Characterization of Virulence Plasmid Diversity Amongst

Clostridium perfringens Type B Isolates

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Abstract

The important veterinary pathogen *Clostridium perfringens* type B is unique for producing the two most lethal *C. perfringens* toxins, i.e., ε toxin and β toxin. Our recent study (Miyamoto *et. al.*, J Bacteriol., 2008, 190: 7178-7188) showed that most, if not all, type B isolates carry a 65 kb, ε toxin-encoding plasmid. However, this ε toxin plasmid did not possess the *cpb* gene encoding β toxin, suggesting 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 ε toxin-encoding plasmid, the β toxin-encoding plasmids of type B isolates were found to carry a *tcp* locus, suggesting 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 indicate 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.
Introduction

Isolates of the Gram-positive, spore-forming anaerobe *Clostridium perfringens* are classified (31) into five different types (A-E), depending upon their production of four (α, β, ε, and ι) lethal typing toxins. All *C. perfringens* types produce α toxin; in addition, type B isolates produce both β and ε toxins, type C isolates produce β toxin, type D isolates produce ε toxin and type E isolates produce ι toxin. Except for the chromosomal α 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 t 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, t 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 their t toxin genes, other potential virulence factors such as lambda toxin and urease. These plasmids also carry a tcp locus, suggesting they may be capable of conjugative transfer. Interestingly, many t 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 t toxin plasmids arose from insertion of an t toxin genes-carrying mobile genetic element near the cpe gene on a tcp-carrying type A plasmid. This insertional event apparently inactivated the cpe gene, so most or all type E isolates now carry silent cpe genes (3, 23).

The ε 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 amongst 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 β and ε 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 β and ε toxins each contribute to lethality in a mouse model involving intravenous injection of type B culture supernatants.

There has only been characterization of 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 plasmid 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 amongst 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 genotype of these isolates (13) was 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) were 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 co-localization using probes for two different ORFs (as described below), DNA plugs were digested with restriction endonucleases ApaI, AvaI, ClaI, KpnI, NcoI, SphI, NheI or XhoI (New England Biolabs) to help distinguish whether these co-localizing signals were due to the different probes hybridizing to two genes carried on the same plasmid or to two genes located on two co-migrating 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 0.5x 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, 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), ureaseC 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 ATCC3626 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: 94°C for 3 min, 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 2x 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: one 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 pCR®2.1-TOPO® vector (Invitrogen) and those inserts were then sequenced at the University of Pittsburgh Core sequencing facility using primers listed in Table 2. Results from these sequencing analyses were deposited in GenBank under Bankit numbers 1272207 and 1271433.

PCR linkage of the cpb gene to IS1151-like sequence in type B isolates. Based upon the ATCC3626 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 primer cpb-F: 5'-CTTGAAGAGTCAACAGATTGAT-3' and IS1151-R: 5'-

GCTGCTAAAGTCTCTACTAG-3'. PCR conditions for these amplifications were: 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 68°C for 4 min, followed by a single extension at 68°C for 10 min. PCR products were run on a 1% gel and stained with ethidium bromide for visualization.
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 x 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 x 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 AccesQuick 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 template for PCR reactions (35 cycles each involving 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-PCR reactions were similarly performed, except for the omission of reverse transcriptase.

**PCR identification of possible circular transposition intermediates carrying the *cpb* or *tpeL* ORFs.** 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* ORF or *tpeL* ORF was assessed using the following primer sets.
TnF (5’-ATACATTAACTAACTTAGAACGTAC-3’) and BetaR (5’GAAAGAAACTGTTATTATCTTAATTG-3’) for \( cpb \) loop; LoopF (5’AACCAATTATAGGATATAGAG-3’) and LoopR (5’GCTACTTACTTAGCTAGTGAAG-3’) for \( tpeL \) loop. PCR conditions for these amplifications were 95\(^\circ\)C for 2 min, 35 cycles of 95\(^\circ\)C for 30sec, 55\(^\circ\)C for 40sec and 68\(^\circ\)C for 2 min and a single cycle of 68\(^\circ\)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 pCR\(^\circ\)2.1-TOPO\(^\circ\) 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.

Results

Characterization of toxin plasmid diversity amongst 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 \textit{cpb2} probe. However, those blots showed a single \textit{cpb2} signal at 65 kb (Fig. 1B), strongly suggesting that most, if not all, type B isolates possess only the \textit{cpb2} gene previously shown to present on their ~65 kb etx-carrying plasmid (29).

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

**Evaluation of whether \textit{cpb} and \textit{etx/cpb2} are present on the same or different plasmids in type B isolate NCTC3110 and CN677.** As shown in Fig. 1, DNA from type B isolates NCTC3110 and CN677 hybridized the \textit{cpb}, \textit{etx} and \textit{cpb2} probes at the same 65 kb pulsed-field gel blot location, indicating either that these two isolates carry these three toxin genes on the same plasmid or (since \textit{etx} and \textit{cpb2} are present on the same 65 kb plasmid in type B isolates (29)) on two distinct co-migrating plasmids of a similar size.

To discriminate between those two possibilities, DNA from NCTC3110 or CN677 was digested with a battery of restriction endonucleases. In this experiment (9, 23, 33), 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 the two genes are present on similar size, but distinct, plasmids (9, 23, 33).

For NCTC3110 DNA, migration of etx-containing DNA and cpb-containing DNA exhibited a different susceptibility pattern of sensitivity to restriction enzyme digestion (Fig. 2A and 2B). In contrast, cpb2-containing DNA showed similar susceptibility to restriction enzyme digestion as did etx-containing DNA (Fig. 2A and 2C), 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 similar size, but different, plasmid 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 amongst 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 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 co-hybridizing 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 co-hybridized 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 co-hybridized to the same blot location containing the cpb plasmid. Finally, most type B isolates hybridized the tcp probes at a third location, distinct from the cpb or etx/cpb2 plasmids, 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 genes (lam) and urease C (ureC) genes in type B isolates.** Lambda toxin, a C. perfringens metalloprotease, can proteolytically activate ε-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 ε-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 DNA 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, DNA 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 amongst 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 DNA 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, e.g, 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 co-hybridized 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 similar-sized plasmids. Those analyses showed (Fig. 4) that lam and ureC migration were
coincidently affected when DNA from type B isolates 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 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.
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 co-hybridized 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 ATCC3626 released by JCVI
identified two IS1151-like sequences upstream of cpb. Therefore, a PCR analysis was performed
using primers based upon the ATCC3626 sequence to investigate whether IS1151-like sequences
reside upstream of the cpb gene in other type B isolates. In this PCR reaction, 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 ATCC3626 (data not shown).

**PCR evaluation of tpeL gene carriage amongst type B isolates.** Inspection of the JCVI ATCC3626 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 amongst other type B isolates. This survey determined that DNA from all 17 surveyed type B isolates supported amplification of a tpeL PCR product (data not shown).

Bioinformatic analysis of the ATCC3626 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-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 4 (NCTC3110, CN677, NCTC8533 and CN3477) 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 7 different type B isolates (Fig. 5C). This ~5.7 kb product matched the expected size of the product that these primers would amplify from ATCC3626 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 ATCC3626.

**Expression of TpeL by C. perfringens type B isolates.** In previous studies (13), we evaluated the expression of perfringolysin O, α toxin, β toxin, beta2 toxin and ε toxin by type B isolates.
However, TpeL toxin was identified (1) since 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 if 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 ATCC3626 sequence also indicated the presence of multiple transposas 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 possible circular transposition intermediates containing those toxin genes have been demonstrated by PCR assays using primers only capable of amplifying a PCR product 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 transposas 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. 7B). When that product was sequenced, the product 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 IScpe3 sequences and sequences present immediately upstream of IScpe3 (Fig. 7E), which is consistent with IScpe3-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 β toxin and ε 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 ~65-90 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 *ι* toxin plasmids of type E isolates (23, 33). This limited *cpb* plasmid diversity observed amongst 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 between type B vs. 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 amongst 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 t 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 amongst type C isolates and tpeL plasmids amongst 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 t 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 this plasmid may also conjugatively transfer among C. perfringens isolates. If a type D isolates 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 β− or ε− 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 ε 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 amongst type E isolates, where it can activate t toxin, as well as type B and D isolates, where it can activate ε toxin (this study, 23, 33). However, it is interesting that lambda toxin gene carriage appears to be more frequent in type B
(75% positive) vs. type D (23% positive) isolates (this study and (33)). Also notable is that type B isolates carry their lam gene on a different plasmid from their etx plasmid, in contrast to lam\(^+\) type D isolates (33), where lam and etx appear to be often present together on the same plasmid. Similarly, the lam gene also appears to be commonly present on the \(\tau\) 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 \(\tau\) 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 this is a relatively unusual C. perfringens virulence plasmid. However, this lam/ure plasmid in type B isolates does carry tcp locus genes, suggesting it may be a third conjugative plasmid in these isolates. Finally, the common ~80 kb size of lam/ure plasmids amongst 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 tpe\(L\) 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.

Acknowledgements

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Figure Legends

Fig. 1. Southern hybridization analyses of pulsed field gels run with DNA 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 panel (B) blot was stripped and reprobed 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.

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 (A) an etx-specific probe, then (B) stripped and reprobed with a cpb-specific probe, before (C) a final stripping and reprobing with a cpb2-specific probe. The migration of molecular size markers is indicated on the left.
Fig. 3. Southern hybridization analyses of pulsed field gels run with DNA extracted from type B isolates NCTC3110, CN677, CN1795, CN1886, CN2003 and CN2414. Probes used include: (A) a rep-specific probe; (B) a tcpH-specific probe (similar results were obtained using a tcpF-specific probe, not shown); (C) a lam-specific probe (similar results were obtained using a ureC probe, data not shown); and (D) an IS1151 probe. The migration of molecular size markers is indicated on the left.

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

Fig. 5. (A) Arrangement of the cpb and tpeL gene locus in C. perfringens type B isolate ATCC3626 (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-R5 primers (Table 2). (C) Long range PCR assay using an internal cpb and an internal tpeL primer to link the tpeL and cpb genes.

Fig. 6. RT-PCR analysis of tpeL expression by type B isolates NCTC3110, CN677, CN3447 and NCTC8533. tpeL expression was analyzed at (A) 8 h or (B) 24 hrs 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.

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 ATCC3626 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.

24
Diagram derived from sequencing the Fig. 7B product that depicts the putative circular transposition intermediate supporting PCR amplification with primers BetaR and TnF with the vertical bars on this diagram indicating the location in the loop of the PCR product amplified using these two primers. (D) PCR amplification of \textit{tpeL} circular intermediates from NCTC8533 using primers LoopR and LoopF. (E) Diagram derived from sequencing the Fig. 7D product that depicts the putative circular transposition intermediate supporting PCR amplification using primers LoopF and LoopR, with the vertical bars on the diagram indicating the location in the loop of the PCR product amplified using these primers.

References


4. **Brynestad, S., and P. E. Granum.** 1999. Evidence that Tn5565, which includes the enterotoxin gene in *Clostridium perfringens*, can have a circular form which may be a transposition intermediate. FEMS Microbiol. Lett. **170:**281-286.


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Table 1: Type B Plasmid Sizes and Plasmid-borne Virulence Gene Location

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<th>tpeL</th>
<th>cpb2</th>
<th>tcpF/H</th>
<th>rep</th>
<th>lam</th>
<th>ureC</th>
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<td>65/90</td>
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</table>

1 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 1930’s-1960’s.

2 Originated from the United Kingdom; geographical and disease origin of the other isolates is unknown except that Bar2 was obtained from an Australian sheep and NCTC3110 is a vaccine production strain isolated in Kenya.

3 Numbers shown refer to the size (in kilobases) of the plasmid or plasmids carrying the specified gene or genes.

4 The etx and cpb genes of NCTC3110 and CN677 are located on a different 65 kb plasmid from the 65 kb plasmid carrying the cpb and tpeL genes.
Table 2: Primers used for internal amplification of tpeL for linkage analysis and sequencing of cpb to tpeL

<table>
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<th>Primer name</th>
<th>Primer sequence (5' to 3')</th>
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Fig. 1

Probes:

- **etx**
- **cpb2**
- **cpb**

Overlay of B and C
Fig. 2

Probe:
cpb
etx
cpb2
Fig. 3

A

Probe:
rep

B

tcpH

C

lam

D

IS1151
Fig. 4.
Fig 5

A. Transposase
B. Swim zinc finger
C. Hypothetical protein

NCTC3110 CN677 CN1793 CN1795 CN3447

Bar2 PS49

1045bp 1098bp 1172bp 1244bp 1245bp

Swim zinc finger transposase conserved hypothetical protein

R1 R2 R3 R4 R5

1 kb 5 kb

1 kb 5 kb
Fig 6

A
DNA
-RT
+ RT
DNA
-RT
+ RT
DNA
-RT
+ RT
DNA
-RT+ RT
NCTC3110 CN677 CN3447 NCTC8533
660 bp
660 bp

B