

Characterization of Virulence Plasmid Diversity among *Clostridium perfringens* Type B Isolates[∇]

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The important veterinary pathogen *Clostridium perfringens* type B is unique for producing the two most lethal *C. perfringens* toxins, i.e., epsilon-toxin and beta-toxin. Our recent study (K. Miyamoto, J. Li, S. Sayeed, S. Akimoto, and B. A. McClane, *J. Bacteriol.* 190:7178-7188, 2008) showed that most, if not all, type B isolates carry a 65-kb epsilon-toxin-encoding plasmid. However, this epsilon-toxin plasmid did not possess the *cpb* gene encoding beta-toxin, suggesting that type B isolates carry at least one additional virulence plasmid. Therefore, the current study used Southern blotting of pulsed-field gels to localize the *cpb* gene to ~90-kb plasmids in most type B isolates, although a few isolates carried a ~65-kb *cpb* plasmid distinct from their *etx* plasmid. Overlapping PCR analysis then showed that the gene encoding the recently discovered TpeL toxin is located ~3 kb downstream of the plasmid-borne *cpb* gene. As shown earlier for their epsilon-toxin-encoding plasmids, the beta-toxin-encoding plasmids of type B isolates were found to carry a *tcp* locus, suggesting that they are conjugative. Additionally, *IS1151*-like sequences were identified upstream of the *cpb* gene in type B isolates. These *IS1151*-like sequences may mobilize the *cpb* gene based upon detection of possible *cpb*-containing circular transposition intermediates. Most type B isolates also possessed a third virulence plasmid that carries genes encoding urease and lambda-toxin. Collectively, these findings suggest that type B isolates are among the most plasmid dependent of all *C. perfringens* isolates for virulence, as they usually carry three potential virulence plasmids.

Isolates of the Gram-positive, spore-forming anaerobe *Clostridium perfringens* are classified (31) into five different types (A to E), depending upon their production of four (alpha, beta, epsilon, and iota) lethal typing toxins. All *C. perfringens* types produce alpha-toxin; in addition, type B isolates produce both beta- and epsilon-toxins, type C isolates produce beta-toxin, type D isolates produce epsilon-toxin and type E isolates produce iota-toxin. Except for the chromosomal alpha-toxin gene (*plc*), all *C. perfringens* typing toxins are encoded by genes resident on large plasmids (11, 22, 23, 32, 33). Large plasmids can also encode other *C. perfringens* toxins, such as the enterotoxin (CPE) or beta2-toxin (8, 9, 14, 35), as well as other potential virulence factors such as urease (12, 23).

The large virulence plasmids of *C. perfringens* are only now being characterized (23, 28, 29, 33). The first analyzed, and still most studied, *C. perfringens* toxin plasmids are the CPE-encoding plasmids of type A isolates (14, 28). In type A isolates, most plasmids carrying the enterotoxin gene (*cpe*) belong to one of two families: (i) a 75.3-kb plasmid with a *cpe* locus containing an *IS1151* element and the *cpb2* gene encoding beta2-toxin or (ii) a 70.5-kb plasmid that lacks the *cpb2* gene and carries a *cpe* locus with an *IS1470*-like sequence instead of an *IS1151* element. Sequence comparisons (28) revealed that these two *cpe*

plasmid families of type A isolates share a conserved region of ~35 kb that includes the transfer of clostridial plasmid (*tcp*) locus, which is related to the conjugative transposon Tn916. Confirming that *cpe* plasmids can be conjugative, mixed mating studies have directly demonstrated transfer of the *cpe* plasmid from type A isolate F4969 to other *C. perfringens* isolates (5). A similar *tcp* locus is also shared by the tetracycline resistance plasmid pCW3 and several other toxin plasmids (2, 23, 28, 29, 33), as discussed below. Mutagenesis analyses demonstrated the importance of several genes in the *tcp* locus for conjugative transfer of pCW3 (2) and, by extension, presumably the *tcp*-carrying, conjugative toxin plasmids, such as the *cpe* plasmid of isolate F4969 (5) and some *etx* plasmids of type D isolates (19).

Although the sequence of an iota-toxin-encoding plasmid has not yet been published, pulsed-field gel electrophoresis (PFGE) and PCR analyses determined that these plasmids are typically larger than the *cpe* plasmids of type A isolates (23). Specifically, iota-toxin plasmids are often ≥100 kb in size, reaching up to a size of ~135 kb. These plasmids of type E isolates often encode, in addition to the iota-toxin, other potential virulence factors such as lambda-toxin and urease. These plasmids also carry a *tcp* locus, suggesting that they may be capable of conjugative transfer. Interestingly, many iota-toxin plasmids appear to be related, sometimes extensively, to the *cpe* plasmids of type A isolates. Consequently, it has been suggested (3, 23) that many iota-toxin plasmids arose from insertion of an iota-toxin gene-carrying mobile genetic element near the *cpe* gene on a *tcp*-carrying type A plasmid. This insertional event apparently inacti-

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TABLE 1. Type B plasmid sizes and plasmid-borne virulence gene locations

Isolate ^a	Size(s) (kb) of plasmid(s) carrying:								
	<i>etx</i>	<i>cpb</i>	<i>tpeL</i>	<i>cpb2</i>	<i>tcpF/H</i>	<i>rep</i>	<i>lam</i>	<i>ureC</i>	<i>IS1151</i>
CN1794	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN1990 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
NCTC3110 ^b	65 ^c	65	65	65	65	65			65
CN677 ^b	65 ^c	65	65	65	65	65			65
CN1793 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN1795	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN1886 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN2003 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN2414 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN2416 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN3425 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN3434 ^b	65	90	90	65	65/80/90	65/90	80	80	65/80/90
CN3447	65	90	90	65	65/80/90	65/90	80	80	65/80/90
NCTC8533 ^b	65	90	90	65	65/80/90	65/90	80	80	65/90
PS49	65	90	90	65	65/80/90	65/90	80	80	65/90
JGS1984	65	90	90	65	65/80/90	65/90			65/90
Bar2	65	90	90	65	65/80/90	65/90			65/90

^a Isolates with a CN prefix originated from the Burroughs-Wellcome collection, which included a number of type B isolates typically collected from diseased animals (usually lambs) during the 1930s to 1960s. Except as otherwise indicated, the geographical and disease origins of the isolates are unknown except that Bar2 was obtained from an Australian sheep and NCTC3110 is a vaccine production strain isolated in Kenya.

^b Originated from the United Kingdom.

^c The *etx* and *cpb* genes of NCTC3110 and CN677 are located on a 65-kb plasmid different from their 65-kb plasmid carrying the *cpb* and *tpeL* genes.

vated the *cpe* gene, so most or all type E isolates now carry silent *cpe* genes (3, 23).

The epsilon-toxin-encoding plasmids of type D isolates show considerable size variations (33), ranging from ~48 kb to ~110 kb. These size variations in type D *etx* plasmids reflect, in part, differences among their toxin gene carriage. The small 48-kb *etx* plasmids present in some type D isolates typically lack either the *cpe* gene or the *cpb2* gene (encoding beta2-toxin), while the larger (>75-kb) *etx* plasmids found in other type D isolates can also carry the *cpe* gene, the *cpb2* gene, or both the *cpe* and *cpb2* genes. Consequently, some type D isolates carry a toxin plasmid encoding only *etx*, other type D isolates carry a toxin plasmid with up to three different functional toxin genes (*etx*, *cpb2*, and *cpe*), and the remaining type D isolates carry their *etx*, *cpe*, and *cpb2* genes on up to three distinct plasmids.

C. perfringens type B isolates uniquely produce both beta- and epsilon-toxins, the two most lethal *C. perfringens* toxins (13). These bacteria are important pathogens of sheep but also cause disease in goats, calves, and foals (26). For unknown reasons, diseases caused by *C. perfringens* type B isolates apparently are restricted to certain geographic regions (24, 25, 26). *C. perfringens* type B enterotoxemias initiate when these bacteria proliferate in the gut, accompanied by toxin production. Those toxins initially affect the intestines but later are absorbed and act systemically. Studies from our group (13) showed that beta- and epsilon-toxins each contribute to lethality in a mouse model involving intravenous injection of type B culture supernatants.

There has been characterization of only one type B virulence plasmid to date. Our recent study (29) showed that most, if not all, type B isolates carry a common *etx* plasmid of ~65 kb that also possesses a *tcp* locus and a *cpb2* gene, although not the *cpb* gene encoding beta-toxin. Interestingly, the type B *etx* plasmid is highly (80%) related to the ~75-kb *cpe*- and *cpb2*-carrying plasmid found in some type A isolates (28). The ~65-kb *etx*

plasmid present in most, if not all, type B isolates is also carried by a minority of type D isolates (29).

The absence of the *cpb* gene from their *etx* plasmids suggested that most type B isolates might carry additional virulence plasmids. Therefore, the current study was performed to better address virulence plasmid carriage and diversity among type B disease isolates.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. As indicated previously (13, 29), the 17 *C. perfringens* type B isolates examined in this study originated mainly from diseased animals (Table 1). The toxin genotypes of these isolates (13) were determined previously using multiplex PCR (15). Each type B isolate was grown overnight at 37°C under anaerobic conditions on SFP agar (Difco Laboratories) containing 0.04% D-cycloserine (Sigma Aldrich) in order to ensure culture purity. Fluid thioglycolate medium (FTG) (Difco Laboratories) or TGY medium (3% tryptic soy broth [Becton-Dickinson] containing 2% glucose [Fisher Scientific], 1% yeast extract [Becton-Dickinson], and 0.1% thioglycolate [Sigma Aldrich]) was used to grow broth cultures.

Pulsed-field gel electrophoresis. *C. perfringens* type B isolates were grown overnight at 37°C in FTG broth. A 0.1-ml aliquot of each starter culture was then inoculated into 10 ml of TGY and grown overnight at 37°C. The overnight TGY cultures were used to prepare genomic *C. perfringens* DNA-containing agarose plugs, as described previously (33).

For isolates showing Southern blot signal colocalization using probes for two different open reading frames (ORFs) (as described below), DNA plugs were digested with restriction endonuclease ApaI, AvaI, ClaI, KpnI, NcoI, SphI, NheI, or XhoI (New England Biolabs) to help distinguish whether these colocalizing signals were due to the different probes hybridizing to two genes carried on the same plasmid or to two genes located on two comigrating plasmids (9, 23, 33). In these experiments, each set of plugs was incubated, with or without a restriction enzyme, in 200 µl of the appropriate buffer solution recommended by the enzyme manufacturer.

Pulsed-field gel electrophoresis was performed with a 1% agarose gel using a CHEF-DR II system (Bio-Rad Laboratories) and 0.5× Tris-borate-EDTA buffer (Bio-Rad Laboratories) at 14°C. The running parameters for undigested DNA were as follows: initial pulse, 1 s; final pulse, 25 s; voltage, 6 V/cm; time, 24 h. The following running parameters were used for DNA digested with restriction enzymes: initial pulse, 1 s; final pulse, 12 s; voltage, 6 V/cm; time, 16 h. After pulsed-field gel electrophoresis, the gel was stained with ethidium bromide,

TABLE 2. Primers used for internal amplification of *tpeL* for linkage analysis and sequencing of *cpb* to *tpeL*

Primer	Sequence (5' to 3')	PCR product (size, bp)
TpelscF	CATTGCCATTTATTTTCAG	(646)
TpelscR	TATTCATTTTGTGGGGTTG	
TpelR1	GATTATAAAGCGAAAACCTTTGAC	R1 (1,245)
TpelF1	ATTCCTATAATTATTTTATTATCC	
SeqR6	GGATAATAAAATAATTATAGGAAAT	R2 (1,244)
SeqF6	CTCTATATCCTATAATTGGTTCC	
SeqR5	GGAACCAATTATAGGATATAGAG	R3 (1,172)
SeqF5	GCATGGAGATAGAGTAGACC	
SeqR4	GGTCTACTCTATCTCCATGC	R4 (1,098)
SeqF4	GTTATTCCTGCAATATCCATAAG	
cpbR3	GTTCTAACTGCTCCTAATGG	R5 (1,045)
cpbF3	CTTATGGATATTGCAAGAATAAC	
N1	TATATGAATTAGAAAATGATGGTG	— ^a
N2	TATTGAATGATAACTCTGAAAAG	—
N3	TCATCTTTAGTATCTCTAAATC	—
N4	TTATTCCTAAAAAGTCTGGTG	—

^a —, sequencing primer.

washed with distilled water, and photographed under UV light. Mid-range or low-range PFG markers (New England Biolabs) were used as molecular size standards, as appropriate.

Southern blot analyses of pulsed field gels. Digoxigenin (DIG)-labeled *etx*, *cpb*, *cpb2*, *tcpH*, *tcpF*, *IS1151*, replication protein gene (*rep*), urease C gene (*ureC*), and lambda-toxin gene (*lam*) probes were prepared with primers described previously (23, 33, 34). Using those DIG-labeled probes, Southern hybridization of pulsed-field gels was performed by our standard techniques (23, 29, 33). DIG labeling and detection reagents were obtained from Roche Applied Science. The CSPD substrate (Roche Applied Science) was used for detection of hybridized probes according to the manufacturer's instructions.

PCR analyses of *lam* and *ureC* genes in *C. perfringens* type B isolates. Carriage of the *lam* gene or *ureC* gene was assessed by PCR, as described previously (23, 33). The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide for visualization.

PCR analyses of *tpeL* carriage and linkage between *cpb* and *tpeL* in type B isolates. To evaluate the presence of the *tpeL* gene encoding the *C. perfringens* large cytotoxin (TpeL) in type B isolates, PCR was performed using the primers (TpelscF and TpelscR) listed in Table 2. Based upon the *C. perfringens* type B strain ATCC 3626 sequence released by the J. Craig Venter Institute (JCVI), a series of primers were then constructed (Table 2) to evaluate, by overlapping PCR, the potential linkage between *tpeL* and *cpb* in type B isolates. PCR conditions for these amplifications were as follows: 94°C for 3 min and 35 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 1.5 min. PCR products were run on a 1% agarose gel and stained with ethidium bromide for visualization.

Long-range PCR studies were also performed to directly connect the *tpeL* and *cpb* genes. DNA was isolated from type B strains NCTC3110, CN677, CN1793, CN1795, CN3447, Bar2, and PS49 using the Master-Pure 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 TpelR1 and cpbF3 (Table 2) (1 µM final concentration). 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 5 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 CN677 and CN1795 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 primers listed in Table 2.

PCR linkage of the *cpb* gene to *IS1151*-like sequences in type B isolates. Based upon the ATCC 3626 sequence released by JCVI, a possible linkage between the *cpb* gene and *IS1151*-2 insertion sequences in other *C. perfringens* type B isolates was investigated by PCR using primers cpb-F (5'-CTTGAAGAGTCAACAGATTGAT-3') and IS1151-R (5'-GCTGCTAAAGTCTCTACTAG-3'). PCR conditions for these amplifications were as follows: 94°C for 3 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 4 min; and a single extension at 68°C for 10 min. PCR products were run on a 1% gel and stained with ethidium bromide for visualization.

Reverse transcription-PCR (RT-PCR) analysis of *tpeL* expression in type B isolates. *C. perfringens* type B isolates NCTC3110, CN677, CN3447, and NCTC8533 were grown overnight at 37°C in FTG. A 0.1-ml aliquot of each of those starter cultures was transferred to 10 ml of TGY medium and grown at 37°C for either 8 h or 24 h. Total *C. perfringens* RNA was extracted from 2 ml of an overnight TGY culture using saturated phenol (Fisher Scientific). After centrifugation (10,000 × g at 4°C for 5 min), the nucleic acid-containing supernatant received cold ethanol. The sample was mixed well and then centrifuged (10,000 × g at 4°C) for 5 min to obtain the RNA pellet. The pellet was washed two times with cold 70% ethanol and then resuspended in 100 µl of DNase-free, RNase-free water. All RNA samples were additionally treated with 2 U of DNase I (Ambion) at 37°C for 30 min. To stop DNase I activity, a DNase I inhibitor (Ambion) was added to the reaction tube. RNA was quantified by absorbance at 260 nm and stored in 50-µl aliquots at -80°C.

RT-PCR was performed with the DNase-treated RNA samples using the AccessQuick RT-PCR system (Promega). Briefly, each RNA sample (50 ng) was reverse transcribed to cDNA at 45°C for 45 min and then used as the template for PCRs (35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a single cycle at 72°C for 10 min) with the gene-specific primers (TpelscF and TpelscR [Table 2]) or housekeeping gene *polC* using primers and conditions as described before (36) to check the quality of RNA samples (data not shown). Control RT-PCRs were similarly performed, except for the omission of reverse transcriptase.

PCR identification of possible circular transposition intermediates carrying the *cpb* or *tpeL* ORF. Template DNAs used for these PCR studies were prepared as described previously (33). By using those template DNAs, PCR amplification of possible circular transposition intermediates containing the *cpb* or *tpeL* ORF was assessed using the primer sets TnF (5'-ATACATTAACCTAGAACGTAC-3') and BetaR (5'-GAAAGAACTGTTATTATCTTAATTG-3') for *cpb* loop and LoopF (5'-AACCAATTATAGGATATAGAG-3') and LoopR (5'-GCTACTTAGCTAGTGAAG-3') for *tpeL* loop. PCR conditions for these amplifications were as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 40 s, and 68°C for 2 min; and a single cycle of 68°C for 10 min. PCR products were run on a 1% agarose gel and stained with ethidium bromide for visualization. PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced at the Genomics and Proteomics Core Laboratory of the University of Pittsburgh, using vector-specific primers M13F and M13R.

Nucleotide sequence accession numbers. Results from the sequencing analyses were deposited in GenBank under accession numbers GU054491 and GU054492.

RESULTS

Characterization of toxin plasmid diversity among type B isolates. To initiate these studies, pulsed-field gels were run under electrophoresis conditions that (i) allow plasmids, but not chromosomal DNA, to enter the gel (9, 12, 23, 28, 33) and (ii) provide an accurate measurement of *C. perfringens* plasmid size (28). We first subjected DNA that had been electrophoresed on a pulsed-field gel to Southern blot hybridization with an *etx* probe (Fig. 1A and Table 1), which confirmed previous observations (29) that *etx* is consistently present on an ~65-kb plasmid in type B isolates.

Previous experiments had also indicated that in type B isolates, a *cpb2* gene is present on the ~65-kb *etx* plasmid (29). To confirm that finding and (more importantly) assess for the first time whether some type B isolates might possess additional plasmids also carrying *cpb2*, DNA on a pulsed-field gel was Southern blotted with a *cpb2* probe. However, those blots showed a single *cpb2* signal at 65 kb (Fig. 1B), strongly sug-

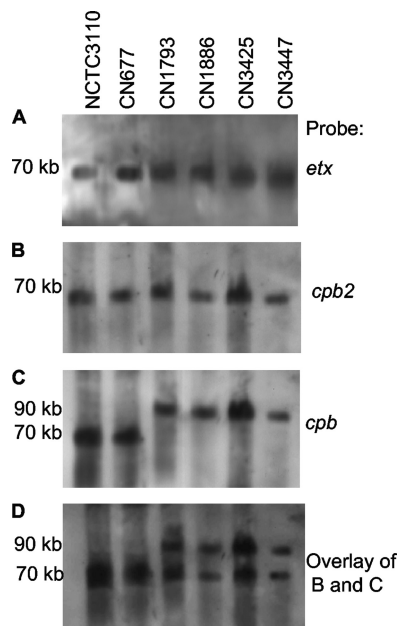


FIG. 1. Southern hybridization analyses of pulsed-field gels run with DNAs extracted from representative type B isolates NCTC3110, CN677, CN1793, CN1886, CN3425, and CN3447. (A) A blot hybridized with an *etx*-specific probe; (B) a similar blot of a different pulsed gel hybridized with a *cpb2*-specific probe; (C) the blot from panel B was stripped and reprobred with a *cpb*-specific probe; (D) overlay of the *cpb*-probed blot and *cpb2*-probed blot of panels B and C. The migration of molecular size markers is indicated on the left, and isolate designations are indicated above the lanes.

gesting that most, if not all, type B isolates possess only the *cpb2* gene previously shown to be present on their ~65-kb *etx*-carrying plasmid (29).

Plasmid carriage of the *cpb* gene among type B isolates has not yet been surveyed (note that, despite their similar names, *cpb* and *cpb2* do not share sequence homology [16]). Therefore, the same Southern blot that had been hybridized with a *cpb2* probe was stripped and rehybridized with a *cpb* probe. This analysis revealed that the *cpb* gene is usually present on an ~90-kb plasmid in type B isolates (Fig. 1C and Table 1). The presence of distinct *etx/cpb2* plasmids and *cpb* plasmids in most type B isolates was clearly demonstrated by overlaying the Southern blots shown in Fig. 1 after separate hybridizations with *cpb2* or *cpb* probes (Fig. 1D). However, two type B isolates, NCTC3110 and CN677, did not fit this general pattern, as they carried their *cpb* gene on an ~65-kb plasmid (Fig. 1C), matching the size of their *etx/cpb2* plasmid.

Evaluation of whether *cpb* and *etx/cpb2* are present on the same or different plasmids in type B isolates NCTC3110 and CN677. As shown in Fig. 1, DNAs from type B isolates NCTC3110 and CN677 hybridized the *cpb*, *etx*, and *cpb2* probes at the same 65-kb pulsed-field gel blot location, indicating that these two isolates carry these three toxin genes either on the same plasmid or (since *etx* and *cpb2* are present on the same 65-kb plasmid in type B isolates [29]) on two distinct comigrating plasmids of similar sizes.

To discriminate between those two possibilities, DNA from NCTC3110 or CN677 was digested with a battery of restriction

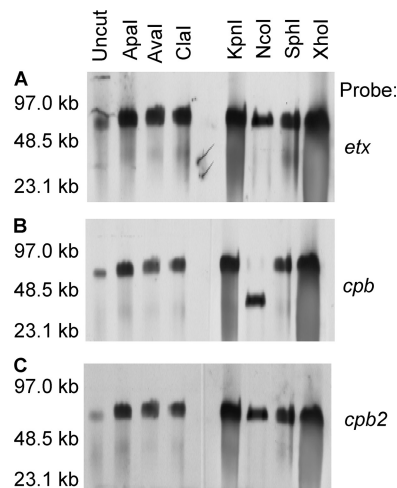


FIG. 2. Southern hybridization analyses of pulsed-field gels run with restriction enzyme-digested DNA extracted from type B isolate NCTC3110. The blot was first hybridized with an *etx*-specific probe (A) and then stripped and reprobred with a *cpb*-specific probe (B) before a final stripping and reprobred with a *cpb2*-specific probe (C). The migration of molecular size markers is indicated on the left.

endonucleases. In this experiment (9, 23, 33), the migration of two genes residing on the same plasmid should consistently exhibit the same response (sensitivity or insensitivity) to digestion using each restriction endonuclease. However, if one gene showed no change in migration after digestion with a particular restriction endonuclease but migration of the second gene was affected by digestion with the same enzyme, this would indicate that the two genes are present on plasmids that are of similar size but distinct (9, 23, 33).

For NCTC3110 DNA, the migration of *etx*-containing DNA and *cpb*-containing DNA exhibited a different susceptibility pattern of sensitivity to restriction enzyme digestion (Fig. 2A and B). In contrast, *cpb2*-containing DNA showed a susceptibility to restriction enzyme digestion that was similar to that of *etx*-containing DNA (Fig. 2A and C), which is consistent with previous conclusions that these two toxin genes are located on the same 65-kb plasmid in type B isolates (29). These results indicated that NCTC3110 carries its *cpb* gene on a plasmid of similar size as, but different from, the one carrying the *etx* and *cpb2* genes.

Similar analysis indicated (data not shown) that the *cpb2* and *etx* genes of CN677 are also present together on ~65-kb plasmid that is distinct from the ~65-kb plasmid carrying the *cpb* gene in this type B isolate.

Carriage of the *rep* gene among plasmids of type B isolates. The Rep protein was shown to be important for replication of *C. perfringens* tetracycline resistance plasmid pCW3 (2). The gene (*rep*) encoding the Rep protein has also been localized to several toxin plasmids in type A, D, and E isolates (23, 28, 33), including the sequenced *etx/cpb2* plasmid of type B isolates (29). Therefore, a Southern blot analysis of pulsed-field gels was performed to assess whether type B isolates might possess additional plasmids, besides their *etx/cpb2* plasmid, that also carry *rep* sequences. This survey demonstrated that, for all surveyed type B isolates, the *rep* probe consistently hybridized to the same blot locations as did the *cpb* probe and the

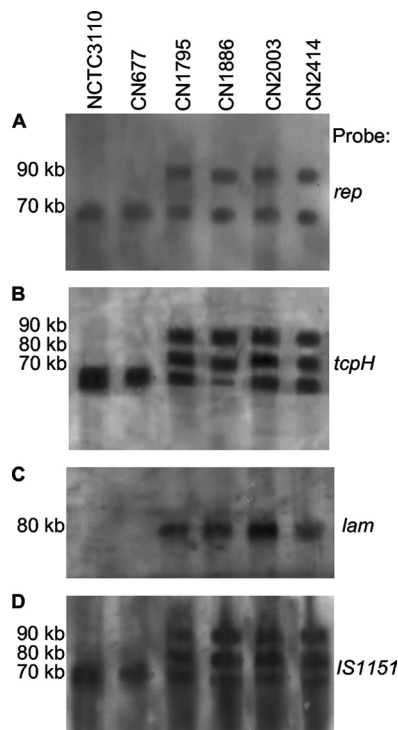


FIG. 3. Southern hybridization analyses of pulsed-field gels run with DNAs extracted from type B isolates NCTC3110, CN677, CN1795, CN1886, CN2003, and CN2414. Probes used were a *rep*-specific probe (A), a *tcpH*-specific probe (similar results were obtained using a *tcpF*-specific probe [not shown]) (B), a *lam*-specific probe (similar results were obtained using a *ureC* probe [not shown]) (C), and an *IS1151* probe (D). The migration of molecular size markers is indicated on the left.

etx probe, suggesting that *rep* is commonly present on *cpb* plasmids, as well as on *etx/cpb2* plasmids of type B isolates (Fig. 3A).

Plasmid *tcp* locus carriage by type B isolates. As mentioned in the introduction, *tcp* genes have been shown to mediate conjugative transfer of *C. perfringens* plasmid pCW3 and are also likely to be responsible for the demonstrated conjugative transfer of the *cpe* plasmids present in some type A isolates (5, 28), as well as the *etx* plasmids of some type D isolates (19).

Since a *tcp* locus was identified on the *etx/cpb2* plasmid of type B isolates (29), a survey was performed to determine whether *tcp* genes might be associated with the *cpb* plasmid or other plasmids in type B isolates. For this survey, a pulsed-field gel Southern blot was hybridized with probes specific for two *tcp* genes (*tcpF* or *tcpH*) known to be required for pCW3 conjugative plasmid transfer (2). In this experiment, all surveyed type B isolates hybridized both *tcp* probes, with most type B isolates cohybridizing the *tcpF* and the *tcpH* probes at multiple blot locations (Fig. 3B and Table 1). For all surveyed type B isolates, these two *tcp* probes always cohybridized to the same blot location where the *etx/cpb2* plasmid had migrated, as expected from previous sequencing and PCR results (29). These *tcp* probes also consistently cohybridized to the same blot location containing the *cpb* plasmid. Finally, most type B isolates hybridized the *tcp* probes at a third location, distinct from that for the *cpb* or *etx/cpb2* plasmid, indicating that those

type B isolates carry a third large (~80-kb) plasmid with conjugative potential.

PCR and Southern blot analyses to evaluate the presence of plasmid-borne lambda-toxin (*lam*) and urease C (*ureC*) genes in type B isolates. Lambda-toxin, a *C. perfringens* metalloprotease, can proteolytically activate epsilon-toxin (20, 30). Since the lambda-toxin gene (*lam*) has been detected in some type D isolates (20, 33), which resemble type B isolates in producing epsilon-toxin, our 17 type B isolates were surveyed by PCR to determine their carriage of the *lam* gene. This PCR testing detected the presence of the *lam* gene in 13 of the 17 (~75%) surveyed type B isolates (data not shown).

To assess whether the *lam* gene is plasmid borne in *lam*⁺ type B isolates, a Southern blot analysis was performed after type B isolate DNA was electrophoresed on pulsed-field gels. As expected, this analysis detected no *lam* probe hybridization with DNAs from the four (NCTC3110, CN677, JGS1984, and Bar2) type B isolates that had tested PCR negative for carriage of the *lam* gene (Fig. 3C and data not shown). In contrast, DNAs from all *lam* PCR-positive type B isolates hybridized with the *lam* probe. This Southern blot analysis further revealed that all the *lam*⁺ type B isolates carried their *lam* gene on a plasmid of ~80 kb (Fig. 3C and Table 1).

Urease has been considered a potential *C. perfringens* virulence factor, and the genes encoding urease were detected on plasmids carried by about 2% of *C. perfringens* isolates in a global survey (12). In several type D and E isolates, urease genes have been localized to large plasmids that also carry the *etx* or iota-toxin genes, respectively (12, 23, 33). While it has been shown previously that *ure* genes are present in a few type B isolates (12), the extent of *ure* gene carriage among type B isolates has not been reported, nor has it been determined whether *ure* genes in type B isolates are plasmid borne and, if so, whether they are carried by toxin-encoding plasmids as in type D and E isolates.

Therefore, our type B isolate collection was surveyed for *ureC* gene carriage by Southern blot analyses of pulsed-field gels. Those analyses found that DNAs from 13 of 17 (~75%) isolates hybridized the *ureC* probe (data not shown). Furthermore, these blots demonstrated a clear association between *ureC* and *lam* carriage; i.e., isolates which were *lam*⁺ were also *ureC*⁺ and vice versa (Table 1). It was also noted that, using DNA from each *lam*⁺/*ureC*⁺ type B isolate, the *ureC* probe and *lam* probes always cohybridized to the same Southern blot location (Table 1).

Given those results, restriction digestion analysis was conducted to test whether these two genes are present, in two representative *lam*⁺/*ureC*⁺ type B isolates, on the same plasmid or on two plasmids of similar size. Those analyses showed (Fig. 4) that *lam* and *ureC* migration were coincidentally affected when DNA from type B isolate NCTC8533 or CN3447 was digested with restriction endonucleases; i.e., for these two type B isolates, if a nuclease affected migration of their *ureC*-carrying DNA, it also consistently affected migration of their *lam*-carrying DNA, and vice versa. These results suggest that the *lam* and *ureC* genes reside on the same ~80-kb plasmid in these type B isolates.

Southern blot and PCR analyses of *IS1151* association with *cpb*-carrying plasmids in type B isolates. *IS1151* insertion sequences are closely associated with several toxin genes in *C.*

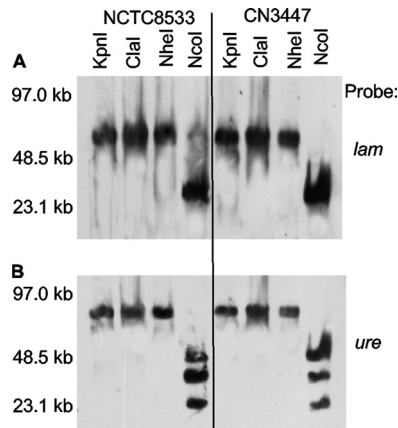


FIG. 4. Southern hybridization analyses of pulsed-field gels run with restriction enzyme-digested DNAs extracted from type B isolates NCTC8533 and CN3447. The blot was first hybridized with a *lam*-specific probe (A) before stripping and reprobng with a *ureC*-specific probe (B). The migration of molecular size markers is indicated on the left.

perfringens (10, 14, 23, 28, 29, 33), including the *etx* genes in type B and D isolates. When analyzed by Southern blot analyses of pulsed-field gels, an *IS1151* probe cohybridized with the location of the *etx* and *cpb* plasmids in all surveyed type B isolates (Fig. 3D and Table 1). In addition, all isolates, except NCTC3110 and CN677, possessed one or two other *IS1151*-carrying plasmids.

Bioinformatic analyses of sequence data for type B strain ATCC 3626 released by JCVI identified two *IS1151*-like sequences upstream of *cpb*. Therefore, a PCR analysis was performed using primers based upon the ATCC 3626 sequence to investigate whether *IS1151*-like sequences reside upstream of the *cpb* gene in other type B isolates. In this PCR, all four surveyed type B isolates (NCTC3110, NCTC8533, CN1793, and CN1795) supported PCR amplification of a product matching the expected size of a product from the *IS1151*-2-*cpb* region of ATCC 3626 (data not shown).

PCR evaluation of *tpeL* gene carriage among type B isolates. Inspection of the JCVI ATCC 3626 sequence revealed this isolate carries the *tpeL* gene, which encodes the recently discovered TpeL toxin (1). Therefore, a PCR assay was performed to assess the presence of the *tpeL* ORF among other type B isolates. This survey determined that DNAs from all 17 surveyed type B isolates supported amplification of a *tpeL* PCR product (data not shown).

Bioinformatic analysis of the ATCC 3626 sequence also indicated that the *tpeL* gene in this type B isolate is located ~3 kb downstream of its *cpb* gene (Fig. 5A). Therefore, an overlapping PCR assay was performed, using a five-pair set of primers (encoding PCR products R1 to R5 [Table 2]), to assess whether a similar genetic arrangement might exist between the *cpb* and *tpeL* genes in other type B isolates. Results from this overlapping PCR assay were consistent with the *cpb* gene being linked to the *tpeL* gene in all four (NCTC3110, CN677, NCTC8533, and CN3447) surveyed type B isolates (Fig. 5B and data not shown). This suggestion received further support from long-range PCR, where internal *tpeL* and *cpb* primers amplified a ~5.7-kb product from seven different type B iso-

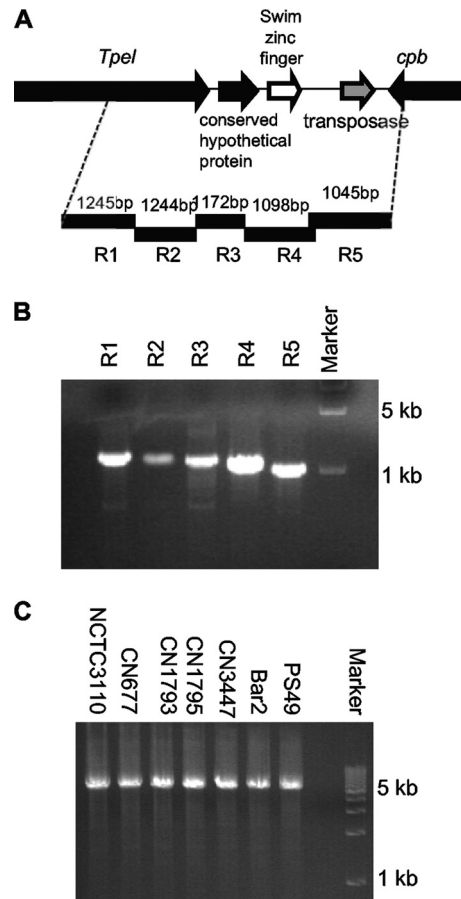


FIG. 5. (A) Arrangement of the *cpb* and *tpeL* gene locus in *C. perfringens* type B isolate ATCC 3626 (based upon sequencing results released from JCVI). Solid black bars indicate the span of overlap PCR products. (B) Overlap PCR analysis of the region extending from *cpb* to *tpeL* in NCTC 3110 using reaction R1 to R5 primers (Table 2). (C) Long-range PCR assay using an internal *cpb* primer and an internal *tpeL* primer to link the *tpeL* and *cpb* genes.

lates (Fig. 5C). This ~5.7-kb product matched the expected size of the product that these primers would amplify from ATCC 3626 DNA. In addition, sequencing confirmed that the ~5.7-kb products from NCTC3110 and CN1795 showed >99.5% similarity to the sequence present in the *tpeL*-*cpb* region of ATCC 3626.

Expression of TpeL by *C. perfringens* type B isolates. In previous studies (13), we evaluated the expression of perfringolysin O, alpha-toxin, beta-toxin, beta2-toxin, and epsilon-toxin by type B isolates. However, TpeL toxin was identified (1) after that earlier study. Therefore, given the current PCR detection of the *tpeL* gene in all surveyed type B isolates (Fig. 5 and Table 1), RT-PCR analyses were performed to evaluate whether the *tpeL* gene is expressed by four representative type B isolates (NCTC3110, CN677, CN3447, and NCTC8533). This experiment showed that three of these isolates had detectable levels of *tpeL* transcription after either 8 h or 24 h of growth in TGY broth (Fig. 6), which opens the possibility that TpeL could contribute to type B isolate virulence.

PCR identification of possible circular transposition intermediates. Inspection of the JCVI ATCC 3626 sequence also

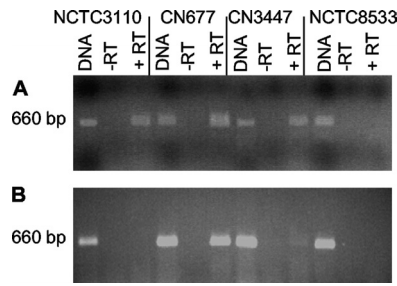


FIG. 6. RT-PCR analysis of *tpeL* expression by type B isolates NCTC3110, CN677, CN3447, and NCTC8533. *tpeL* expression was analyzed at 8 h (A) or 24 h (B) of growth in TGY medium at 37°C with RT (+RT) or without RT (-RT). The migration of molecular size markers is indicated on the left, and isolate designations are indicated above the lanes.

indicated the presence of multiple transposases in the *cpb-tpeL* locus of that isolate. Our overlapping PCR results (Fig. 5B) strongly suggested that this arrangement is conserved in many other type B isolates as well. This finding may be significant since most other plasmid-borne *C. perfringens* toxin genes are also closely associated with insertion sequences (6, 14, 23, 28, 33) and since possible circular transposition intermediates containing those toxin genes have been demonstrated by PCR assays using primers capable of amplifying a PCR product only from DNA sequences that form a short circular loop (4, 23, 33).

Therefore, PCR primers in opposite orientations were designed to evaluate whether circular transposition intermediates might be formed by the transposases present in the *cpb-tpeL* locus (Fig. 7A). Primers BetaR and TnF consistently amplified a strong ~0.6-kb PCR product from several type B isolates

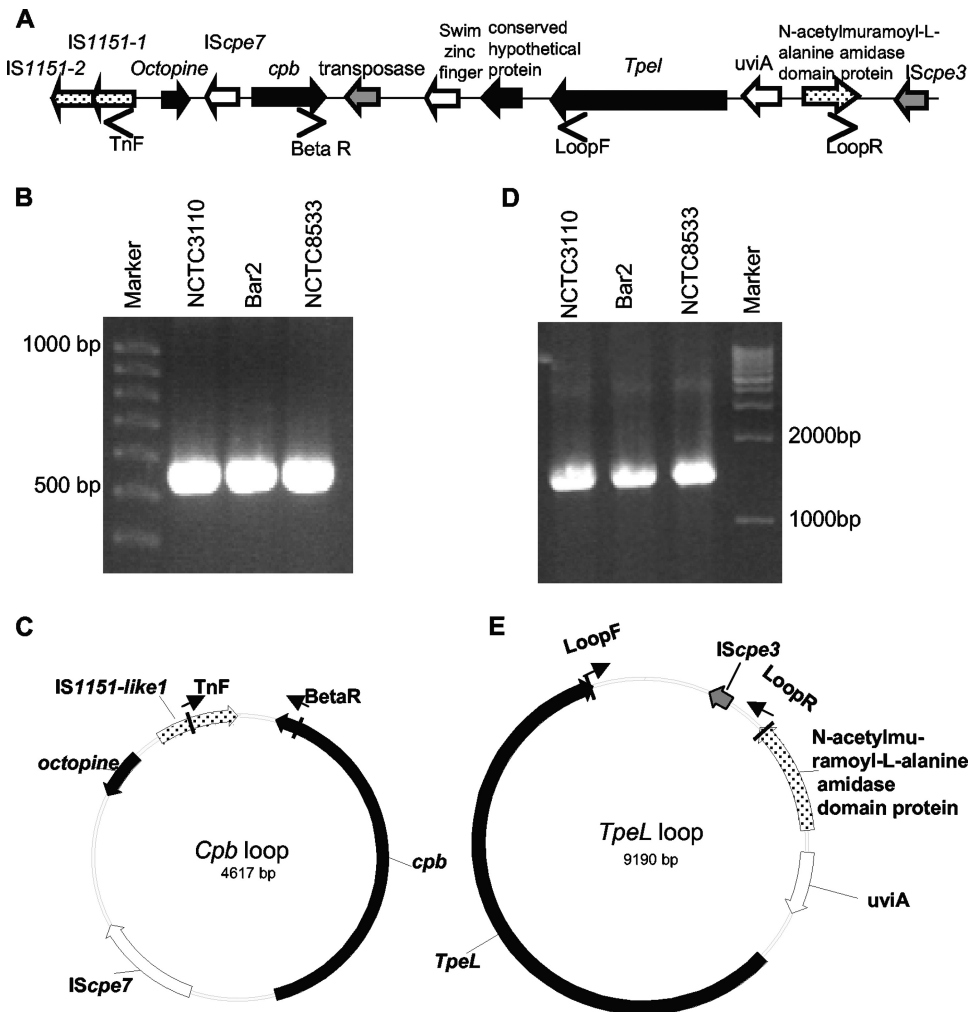


FIG. 7. Detection of potential circular transposition intermediates carrying the *cpb* or *tpeL* gene in type B isolates. (A) Diagram of the *tpeL-cpb* locus in type B isolates (based on sequencing for ATCC 3626 released by JCVI). (B) PCR amplification of *cpb* circular intermediates using the primers BetaR and TnF. The positions of molecular size markers are indicated on the left. (C) Diagram derived from sequencing the product from panel B that depicts the putative circular transposition intermediate supporting PCR amplification with primers BetaR and TnF, with the vertical bars indicating the location in the loop of the PCR product amplified using these two primers. (D) PCR amplification of *tpeL* circular intermediates from NCTC8533 using primers LoopR and LoopF. (E) Diagram derived from sequencing the product from panel D that depicts the putative circular transposition intermediate supporting PCR amplification using primers LoopF and LoopR, with the vertical bars indicating the location in the loop of the PCR product amplified using these primers.

(Fig. 7B). When that product was sequenced, it was shown to contain *cpb* and IS1151-1 sequences, a result consistent with IS1151-mediated mobilization of a circular transposition intermediate containing *cpb* and several adjacent ORFs (Fig. 7C). Similarly, PCR primers LoopF and LoopR consistently amplified a strong ~1.6-kb product (Fig. 7D) from several type B isolates. Sequencing of that product detected 3' *tpeL* and downstream sequences, as well as IS*Spe3* sequences and sequences present immediately upstream of IS*Spe3* (Fig. 7E), which is consistent with IS*Spe3*-mediated mobilization of a *tpeL*-containing circular transposition intermediate.

DISCUSSION

Type B isolates are unique among *C. perfringens* isolates in their ability to produce both beta-toxin and epsilon-toxin. Since these are the two most lethal of the 17 known *C. perfringens* toxins (26), type B isolates are potentially the most virulent of all *C. perfringens* isolates. Therefore, the limited study of the virulence genetics of type B isolates is somewhat surprising. An older pulsed-field Southern blot study (21) had determined that two type B isolates carry their *etx* genes on plasmids, but that previous study was unable to localize the *cpb* gene to plasmids in those two type B isolates. Another pulsed-field Southern blot study by the same group later identified the presence of urease genes in three different type B isolates (7, 12). The most in-depth study of type B virulence genetics to date has been our recent study demonstrating that most, if not all, type B isolates share a common ~65-kb plasmid carrying both *etx* and *cpb2* genes (29).

The current study provides significant new insights into the virulence genetics of type B isolates. To our knowledge, this study has demonstrated for the first time that the *cpb* gene is plasmid borne in most (if not all) type B isolates, as shown earlier for a few type C isolates (21, 32). While most type B isolates were found to carry an ~90-kb *cpb* plasmid, type B isolates NCTC3110 and CN677 possessed ~65-kb *cpb* plasmids. It might be noted these two unusual type B strains had completely independent origins, being isolated 16 years apart at different locations; i.e., CN677 came from the United Kingdom, while NCTC3110 was from Kenya.

Compared to other previously characterized *C. perfringens* toxin plasmids (23, 28, 29, 33), the ~65 to 90-kb *cpb* plasmids in type B isolates are of medium size and show notably less size heterogeneity than the previously characterized *etx* plasmids of type D isolates or the iota-toxin plasmids of type E isolates (23, 33). This limited *cpb* plasmid diversity observed among type B isolates is reminiscent of our earlier determination that most, if not all, type B isolates carry the same 65-kb *etx/cpb2* plasmid. Together, these observations raise the possibility that type B isolates are rare, at least in part, due to virulence plasmid incompatibility issues; i.e., perhaps only certain combinations of *cpb* and *etx* plasmids can be maintained in a single *C. perfringens* cell. This possibility will be further addressed by future studies comparing *cpb* plasmid size and gene content heterogeneity in type B versus type C isolates. If greater *cpb* plasmid heterogeneity is detected among type C isolates, future studies should directly examine potential *etx* and *cpb* virulence plasmid incompatibility issues, a subject that has not (to our knowledge) received any study to date.

Besides their *cpb*, *etx*, and *cpb2* genes, most type B isolates also carry several other potential virulence genes on plasmids, including the *tpeL*, *ureC*, and *lam* genes. Several of these virulence genes mapped to the same plasmid in type B isolates; i.e., the *cpb* and *tpeL* genes reside on one plasmid, while the *ureC* and *lam* genes are present together on a second plasmid. Overall, most type B isolates were found to carry at least three known or potential virulence plasmids, identifying these strains as potentially among the most plasmid dependent of all *C. perfringens* isolates in terms of virulence. By comparison, type A isolates carrying a plasmid-borne *cpe* gene and type E isolates typically possess only one or two virulence plasmids (14, 23, 28); type D isolates are more varied in their virulence plasmid carriage, but only a subset of type D isolates resemble type B isolates by carrying three potential virulence plasmids (33).

Type B isolates may be able to maintain numerous known or potential plasmid-borne virulence genes because these genes are distributed onto several different virulence plasmids. However, since the virulence plasmids of type B isolates often contain homologous regions, such as the *tcp* locus, it is interesting that type B isolates can stably maintain multiple virulence plasmids without apparent recombination problems. Perhaps this plasmid stability reflects the relatively low recombination activity associated with pathogenic *Clostridium* species (18).

Insertion sequences have been previously identified in the iota-toxin gene locus of type E isolates, the *cpe* locus of type A isolates, and the *etx* locus of type B and D isolates (6, 23, 29, 33). On that basis, it was suggested (6, 23, 29, 33) that the common presence of insertion sequences near *C. perfringens* toxin genes facilitates mobilization of these toxin genes, possibly explaining how the *etx* and iota-toxin genes came to reside on several different plasmids or, in the case of *cpe*-positive isolates, on several different plasmids or the chromosome. Consistent with that hypothesis, possible circular transposition intermediates have been detected previously for the *etx*, *cpe*, and iota-toxin genes (6, 23, 33). Similarly, the current study detected several insertion sequences in the plasmid-borne *cpb-tpeL* locus of type B isolates, as well as possible circular transposition intermediates carrying those two toxin genes. Those findings are consistent with insertion sequence-mediated mobilization of *tpeL* and *cpb*, possibly helping to explain how these toxin genes came to reside on two different plasmids in type B isolates. More complete appreciation of the potential contribution of IS-mediated mobilization of *cpb* or *tpeL* genes to virulence plasmid diversity awaits the full characterization of *cpb* plasmids among type C isolates and of *tpeL* plasmids among type A and type C isolates.

As already mentioned, this study detected a *tcp* locus on the *cpb-tpeL* plasmid of type B isolates. This finding further supports the presence of a *tcp* locus as a common feature of *C. perfringens* toxin plasmids, since *tcp* loci have been identified previously on most iota-toxin plasmids of type E isolates, *cpe* plasmids of type A isolates, and *etx* plasmids of type B and D isolates (23, 28, 29, 33). The *tcp* locus has been shown to mediate transfer of *C. perfringens* tetracycline resistance plasmid pCW3 (2), so the presence of a *tcp* locus on the *cpb-tpeL* plasmid of type B isolates suggests that this plasmid may also conjugatively transfer among *C. perfringens* isolates. If a type D isolate acquired, by horizontal spread, a *cpb-tpeL* plasmid, it

would be converted to type B. However, that process must be rare given the scarcity of B isolates among the general *C. perfringens* population; e.g., no type B isolates were detected in Pittsburgh area soils (24). Potential conversion of type C or D isolates to type B by conjugative acquisition of toxin plasmids is probably limited, in part, by the relatively low-level environmental presence of type C and D isolates (24), but the potential plasmid incompatibility issues raised earlier could be another factor limiting the horizontal spread of beta- or epsilon-toxin plasmids to create type B isolates.

The third potential virulence plasmid carried by most type B isolates likely encodes lambda-toxin, a metalloprotease that is capable of proteolytically activating epsilon-toxin and iota-toxin (17, 27). Consistent with proteolytic activation being the primary biologic role of lambda-toxin, it is now clear that the *lam* gene is relatively common among type E isolates, where it can activate iota-toxin, as well as among type B and D isolates, where it can activate epsilon-toxin (references 23 and 33 and this study). However, it is interesting that lambda-toxin gene carriage appears to be more frequent in type B (75% positive) than in type D (23% positive) isolates (reference 33 and this study). Also notable is that type B isolates carry their *lam* gene on a plasmid different from their *etx* plasmid, in contrast to *lam*⁺ type D isolates (33), where *lam* and *etx* can localize to plasmids of the same size. Similarly, the *lam* gene also appears to be commonly present on the iota-toxin plasmids of type E isolates (23).

The *lam*-carrying plasmid of type B isolates also possesses genes encoding another potential virulence factor, urease. A linkage between *lam* and *ure* genes has been noted previously; i.e., most type E isolates carry both *lam* and *ure* genes on their iota-toxin plasmid (23). Whether the common presence of *lam* and *ure* genes on the same plasmid in both type B and E isolates is indicative of a common evolutionary origin or some cooperative functional role will require further study. Interestingly, the type B plasmid carrying *lam* and *ure* lacks the *rep* gene, suggesting that this is a relatively unusual *C. perfringens* virulence plasmid. However, this *lam/ure* plasmid in type B isolates does carry *tcp* locus genes, suggesting that it may be a third conjugative plasmid in these isolates. Finally, the common ~80-kb size of *lam/ure* plasmids among the surveyed type B isolates is notable, perhaps offering yet another suggestion that only certain plasmid combinations can be maintained in a single *C. perfringens* type B isolate due to incompatibility issues.

In summary, since (i) a recent study indicated that both epsilon-toxin and beta-toxin are important for type B virulence (13); (ii) lambda-toxin is known to activate epsilon-toxin (27); (iii) we show that TpeL, a potent cytotoxin (1), is expressed by type B isolates; and (iv) this and previous studies (29) have now shown that *etx*, *cpb*, *lam*, and *tpeL* genes are all plasmid borne in type B isolates, the pathogenicity of type B isolates is clearly heavily dependent upon virulence plasmids. Continued study of these plasmids should provide additional insights into their genetics, evolution, diversity, and role in virulence.

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