fig. 1C and Table 1, pulsed-field Southern blot analyses demonstrated that, in all surveyed *tpeL*-positive type C isolates, the *tpeL* gene resides on plasmids of ~65 kb to ~95 kb in size. For two *tpeL*-positive isolates, i.e., CN5383 and Bar3, the *tpeL* probe clearly hybridized to a plasmid different from the *cpb*-carrying plasmid. However, in the other *tpeL*-positive type C isolates, the *cpb* and *tpeL* probes cohybridized to the same Southern blot location, corresponding to an ~65-kb plasmid, an ~90-kb plasmid, or an ~95-kb plasmid.

PCR linkage of the *tpeL* and *cpb* genes in some *tpeL*-positive type C isolates. Bioinformatic analysis of the JCVI JGS1495 genome sequence indicated that the *tpeL* gene in this type C isolate is located ~3 kb downstream of its *cpb* gene, similar to all characterized type B *cpb* plasmids (28). Based upon that observation, an overlapping PCR assay using a five-pair set of primers (encoding PCR products R1 to R5) (Fig. 5A) (31) was designed to assess whether similar genetic linkages might exist between the *cpb* and *tpeL* genes in other *tpeL*-positive type C isolates. The results obtained from this overlapping PCR assay were consistent with the *cpb* gene being linked to the *tpeL* gene in NCTC10719, JGS1495, and CN886 (Fig. 5B). However, these analyses did not support the *cpb* gene being proximal to *tpeL* in Bar3 or CN5383, consistent with the PFGE Southern blot results (Table 1) showing that *cpb* and *tpeL* are located on different plasmids in those two type C isolates.

Those overlapping PCR results were confirmed by a long-range PCR using one primer to internal *tpeL* ORF sequences and a second primer to internal *cpb* ORF sequences (Fig. 5A). This PCR amplified (Fig. 5C) a product of the expected ~3-kb size from six different type C isolates that had cohybridized *cpb* and *tpeL* probes to the same Southern blot location in Fig. 1C. Furthermore, sequencing confirmed that this long-range PCR product matched the JCVI sequence for the JGS1495 region extending from *tpeL* to *cpb* and that this region is 99% homologous with the *tpeL*-to-*cpb* region in type B isolates. However, the same long-range PCR failed to amplify any product using DNA from Bar3 or CN5383 (Fig. 5C and data not shown), consistent with the PFGE Southern blot results (Table 1) showing that *cpb* and *tpeL* are located on different plasmids in those two type C isolates.

Carriage of the *rep* gene among plasmids of type C isolates. Previous studies (2) have shown that the Rep protein is important for replication of *C. perfringens* tetracycline resistance plasmid pCW3. In addition, several other reports (19, 26, 31, 32) detected the presence of the Rep-encoding gene (*rep*) on toxin plasmids in type A, B, D, and E isolates. Therefore, a Southern blot analysis of pulsed-field gels was performed to assess whether the plasmids in type C isolates might also carry *rep* sequences. This experiment revealed that, for all surveyed type C isolates, the *rep* probe consistently hybridized to the same blot location as the *cpb* probe, suggesting that *rep* is commonly present on the *cpb* plasmid in type C isolates (Fig. 1D and Table 1). For *cpe*-positive type C isolates, there was also consistent cohybridization of the *rep* and *cpe* probes, suggesting that the *cpe* plasmids of type C isolates also carry *rep* sequences. The *rep* and *cpb2* probes cohybridized to the same pulsed-field Southern blot location using DNA from 2 of 3 *cpb2*-positive type C isolates (Table 1).

Plasmid carriage of the *tcp* locus in type C isolates. The *tcp* locus has been shown to mediate conjugative transfer of *C. perfringens* plasmid pCW3 and is also likely responsible for the demonstrated conjugative transfer of the *cpe* plasmids of type A isolates and the *etx* plasmids of type D isolates (2, 5, 14). Therefore, the current study surveyed whether *tcp* genes might be associated with plasmids in type C isolates. In this survey, a

pulsed-field gel Southern blot was hybridized with probes specific for either of two *tcp* genes (*tcpF* or *tcpH*) required for pCW3 conjugative plasmid transfer (2). Results from this experiment showed that all surveyed type C isolates hybridized both *tcp* probes, with most type C isolates cohybridizing the *tcpF* and *tcpH* probes at multiple blot locations (Table 1 and data not shown), suggesting that type C isolates carry multiple plasmids with conjugative potential. Of particular note, *tcp* probes consistently cohybridized to the same blot location containing the *cpb* plasmid and, when present, the *cpe* plasmid for all surveyed type C isolates, suggesting the conjugative potential of these toxin plasmids.

Southern blot and PCR analyses to evaluate an association between IS*1151*- and *cpb*-carrying plasmids in type C isolates. Previous studies have shown that IS*1151* insertion sequences are closely associated with several toxin genes in *C. perfringens*, including the *etx* genes in type B and D isolates (27, 31, 32). Therefore, Southern blot analyses of pulsed-field gels using an IS*1151* probe were performed with type C isolates. Those studies showed hybridization of the IS*1151* probe to the same blot location containing the *cpb* plasmid in all surveyed type C isolates (Fig. 1E and Table 1). In addition, isolates CN3753, CN3763, and CN5388 possessed a second IS*1151*-carrying plasmid. For isolates CN3763 and CN5388, this second IS*1151*-carrying plasmid comigrated with the *cpe* plasmid, consistent with recent identification of IS*1151* sequences located near the *cpe* gene of most *cpe*-positive type C isolates (20).

PCR linkage of the *cpb* gene with IS*1151* or the *dcm* gene in type C isolates. Analysis of the JCVI-released JGS1495 genome sequence identified *dcm* and two IS*1151*-like sequences present near the *cpb* gene (Fig. 6A). Therefore, overlapping PCR studies, using a five-pair set of primers (encoding PCR products F1 to F5) (Table 2 and Fig. 6A) designed from JGS1495 *cpb* locus sequences, were performed to evaluate whether *dcm* and IS*1151*-like sequences might also be located near the *cpb* gene in other *C. perfringens* type C isolates. Results from this overlapping PCR assay supported the proximity of IS*1151*-like sequences near the *cpb* gene in at least 4 of the 5 (JGS1495, CN5388, CN3711, and CN2078) surveyed type C isolates (Fig. 6B). Among those 4 isolates, their IS*1151*-*cpb* locus was clearly adjacent to *dcm* sequences. For another surveyed type C isolate, IS*1151*-like sequences appeared to be present near a *dcm* gene, although overlapping PCR was unable to link IS*1151* with the *cpb* gene in that isolate.

PCR studies to evaluate whether the variable and conserved regions of pCPF5603 or pCPF4969 are present in type C isolates. As mentioned in the introduction, two major families of *cpe* plasmids have been identified in type A isolates, as represented by prototype plasmids pCPF5603 and pCPF4969 (26). Complete sequencing of pCPF5603 and pCPF4969 showed that both *cpe* plasmid families carry *tcp* genes (26). Since we detected the presence of *tcp* genes in the surveyed type C isolates (Table 1), overlapping PCR analyses were performed to evaluate whether type C isolates might carry plasmids related to pCPF5603 or pCPF4969. However, primers to adjacent ORFs in the variable regions of pCPF5603 or pCPF4969 failed to amplify products from any type C isolates (not shown). In contrast, PCR products of the expected size were amplified for the entire *tcp* locus, using DNA from type C isolates CN2078 and CN3763 and primers specific for adjacent ORFs present in the conserved region of pCPF5603 and pCPF4969 (Fig. 7).

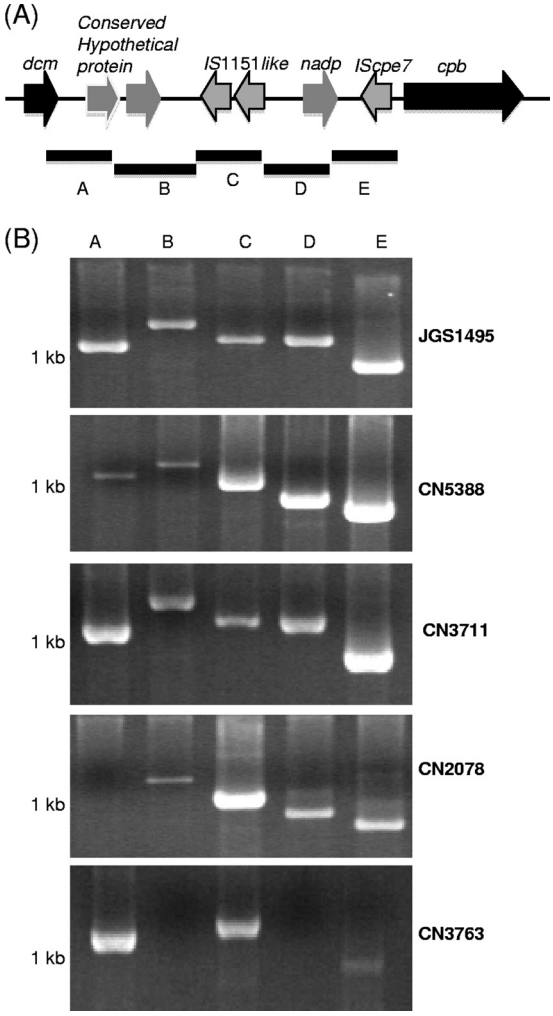

FIG. 6. Overlapping PCR linkage of the *cpb* gene with IS*1151* and the *dcm* gene in some type C isolates. (A) Arrangement of the *cpb* locus in *C. perfringens* type C isolate JGS1495 (based upon sequencing results released from JCVI). Boxes A to E in panel A depict the overlapping PCRs used to link *dcm* and *cpb* in panel B. (B) Overlapping PCR analyses of the JGS1495 region extending from the *dcm* to *cpb* gene using DNA from type C isolates JGS1495, CN5388, CN3711, CN2078, and CN3763 and reaction primers A to E (Table 2). Migration of 1-kb size markers is shown at the left of the gel.

DISCUSSION

Among all *C. perfringens* isolates, only type B and C isolates carry the *cpb* gene. A recent study established that the *cpb* gene in type B isolates is plasmid borne (31). Furthermore, that study detected only limited diversity of *cpb* plasmids among 17 type B isolates, i.e., each of those surveyed type B isolates was found to carry a *cpb* plasmid of either ~65 kb or ~90 kb. Those *cpb* plasmids of type B isolates consistently possessed *tcp* genes, suggesting their conjugative potential, as well as a *rep* gene and IS*1151* sequences. In addition, those ~65-kb and

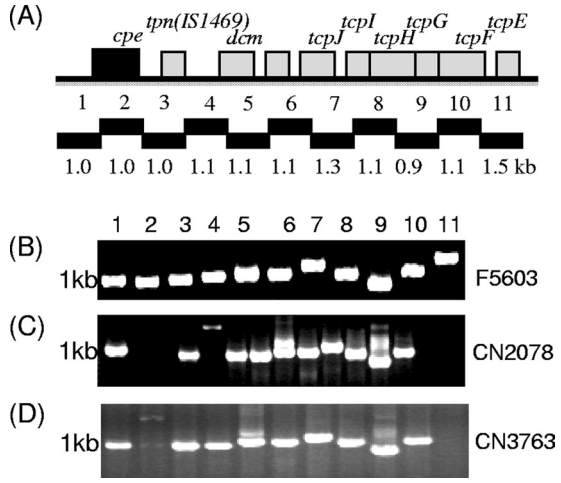

FIG. 7. Overlapping PCR analysis to evaluate the presence of the pCPF5603 conserved region in type C isolates. (A) Diagram depicting the organization of the pCPF5603 conserved region (26) and showing the overlapping PCRs included in this previously developed assay (26). (B) Overlapping PCR analysis of the conserved region, using DNA from type A isolate F5603 (the host for pCPF5603) as a control. Similar overlapping PCR analyses of type C isolates CN2078 (C) and CN3763 (D) are shown. Migration of 1-kb molecular size markers is indicated on the left.

~90-kb *cpb* plasmids of type B isolates were all found to carry a *tpeL* gene ~3 kb upstream from their *cpb* gene.

The current study has now determined that the *cpb* gene is also plasmid borne in most, if not all, type C isolates. Since expression of the *cpb* gene was previously shown to be necessary for the pathogenicity of type C isolates (33), this mapping of the *cpb* gene to plasmids indicates that plasmids are important for the virulence of type C isolates. Furthermore, this study identified some similarities between the *cpb* plasmids of type B and C isolates. For example, the *cpb* plasmids in all surveyed type C isolates also appear to carry *tcp*, *rep*, and IS*1151*, as reported for type B *cpb* plasmids. In addition, no *cpb* plasmids of type B isolates, or *cpb* plasmids of type C isolates, that carry the *cpb2* gene have yet been identified. While the *cpb2* gene is present in most, if not all, type B isolates (27), it is located on their *etx* plasmid rather than on their *cpb* plasmid.

However, the current study also determined that the *cpb* plasmids of the surveyed type C isolates are considerably more diverse than the *cpb* plasmids characterized to date among type B isolates (31). For example, the *cpb* plasmids in the 17 studied type C isolates exhibited greater size diversity than the ~65-kb or ~90-kb *cpb* plasmids present in characterized type B *cpb* plasmids; i.e., the current results suggest that some type C isolates apparently carry the same ~65-kb and ~90- to 95-kb *cpb* plasmids found in all surveyed type B isolates, but other type C isolates possess *cpb* plasmids of ~75 kb, ~85 kb, or ~110 kb. Furthermore, more variation in carriage of other toxin genes apparently exists among type C *cpb* plasmids than among type B *cpb* plasmids. For example, only the ~65- and ~90- to 95-kb *cpb* plasmids of type C isolates carry the *tpeL* gene found on all type B *cpb* plasmids characterized to date. Similarly, while no *cpe*-positive type B isolates have yet been identified, the ~75- and ~85-kb *cpb* plasmids present in some type C isolates apparently also carry the *cpe* gene.

The current discovery of more *cpb* plasmid diversity among the surveyed type C isolates than among type B isolates (31) is reminiscent of the greater *etx* plasmid diversity observed among type D isolates than among type B isolates. Specifically, while all 17 surveyed type B isolates were found to carry the same ~65-kb *etx* plasmid (27, 31), a similar survey of type D isolates revealed a diversity of *etx* plasmids (32). For example, only a few type D isolates were identified that carry the *etx* plasmid present in most, if not all, type B isolates (27). Since the current results revealed that only some type C isolates carry the same *cpb* plasmids present among type B isolates, these findings about *cpb* and *etx* plasmid carriage may collectively indicate that only certain toxin plasmid combinations can be stably maintained within a single *C. perfringens* cell. An inability to stably maintain and inherit different plasmids within a single *C. perfringens* cell would indicate that these plasmids are incompatible, possibly due to either replication or partitioning interference (4).

Since the current study localized both *cpe* and *tpeL* to plasmids among the surveyed type C isolates, plasmid incompatibility issues could similarly help to explain why none of these type C isolates carry both *cpe* and *tpeL* genes. Specifically, the *cpe* gene was detected in type C isolates carrying *tpeL*-negative, *cpb*-positive plasmids of ~75 kb, 85 kb, or 110 kb but not in those type C isolates carrying the *tpeL*-positive, *cpb*-positive plasmids of ~65 kb or ~90 to 95 kb. Since the current study found that those same ~65-kb or ~90- to 95-kb *cpb* plasmids also appear to be present in most, if not all, type B isolates, incompatibility between the ~65-kb or ~90- to 95-kb *cpb* plasmids and some or all *cpe* plasmids could possibly explain why *cpe*-positive type B isolates have not yet been identified.

Extrapolating further, incompatibility issues possibly offer an explanation for why other toxin plasmid combinations have never been observed in a single *C. perfringens* cell. For example, no *C. perfringens* isolate that carries both an iota toxin-encoding plasmid and a *cpb* plasmid has yet been found. However, it is clear that some toxin plasmid combinations can be stably maintained since type B isolates carry their *cpb* and *etx* genes on separate plasmids (31). Similarly, the current study found that some type C isolates carry *cpb* and *tpeL* on separate plasmids. These observations suggest that *C. perfringens* toxin plasmid incompatibility issues represent a research topic worthy of future study. Similar plasmid incompatibility issues may also affect the virulence of other pathogenic clostridia. For example, although plasmids exist that encode only botulinum neurotoxin serotype A or B (13, 23), all surveyed *Clostridium botulinum* stains producing both neurotoxin serotypes B and A carry these two neurotoxin genes on the same plasmid (13, 22). This observation might open the possibility that plasmids encoding only serotype B or serotype A cannot be stably maintained together in the same *C. botulinum* cell.

Combining the current and previous results may also permit speculation regarding the origin of type B isolates. Both type C and type D isolates are present in soil (21), where they might occasionally come in physical contact. As mentioned above, the current study found that nearly all *cpb* plasmids of type C isolates carry *tcp* sequences, which are known to mediate conjugative transfer of other *C. perfringens* plasmids (2). Similar *tcp* sequences are also present on nearly all *etx* plasmids found in type D isolates (32). Therefore, close contact in soil (or

possibly other locations) could allow conjugative toxin plasmid exchange between a type C isolate and a type D isolate. This transfer of a *cpb* or *etx* plasmid between type C and D isolates may often be rapidly followed by loss of one of these two toxin plasmids due to plasmid incompatibility issues, as discussed above. However, stable maintenance of both *etx* and *cpb* plasmids in a single *C. perfringens* isolate, creating a type B isolate, may occur when this transfer involves introduction of the ~65-kb or ~90- to 95-kb *tpeL*-positive *cpb* plasmid carried by many type C isolates into a type D isolate carrying an ~65-kb *etx* plasmid, or vice versa. This hypothesis, which must be experimentally tested, offers an explanation for why type B isolates are the least common of all *C. perfringens* isolates, i.e., they would stably arise only from matings between specific subpopulations of type C and type D isolates.

Besides shedding light on the possible evolution and diversity of *C. perfringens* toxin plasmids, the current results suggest a few additional insights. First, the current study found that the *cpb* gene of many type C isolates is located near several putative transposase ORFs (including two IS*1151*-like sequences) and the *dcm* gene. This finding supports previous suggestions that the *dcm* region of toxin plasmids may represent a hot spot for insertion of toxin gene-carrying mobile genetic elements, particularly those associated with IS*1151* sequences. Previous observations have established the presence of IS*1151* sequences and several other plasmid-borne toxin genes of *C. perfringens*, including iota toxin-encoding genes, *cpe* genes, and *etx* genes, near *dcm* genes (19, 26, 27, 32). Second, it has become clear that many *C. perfringens* toxin plasmids are related. For example, the pCPF5603 *cpe* plasmid family, the pCPF4969 *cpe* plasmid family, the ~65-kb *etx* plasmid family, and many plasmids encoding NetB toxin (18) show extensive homology (26, 27). However, our overlapping PCR results suggest that the *cpb* plasmid found in type C isolates may substantially differ from those other toxin plasmids.

Finally, the current results offer another intriguing observation of potential virulence significance. Specifically, no associations were readily apparent between the origin of the surveyed type C isolates and their plasmid size variations, with one possible exception. All three human enteritis necroticans (pigbel) isolates surveyed in this study were found to carry a 110-kb, *tpeL*-negative *cpb* plasmid. Furthermore, these pigbel isolates were the only surveyed type C isolates found to carry this *cpb* plasmid. Therefore, although pigbel isolates are difficult to obtain, future studies should further examine whether a specific relationship exists between the 110-kb *cpb* plasmid and pigbel disease. If so, this *cpb* plasmid might encode other accessory virulence factors that contribute to this important human enteric disease. Sequencing of this 110-kb *cpb* plasmid, along with other *cpb* plasmids, may provide further insight into potential plasmid-borne virulence genes and should be pursued in the future.

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