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Clostridium perfringens type A–E toxin plasmids

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Abstract

Clostridium perfringens relies upon plasmid-encoded toxin genes to cause intestinal infections. These toxin genes are associated with insertion sequences that may facilitate their mobilization and transfer, giving rise to new toxin plasmids with common backbones. Most toxin plasmids carry a transfer of clostridial plasmids locus mediating conjugation, which likely explains the presence of similar toxin plasmids in otherwise unrelated *C. perfringens* strains. The association of many toxin genes with insertion sequences and conjugative plasmids provides virulence flexibility when causing intestinal infections. However, incompatibility issues apparently limit the number of toxin plasmids maintained by a single cell.

Keywords

plasmid-encoded toxin; clostridia; gastrointestinal disease; conjugation; plasmid evolution

1. Introduction to *Clostridium perfringens*

C. perfringens has a ubiquitous environmental distribution but also ranks amongst the most important pathogens of humans and domestic animals. The virulence of this bacterium is largely attributable to its ~17 toxin arsenal. However, individual strains produce only subsets of this toxin repertoire, which forms the basis for a toxinotyping classification scheme that consigns isolates to five types (A–E), based upon their production of four typing toxins (Table 1). In addition, several toxins not used for toxinotyping are important for pathogenicity, as will be discussed later [1, 2].

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C. perfringens causes a panoply of illnesses ranging from histotoxic infections, such as clostridial myonecrosis (gas gangrene), to intestinal infections. The ability of *C. perfringens* to cause infections originating in the intestines is often dependent upon possession of toxin plasmids, which are the main focus of this review.

2. *C. perfringens* toxin plasmids and intestinal disease

When producing certain plasmid-encoded toxins, each *C. perfringens* type (and sometimes even specific subtypes) can cause intestinal infections, as shown in Table 2. These infections include enteritis and enterotoxemias, the latter characterized by toxins produced in the intestines, which then transit into the circulation to affect extra-intestinal organs. The ability of each *C. perfringens* type/subtype to cause intestinal diseases will now be briefly reviewed, along with a brief description of the plasmids relevant to those illnesses.

2.1 Type A *C. perfringens*

2.1.1 *C. perfringens* enterotoxin (CPE) plasmids—Type A strains producing CPE are the second most common cause of bacterial food poisoning in the United States, with ~1,000,000 cases/yr at an estimated economic cost of >$300 million USD/yr [3, 4]. Additionally, CPE-producing type A strains are associated with 5–15% of nonfoodborne human intestinal diseases, including antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD) [5]. The enterotoxin gene (*cpe)* can be located chromosomally or on plasmids, with ~70% of food poisoning strains harboring a chromosomal copy of *cpe*, whereas the remaining ~30% of food poisoning strains, and virtually all AAD/SD strains, carry a plasmid-borne *cpe* gene [6, 7]. All of these strains cause disease when *C. perfringens* sporulates in the intestine and produces CPE (see below). During this *in vivo* sporulation, CPE accumulates in the cytoplasm and is finally released into the intestinal lumen when the mother cell lyses [6].

Substantial evidence supports CPE involvement in human intestinal disease. For example: 1) administration of CPE to human volunteers caused the classical diarrhea observed during natural disease [8]; 2) CPE is detectable in the feces of individuals with *C. perfringens* type A infection [9]; 3) CPE antisera can inhibit intestinal pathology in experimental animal models [10]; and 4) purified CPE damaged human ileal tissue *ex vivo* [11]. Perhaps the most persuasive evidence for the pathogenic role of CPE was provided by fulfilling molecular Koch's postulates for strain SM101 (a type A, chromosomal *cpe*, food poisoning strain) and F4969 (a type A, plasmid *cpe*, SD strain), which showed that CPE is essential for these two strains to cause histological damage and fluid accumulation in rabbit ileal loops [12].

CPE, an ~35 kDa single polypeptide, consists of a C-terminal binding domain and an N-terminal domain that mediates oligomerization and membrane insertion [6]. CPE action starts when this toxin binds to claudins, including claudin-3, -4, -6, -7, -8, -14, on the apical surface of small intestinal or colonic cells [13–19]. This binding localizes CPE in a small ~90 kDa complex, which then oligomerizes [20] into an ~500 kDa hexameric prepore named CH-1 that forms on the plasma membrane surface [17, 21, 22]. The toxin then uses its amphipathic region named TM1 to insert into membranes and form a pore of 0.5 – 1.0 nm [23]. Both the small complex and CH-1 contain receptor and nonreceptor claudins, as

well as CPE [17]. A secondary CPE large complex, named CH-2, can form that contains receptor and nonreceptor claudins, as well as another tight junction protein named occludin [17]. Formation of the CH-1 pore leads to an influx of Ca^{2+} into the cell and a K^+ efflux. The Ca^{2+} influx activates calpain, which can lead to apoptosis (low toxin dose) or necrosis (high toxin dose) [24, 25]. During *in vivo* disease, CPE-induced cell death leads to the intestinal lesions that trigger fluid accumulation and diarrhea [10, 18]. Upon prolonged contact with the intestines, CPE can be absorbed into the circulation and cause enterotoxemia, affecting organs such as the liver or kidneys [26]. This enterotoxemia may explain fatalities that occurred during two food poisoning outbreaks in psychiatric hospitals [27, 28]. In mouse models of CPE enterotoxemia, this leads to increased serum K^+ and hyperkalemia, which then causes cardiac arrhythmia and death [26].

During type A foodborne illness involving CPE, *C. perfringens* spores that survive the cooking process germinate in food, multiply and then are ingested [6]. Spore resistance against cooking and other stresses is influenced by which Ssp4 small acid-soluble protein variant is produced by the infecting strain [29, 30]. Foodborne strains carrying a chromosomal *cpe* gene typically make a Ssp4 variant that binds strongly to spore DNA and thus imparts exceptional heat and chemical resistance properties to *C. perfringens* spores, while strains carrying a plasmid-borne *cpe* gene produce a different Ssp4 variant that binds DNA less tightly, resulting in decreased spore resistance properties [29, 30]. These differences in spore resistance properties help to explain why the chromosomal *cpe* strains are more commonly implicated in food poisoning than the plasmid *cpe* strains.

Both chromosomal and plasmid-borne *cpe* genes are only expressed when *C. perfringens* sporulates; during disease, this sporulation occurs in the intestines. CPE production during sporulation is dependent upon three sporulation-specific sigma factors named SigF, SigE, and SigK. SigK and SigE bind to promoters upstream of *cpe* genes and positively-regulate toxin expression, while SigF indirectly controls CPE expression by controlling the production of SigE and SigK [31, 32]. CPE production and sporulation are also positively regulated by the Agr-like quorum sensing (QS) system [33] and the CcpA protein [34]. In contrast, the *virX* small RNA negatively regulates *cpe* expression during early sporulation [35].

Two *cpe*-carrying plasmids of type A strains were the first fully-sequenced *C. perfringens* toxin plasmids (Fig. 1B) [36]. pCPF4969, a plasmid of 70.5 kb from type A strain F4969, carries a functional copy of the *cpe* gene but lacks genes encoding any other toxins or putative toxins. In contrast, the *cpe*-encoding toxin plasmid pCPF5603 in type A SD strain F5603 is ~75.3 kb and also carries the *cpb2* gene encoding the *C. perfringens* beta2-toxin (CPB2) toxin (Table 3). The plasmid-borne *cpe* gene in both strains is flanked by a 5′ IS*1469*, though the 3′ end of *cpe* can be flanked by either IS*1151* (for pCPF5603) or IS*1470* (for pCPF4969) [36].

Both pCP5603 and pCP4969 share nearly 50% homology. Of note, both plasmids carry a complete *tcp* (transfer clostridial plasmids) gene locus, which is known (as discussed later) to mediate conjugative plasmid transfer of other *C. perfringens* plasmids [36]. The presence

of this *tcp* locus likely explains the demonstrated ability of pCPF4969 to conjugatively transfer and predicts that pCP5603 should also be conjugative [37].

Variable regions of these two prototype *cpe*+ type A plasmids include genes exclusively encoded on pCPF4969, i.e., genes encoding a putative bacteriocin, a biosynthetic operon for the production and secretion of peptide-based lantibiotics, and two component regulatory systems, including one resembling the VirS/VirR system. Conversely, pCPF5603 differs from pCPF4969 not only by encoding CPB2 but also carrying several genes encoding proteins involved in carbohydrate and lipid metabolism [36] (Fig. 1).

Additional studies [36] revealed that pCPF5603 and pCPF4969 represent the vast majority of *cpe*-encoding plasmids found in type A strains, particularly AAD and SD strains. However, some different *cpe*-encoding plasmids have been identified in type A soil strains, although they have not yet been sequenced [38]. CPE can also be encoded for by plasmids of types C, D, and E (to be discussed in later sections) [39–44].

2.1.2 NetB plasmids—Certain type A strains cause necrotic enteritis (NE), a debilitating intestinal disease that affects several poultry species. This disease has been demonstrated to involve the Necrotic Enteritis Beta-like toxin (NetB) (see below) [45, 46]. Clinically, birds with the acute or peracute form of the disease present with diarrhea, ruffled feathers, anorexia and depression; sudden death without clinical signs being observed can occasionally occur. Most peracute cases result in death within hours of the onset of symptoms, with flock mortality levels reaching 50%. Birds may develop subclinical disease which is characterized clinically by a drop in production with little or no diarrhea. Although mortality rates for this form of the disease are typically very low, feed conversion is negatively affected by the disease, resulting in significantly longer than normal grow-out periods. Pathologically, all three forms of NE are characterized by multifocal to coalescent intestinal necrosis, and are frequently covered by a pseudomembrane [47–49].

NetB has been implicated as the major toxin involved in avian NE through several lines of evidence. First, *C. perfringens* alpha toxin null mutants constructed in NE strains retained full virulence during experimental challenge [50]. Second, a *netB* null mutant failed to produce lesions during experimental challenge in poultry, whereas the wild-type parent strain produced disease in 45% of the challenged animals [45]. This effect was reversed by complementation of the *netB* gene. Finally, numerous studies have assayed strains from NE outbreaks, identifying 60–90% of outbreak strains as NetB-positive [51–54].

NetB is expressed as a 323 amino acid protein that is processed prior to secretion to remove a 30 amino acid signal sequence generating a mature 33 kDa protein [45]. The crystalline structure of NetB has been solved, identifying this protein as a member of the β-pore-forming toxin (PFT) family [55, 56]. Although complete structure-function analysis of NetB has not been performed, several site directed mutants generated in the predicted rim domain of the protein showed a reduced binding phenotype using chicken hepatocellular carcinoma cells [55].

As with all β-PFT's, NetB-induced cell death is induced through the formation of unregulated ion channels in the membrane of susceptible cells; NetB pores have a pore diameter of ~1.4–1.6 nm. Formation of the pore begins with binding of the monomeric toxin to a currently unknown receptor. Binding is followed by oligomerization of the toxin into a prepore, a process linked to direct interactions of the toxin with cholesterol [55]. The prepore then inserts into the host cell membrane, likely utilizing an amphipathic domain identified in crystallization studies [55, 56]. The specific ions traversing the pore are not currently known, but initial experiments and model predictions suggest that the ion channel may be cation-selective [55, 56]. LD_{50} levels for NetB have not been determined; however, reports in the literature indicate doses as low as 2.5 μg/ml of NetB are capable of causing LMH cell rounding and lysis *in vitro*.

Studies have determined that *netB* expression is under the control of the VirS/VirR two component system. In this work, two VirR-binding boxes were identified directly upstream of the *netB* promoter. Testing of a *virR* null mutant demonstrated reduced NetB production as compared to wild type, which was restored by complementation [57]. Genes under control of VirS/VirR system are often also under control of the Agr-like QS system in *C. perfringens* [33, 58, 59]; however, it remains to be proven if the Agr system also regulates NetB production.

Type A necrotic enteritis strains typically carry 2 to 5 highly conserved, low copy number plasmids ranging in size from 50 to 100 kb in size [60]. The *netB* gene maps to a 42 kb pathogenicity locus called NEloc1 that is present on a plasmid of 80–85 kb in size, with the sequenced plasmid, pJIR3535, being 82 kb [60–62]. This plasmid is distinct from the plasmid carrying CPB2 in CPB2-positive type A avian necrotic enteritis strains, although there is a large common region. A 5.6 kb putative pathogenicity locus, named NEloc3, was also shown to be plasmid-borne, mapping to a second, 70 kb plasmid (Table 3). All of the sequenced large plasmids from type A NE isolates have been shown to carry the *C. perfringens tcp* locus (Fig. 1). Conjugative conversion of strains has been demonstrated experimentally using type A strain EHE-NE18 [61]. In this work, transfer of all three large plasmids present in EHE-NE18, including the plasmids harboring *netB* and *tet*(P) genes, to a recipient strain was demonstrated.

2.1.3 CPB2 plasmids—Many type A strains encode another toxin named beta-2 toxin (CPB2). CPB2 has also been found in types B, C, D and E *C. perfringens* isolates. CPB2 is expressed as a 31 kDa prototoxin that is subsequently cleaved during secretion into the mature 28 kDa toxin [63]. This toxin is active *in vitro*, causing cell rounding and death of both I407 and CHO cell lines at CPB2 concentrations >20 μg/ml [63]. It is not currently known how CPB2 causes cell death, but disruption of the cellular membrane or pore formation has been proposed as a possible explanation [64].

CPB2 has an unclear etiological role in disease since, to date, molecular Koch's postulates have not been reported in the literature for this toxin. Indirect evidence supporting CPB2 having a role in disease comes mainly from the isolation of *cpb2*-positive strains from diseased animals. However, many normal flora isolates from healthy animals also carry this gene, making it challenging to draw conclusions about CPB2 contributions to disease.

Gilbert *et al.*[63] did report that 3 μg of purified CPB2 delivered intravenously to mice was lethal. Additionally, one study found more pronounced disease from a *cpa+/cpb2+* strain as compared to *cpa+/cpe+* or *cpa+* strains in a bovine ligated intestinal loop model, possibly suggesting CPB2 and CPA have synergistic effects *in vivo* [65, 66].

The regulation of CPB2 expression has been examined in *C. perfringens* strain 13. In this type A stain, CPB2 expression is under the control of the VirS/VirR two-component system. Furthermore, VirS/VirR regulation appears to be indirect, involving a sRNA named VR-RNA [67]. The Agr QS system was also shown to have regulatory effects on CPB2 expression in a type A strain, although not in two type B strains [33, 68].

In *C. perfringens* strains, CPB2 is encoded on plasmids ranging in size from 45–97 kb [40, 41, 43, 63, 69]. CPB2 maps to the same plasmid harboring epsilon toxin (ETX) in type B and D strains, and the same plasmid as CPE in some type A strains, but has not been found on the same plasmid as CPB in type C strains [40, 69, 70] (Table 3). Furthermore, the *cpb2* gene has been mapped to plasmids independent of the iota toxin plasmids in type E strains [43]. Only some of the *cpb2* plasmids in type E strains carry a *tcp* conjugation locus. In type A chicken necrotic enteritis strains, the *cpb2* gene is located on a conjugative plasmid that is distinct from the plasmid that carries the *netB* gene [61].

2.1.4 BEC plasmids—Non-CPE-producing strains of type A *C. perfringens* were recently implicated in two food-borne gastroenteritis outbreaks in Japan [71]. Culture supernatants from those isolates were able to cause fluid accumulation in rabbit ileal loops. An enterotoxic protein was purified and its N-terminal region was sequenced. Genome sequencing revealed the presence of a binary toxin that matched the N-terminal sequence of the purified enterotoxic protein; this toxin was found to share approximately 43% identity to the binding and enzymatic components of iota toxin (discussed in 2.5.1). This novel toxin, named binary enterotoxin of *C. perfringens* (BEC), was found to ADP-ribosylate actin (similarly as other binary toxins of clostridial species) via the BECa subunit and also caused fluid accumulation in a suckling mouse model. Lastly, an isogenic *becB* (the gene encoding the BEC binding subunit) null mutant lost fluid-accumulating activity in the suckling mouse model, suggesting this binary toxin is the major mediator of gastrointestinal disease for the newly identified *C. perfringens* isolates [71].

BEC was found to be encoded on large plasmids of ~54 kb (Table 3). Sequencing showed these plasmids encode 39–55 potential ORFs, 16 of which have an assigned function. The BEC-encoding plasmids, pCP-OS1 and pCP-TS1, share significant homology with pCP13 over a span of ~38 kb and contain a number of partitioning and replication-associated genes common to pCP13 [72]. Like pCP13, these plasmids lack a *tcp* locus. Interestingly, and similarly as other plasmids of *C. perfringens*, these two plasmids encoded a putative transposon resolvase, which may play a role in transfer of the *becAB* genes to other plasmids and/or strains (Fig. 1) [71].

2.2 Toxin plasmids of type B *C. perfringens*

While also associated with hemorrhagic enteritis in goats, calves, and foals, type B strains are primarily known as the etiological agent of lamb dysentery [73]. Infection begins with

transfer of the organism either directly from the dam or the environment to the lamb, usually within the first few days of life. Once in the intestine, the type B strain rapidly divides and produces toxins, resulting in enteritis with extensive necrosis and hemorrhage of the small intestine and enterotoxemia. Occasionally, focal symmetrical encephalomalacia is also present. In acute cases, clinical signs consist mainly of hemorrhagic diarrhea, abdominal pain, neurological signs, and as disease progresses, recumbency and death occur within 24 h of clinical signs onset. In peracute cases, no signs are observed and sudden death is the only indicator of disease. During type B outbreaks, infection rates can exceed 30%, with lethality rates approaching 100%. However, outbreaks of type B disease are rare and restricted to the United Kingdom, South Africa and the Middle East [73–76].

Both beta toxin (CPB) and epsilon toxin (ETX) appear to be important for the lethal enterotoxemias caused by type B strains [77]. This conclusion is based upon results where supernatants from 19 type B isolates were treated with or without trypsin (note that trypsin is necessary for ETX toxin activation) and in the presence or absence of toxin neutralizing antibodies, before those supernatants were injected intravenously into mice in a lethality model of type B enterotoxaemia. In this work, a positive correlation was noted between LD_{50} and CPB levels present in type B culture supernatants. Furthermore, a neutralizing MAb against CPB (but not ETX or CPA) reduced the lethality of non-trypsin treated culture supernatants. In contrast, antibodies against both CPB and ETX were necessary to prevent the lethality of trypsin-treated culture supernatants, despite the trypsin-labile nature of CPB [78].

2.2.1 CPB plasmids—CPB is the second most potent of the *C. perfringens* toxins, with an LD_{50} of 400 ng/kg body weight in mice [79]. This toxin is expressed as a 336 amino acid polypeptide containing a 27 amino acid leader sequence that is cleaved during secretion to generate a mature protein of ~35 kDa. The CPB structure has not been resolved, but this toxin is predicted to be a β-PFT based on sequence homology to other toxins with related amino acid sequences and known structures. Cell death caused by CPB occurs through the creation of unregulated ion channels in host cell plasma membranes. Pore formation begins with monomeric CPB binding to susceptible cells via an unknown receptor. During acute infection, CPB can bind to vascular endothelial cells in the intestine and it has been speculated, although not yet proven, that this causes thrombosis which might be responsible for the intestinal necrosis characteristic of the disease [80]; whether direct CPB damage to enterocytes is involved in disease is less clear. Once bound, CPB oligomerizes on the host cell surface into heptameric or hexameric prepores [81]. These prepores then rapidly insert into the plasma membrane using their amphipathic transmembrane domain, resulting in pore formation. The CPB channels have a pore size of approximately 12Å and allow the rapid efflux of K^+ and influx of Na^+, Ca^{2+} and Cl^-, resulting in cellular swelling and lysis [81, 82]. A recent study suggested that CPB-induced cell death involves programmed necrosis [83].

Studies of CPB production regulation by type B strains are limited. Using AgrB mutants and complementing strains, the Agr QS system was shown to regulate CPB production by type B strains CN1793 and CN1795. The defect in CPB production by the AgrB mutants of type B strains involved a decrease in *cpb* transcription [68].

C. perfringens type B strains carry their CPB- and ETX-encoding genes on separate plasmids [69]. The *cpb* gene in type B strains is often carried on a plasmid of 90kDa, although a few isolates possess a 65 kDa *cpb* plasmid that is distinct from the 65 kDa plasmid carrying the *etx* gene in some type B strains (see below) (Table 3). Furthermore, the *cpb* plasmid can carry additional toxin genes encoding CPB2, the large clostridial cytotoxin (TpeL) or both of those toxins. The *tcp* locus is present on most or all *cpb* plasmids, indicating that these plasmids are likely to be conjugative. On type B plasmids, IS*1551*-like sequences are present upstream of the *cpb* gene; in addition, *cpb*-carrying circular DNA forms have been identified, indicating that the *cpb* gene can be excised from plasmids and suggesting that it may be carried on a transposable genetic element [69].

2.2.2 ETX plasmids—ETX, the most potent *C. perfringens* toxin, is also produced by type B (as well as type D) strains. ETX is secreted as a nearly-inactive, ~33 kDa prototoxin [84–86]. In the intestines, proteases such as trypsin and chymotrypsin remove the 13 N-terminal amino acids (trypsin) [84] and, as required for activation and cytotoxic activity, the 23-(trypsin) or 29-(chymotrypsin) C-terminal amino acid residues [85, 86]. This activation results in a nearly 1000-fold decrease in mouse LD_{50} [87] and increased cytotoxicity in cultured Madine-Darby Canine Kidney (MDCK) cells [88, 89]. ETX action is similar to that of other pore-forming toxins [90] in that the activated ETX binds to an unknown receptor on the surface of cells present in anatomical niches such as the intestines of goats, or the kidney and other organs of other mammals [1, 75, 76, 91]. Once bound, ETX oligomerizes into a surface prepore [89]. After insertion of transmembrane loops to form a pore, ion dysregulation causes eventual host cell death [92].

In contrast to the regulation of CPB production in type B strains, isogenic AgrB null mutants of two type B strains still produced wild-type levels of ETX, indicating the the Agr-like QS system does not regulate expression of all plasmid-encoded toxins in all strains of *C. perfringens* [68]. However, the Agr-like QS system does regulate ETX production in type D strains (to be discussed later) [93].

One *etx*-carrying plasmid, i.e., pCP8533etx from the type B strain CN8533, has been completely sequenced [70]. This *etx* plasmid is ~65 kb and also carries the *cpb2* gene, like pCPF5603 (Table 3). In fact, pCP8533etx and pCPF5603 share approximately 80% of the same ORFs, with pCP8533etx lacking the ORFs on pCPF5603 discussed in 2.1.1 that encode for proteins involved in carbohydrate and lipid metabolism [36, 70] (Fig. 1).

Diversity amongst *etx* plasmids in other type B strains has been addressed by overlapping PCR and by pulsed-field gel electrophoresis (PFGE) with Southern blotting using an *etx*-specific probe. Those studies detected the presence of a similar, if not identical, ~65 kb *etx* plasmid in all surveyed type B isolates (Table 3) [69]. The plasmid pCP8533etx also carries the *tcp* gene locus required for conjugative transfer of *C. perfringens* plasmids, suggesting that type B *etx*-carrying plasmids, can undergo conjugative transfer between isolates, as proven for the *etx* plasmids of type D strains CN3718 and CN1020 [69, 70, 94]. Finally, an IS*1151*-like insertion sequence and a transposase gene are located adjacent to the *etx* gene, providing evidence that mobile DNA intermediates may have played a role in the evolution of *etx* plasmids of type B *C. perfringens* strains [69, 70].

2.3 Type C C. perfringens

2.3.1 CPB plasmids—*C. perfringens* type C infections, which occur in both humans and several animal species (horses, sheep, cattle, and pigs amongst others), manifest as necrotic enteritis that may be accompanied by enterotoxemia. The majority of type C disease is observed in neonatal animals, presenting in an acute or peracute form and characterized by severe abdominal pain, bloody diarrhea and depression. Occasionally, neurological signs are also observed. The rapidity of disease is likely due to the lack of competing flora and the trypsin-inhibiting effects of colostrum, which create an ideal environment for disease by protecting trypsin-labile CPB. Affected animals are colonized within a few hours of birth, likely from contact with contaminated fecal material shed by the dam. Outbreaks of the disease occur in unvaccinated herds, with rapid onset of signs and lethality rates in excess of 50%. In addition to affecting neonates, chronic disease is also seen in unvaccinated mature sheep and horses, where it presents as a chronic blood-free diarrhea that leads to dehydration [95].

In humans, type C disease presents as enteritis necroticans (EN), also referred to as Darmbrand or Pigbel, which is a severe intestinal infection marked by the presence of abdominal pain, bloody stool, vomiting and, in severe cases, a rapid toxemia causing death within 48 hours [96, 97]. The majority of EN cases happen in developing countries, where limited diets and consumption of staple foods rich in trypsin inhibitors, such as sweet potato, provide an ideal environment for epidemic disease, and support the importance of trypsin as a natural host defense against EN. EN occurs occasionally in developed countries, where it is generally restricted to people with pancreatic dysfunction, such as diabetics [98, 99].

Strong evidence supports the role of CPB as the major toxin for type C infections. Early studies demonstrated that neutralization of CPB in type C culture supernatants was necessary and sufficient to protect mice from lethal intravenous challenge with those samples [100]. Later studies utilizing highly purified CPB mixed with trypsin inhibitor reproduced the intestinal lesions typical of type C disease seen in rabbit intestinal loops. Further, pre-incubation of purified CPB with monoclonal antibodies specific for CPB blocked the pathological effects of the purified toxin [101, 102]. The most persuasive evidence supporting CPB's role as the primary toxin in type C disease comes from fulfilling molecular Koch's postulates. Isogenic toxin knockout mutants of strain CN3685, which produces chromosomally-encoded alpha toxin (CPA) and perfringolysin O (PFO) as well as CPB, demonstrated full pathogenicity of the *cpa* and *pfo* mutants, whereas the *cpb* KO mutant was completely attenuated unless the mutation was reversed [101]).

Several type C human EN strains produce CPE in addition to CPB [39, 103]. Recent work using sporulating culture lysates (SCLs) demonstrated that inactivating either CPB or CPE production by directed mutation rendered the SCLs of EN strain CN3758 unable to cause intestinal lesions in rabbit small intestinal loops, whereas SCLs of wild-type CN3758 produced fulminant disease. Quantification of toxin in wild-type CN3758 SCLs detected a relatively low presence of CPB and CPE, explaining why SCLs from mutants producing either CPE or CPB alone failed to cause disease. Consistent with that conclusion, challenge of rabbit loops with low doses of purified CPB or CPE mirrored the mutant SCL results,

further demonstrating synergistic effects for these two toxins when present together in the small intestine at low concentrations [104].

The VirS/VirR system, which regulates the expression of CPA and PFO [105, 106], also modulates CPB expression in type C strains. Initial *in vitro* experiments demonstrated a rapid increase in CPB production in the presence of cultured Caco-2 cells [107]. Involvement of the VirS/VirR system was shown when an isogenic *virR* null mutant failed to increase CPB production in the presence of Caco-2 cells; complementation of *virR* rescued the mutant. Later work showed that, in contrast to the virulence of wild-type CN3685, an isogenic *virR* null mutant was attenuated in rabbit intestinal loops. Furthermore, Western blot analysis of intestinal fluid detected *in vivo* CPB production by the parent but not the mutant. Complementation completely restored the *virR* mutant's virulence and wild-type CPB production levels, indicating that VirS/VirR is necessary for virulence because it controls *in vivo* CPB production. These strains were also tested for lethality in a mouse ID challenge model. Again, a significant reduction in mortality was observed in mice treated with the *virR* mutant compared to challenge with the wild-type strain [108]. In addition to the VirS/VirR system, type C strains also utilize the Agr-like QS system for virulence. Western blotting of intestinal fluid samples collected from rabbit small intestinal loops challenged with CN3685 or a *agrB* null mutant demonstrated that the Agr-like QS system is required for *in vivo* CPB expression by type C strains [59].

The *cpb* gene in type C strains is encoded on large plasmids ranging from 65–110 kb in size. Other toxins may be encoded on the same plasmid as CPB, such as TpeL or CPE, but these three toxin genes have not yet been found together on the same plasmid [39, 40]. To date, no plasmid in type C strains have been shown to encode both CPB and CPB2.

Interestingly, CPB and CPE are located on the same ~85 kb plasmid in some type C strains, but on different plasmids (110 kb and 65–75 kb for CPE and CPB respectively) in other strains [39] (Table 3). The presence of IS sequences flanking both the CPB and CPE toxin genes provides a potential mechanism for the generation of single plasmids harboring both toxins, where one toxin gene has been mobilized and inserted onto a plasmid already harboring the other toxin gene.

Like most other toxin plasmids, CPB plasmids of type C strains typically possess a *tcp* region, indicating their potential for conjugative transfer. Additionally, IS*1151* sequences are associated with the *cpb* gene, suggesting that type C strains arise when the *cpb* gene inserts into a plasmid in a type A strain, converting it to type C [40]. In support of this theory, circular *cpb* containing transposon intermediates have been identified in CPB positive strains, demonstrating the potential mobility of the *cpb* gene [39].

2.4 Type D C. perfringens

2.4.1 ETX plasmids—Type D infections occur mostly in sheep and goats, with occasional cases observed in cattle and other animal species. The acute, sub-acute and chronic cases are clinically characterized by neurological and respiratory alterations, although hemorrhagic diarrhea can be observed in goats. Peracute cases can present with sudden death without clinical signs being observed in all species. Pathologically, the disease in all species is

mainly characterized by pulmonary and cerebral edema, the latter being observed mostly in a perivascular location which is considered to be a rather specific diagnostic feature. In goats, necrotizing and hemorrhagic enterocolitis is characteristic of the sub-acute and chronic form of the disease [75, 76, 90].

ETX, described in 2.2.2, is the major toxin required for the virulence of type D strains of *C. perfringens* when mice, goats, or sheep are challenged intraduodenally with washed type D cells [91]. It has been proposed that the Agr system signals through the VirS/VirR two-component regulatory system. However, while ETX expression was found to be positively regulated by the Agr-like QS system in type D strain CN3718, inactivating VirS/VirR in this strain had no effect on ETX production levels, indicating that the Agr-like QS system can sometimes regulate gene expression independently of VirS/VirR [93]. In addition to Agr, the regulator CodY also positively regulates ETX production by binding to sequences directly upstream of the *etx* gene in strain CN3718 [109].

In contrast to the single *etx* plasmid found in type B strains, the *etx* plasmids of type D strains show considerable variability, ranging in size from ~45 kb to ~110 kb, based upon PFGE Southern blotting with an *etx*-specific probe [41]. The diversity of *etx* plasmid size in type D strains correlates with carriage of *cpe* and/or *cpb2* genes, i.e., *etx* is typically carried on plasmids of ~48 kb (though rarely 75 kb) in *cpe−/cpb2−* strains, but *etx* is carried on larger plasmids of either 75 or 110 kb in *cpe+/cpb2+* strains (Table 3). Most *etx* plasmids of type D strains carry a functional *tcp* conjugative transfer locus [41, 94], and conjugative transfer of *etx* plasmids has been demonstrated for type D strains CN1020 and CN3718 [94]. Interestingly, a few type D strains carry the same ~65 kb *etx* and *cpb2* plasmid that is also found in most or all type B strains of *C. perfringens* [41, 69, 70] (Table 3).

2.5 Type E C. perfringens

2.5.1 Iota toxin plasmids—Iota toxin (ITX) is a typing toxin produced only by type E strains [1, 110], which have been implicated in enteritis in rabbits, lambs, and cattle. However, the role of ITX in these type E-associated diseases has not yet been carefully examined [1]. Nor has the regulation of ITX production been explored [43, 44].

ITX is a member of the binary toxin family that also includes BEC of *C. perfringens*, CDT of *C. difficile*, *C. botulinum* C2 toxin, and *C. spiroforme* toxin CST [110, 111]. ITX is comprised of an enzymatic subunit (Ia) and a binding component (Ib) [110, 111]. The *itx* genes are transcribed in an operon. The regulation of ITX production remains unexplored [43, 44]. ITX has cytotoxic activity in numerous cell culture models [110, 112, 113]. The action of this toxin begins when Ia and Ib propeptides are activated by proteases such as alpha-chymotrypsin, pepsin, proteinase K, subtilisin, and thermolysin [114]. The activated Ib toxin subunit then binds to a surface-localized receptor named lipolysis-stimulated lipoprotein receptor [115] and, possibly, to the mammalian protein CD44 [116]. Once bound to its receptor, ITX-Ib oligomerizes to form a heptamer, which then binds the Ia enzymatic subunit [117–119]. The holotoxin is endocytosed [119, 120] and, after escaping into the cytoplasm, Ia then ADP-ribosylates actin, leading to a disassembly of the cellular cytoskeleton and cell death [112, 113].

The ITX plasmids of type E strains have been well characterized, with two major kinds of ITX toxin plasmids identified in these strains. These include plasmids of either 97 or 135 kb that encode ITX (*iap* and *ibp*), as well as urease and lambda-protease. This 97/135 kb ITX plasmid family also encodes silent *cpe* sequences (Table 3) [43, 44]. The backbone of these plasmids can resemble pCPF5603, where an IS*1151*-like insertion sequence is located immediately adjacent to the ITX encoding genes and the silent *cpe* sequences [43, 44, 121]. This may explain the evolution of these ITX plasmids, as discussed later. The ITX plasmids present in these type E plasmids carry a *tcp* locus, which suggests they can transfer horizontally [43, 44].

A more recent study identified type E strains carrying an ~65 kb plasmid (named pCPPB-1) that carries a variant *cpe* locus and a variant *iap/ibp* operon. This ITX plasmid also possesses a *tcp* locus and is related to the pCFP4969 *cpe* plasmid (Table 3). For example, pCPPB-1 shares several features of pCPF4969 including encoding a VirR/VirS-like two-component regulatory system, a bacteriocin, and enzymes involved in the synthesis and secretion of lantibiotics (Fig. 1). Several isolates carrying pCPPB-1 were found in the feces of healthy individuals or the environment, though a clear role for the variant CPE or ITX in the pathogenesis of type E strains has not been identified [36, 43, 44, 121].

Type E strains carrying silent *cpe* sequences also commonly possess a second plasmid of 75–97 kb that carries the *cpb2* gene. This plasmid also carries IS*1151* sequences, but does not always carry the *tcp* locus needed for conjugative transfer [43].

3. *C. perfringens* plasmid biology

3.1 Conjugation of *C. perfringens* toxin plasmids

Conjugative transfer has been demonstrated for several *C. perfringens* toxin and antibiotic resistance plasmids [37, 61, 94]. All known conjugative plasmids of *C. perfringens* have a novel conjugation region called the *tcp* locus [122]. Using the paradigm conjugative *C. perfringens* plasmid pCW3, a 47 kb tetracycline resistance plasmid [61], mutagenesis studies demonstrated that the *tcp* locus is essential for conjugative transfer [61, 123–125]. A model for the *C. perfringens* conjugation system has been proposed based on functional studies of several Tcp proteins [122, 126]. The presence of the *tcp* genes on most studied *C. perfringens* toxin plasmids suggests that this model represents a conserved mechanism of transfer that contributes to the spread of toxin genes and resistance determinants in *C. perfringens* [122].

Conjugative plasmid transfer involves a type IV secretion system (T4SS) that has recently been structurally resolved for Gram-negative systems [127]. Recent studies have classified the T4SS, or mating pair formation complex, encoded by the *tcp* locus of pCW3 as belonging to the MPF_{FA} class, which includes Tn*916* and ICEB*s*1 from *Bacillus subtilis* [128]. This classification is consistent with the original finding of similarity between Tcp proteins and products of the conjugation region of Tn*916* [129]. The Gram-positive T4SS are predicted to be minimized systems and, unsurprisingly, lack homologs for proteins that form the outer membrane core complex in Gram-negatives [130, 131]. Conserved protein

families have been identified in both the Gram-negative and Gram-positive systems, including the pCW3 system (Fig. 2) [128, 130, 131].

Domains from proteins that form the inner membrane complex in Gram-negative T4SS, i.e., VirB6 and VirB8, were identified in TcpH and TcpC, respectively, suggesting that they form the core of the T4SS in *C. perfringens* [125, 129, 132]. Essential for pCW3 transfer, TcpH was identified as an integral membrane protein with eight putative transmembrane domains (TMDs), which localizes TcpH to the cell envelope at the poles of *C. perfringens* donor cells [129]. Based on its similarity to VirB6 proteins, TcpH is postulated to play a similar core role in T4SS assembly and stabilization [132]. The VirB6 domain, the N-terminal domain and the $_{242}$VQQPW$_{246}$ conserved motif were shown to be essential for TcpH function [132]. The N-terminal domain mediates TcpH interactions with itself, TcpA and TcpC, while the VirB6 domain is crucial for interaction with the other postulated core component, TcpC [126, 132].

TcpC was identified as a 359 amino acid biotopic membrane protein that is required for efficient transfer of pCW3 and which is localized to the cell envelope by two essential N-terminal TMDs [125]. Structural resolution of the stable TcpC$_{99–359}$ derivative lacking these TMDs identified two linked structural domains that each had an unexpectedly similar fold to biotopic VirB8-like proteins. TcpC is the first VirB8-like protein to have two domains with a VirB8 fold and represents a novel class of this family of proteins [133]. TcpC was shown to interact with itself, TcpA, TcpG and TcpH, which is consistent with its postulated role as an assembly and scaffolding protein, similar to other VirB8-like proteins [125, 126]. The TMDs were shown to be essential for TcpC interactions, probably due to their role in localization and oligomerization [125]. The central domain, which is buried within the trimeric TcpC structure, was involved in TcpC self-interaction and interactions with TcpG. Deletion of the C-terminal domain completely abolished interactions with TcpA, TcpG and TcpH, a result consistent with its localization on the external surface of the TcpC trimer.

TcpF has a putative VirB4-like ATPase domain and therefore is related to a protein family that is a signature of all T4SS [129]. TcpF was shown to be essential for conjugative transfer of pCW3 and is predicted to energize the *C. perfringens* conjugation system. Immunofluorescence studies showed that TcpF co-localizes with TcpH at the poles of *C. perfringens* donor cells, suggesting that it forms part of the pCW3 T4SS, although no protein-protein interactions have been identified between TcpF and the other Tcp proteins [132].

Two gene products encoded by the *tcp* locus, TcpG and TcpI, were identified as putative peptidoglycan hydrolases [129], which are postulated to be important for the assembly of the T4SS in the Gram-positive cell envelope [134]. TcpG is required for efficient conjugative transfer of pCW3, but TcpI is not required [123]. TcpG was shown to have peptidoglycan hydrolyzing activity on purified peptidoglycan from *C. perfringens* and has two functional catalytic domains that are required for activity. Interactions between TcpG, TcpC and TcpA are postulated to direct the localized assembly of the transfer apparatus in *C. perfringens* donor cells [123, 126].

Three hypothetical proteins are encoded on the *tcp* locus: TcpD, TcpE and TcpJ [129]. The only homology identified was for TcpE, which has 27% sequence identity to ORF17-like proteins of unknown function that are only present in the MPF_{FA} class of T4SS [128, 129]. TcpD and TcpE are essential for pCW3 transfer, whereas TcpJ is not required (J.A. Wisniewski, W.L. Teng, T.L. Bannam and J.I. Rood, unpublished). The functional role of these novel proteins in *C. perfringens* conjugative transfer remains to be determined.

A family of single-stranded DNA translocases known as type IV coupling proteins (T4CP) are associated with T4SS systems that have the ability to transfer DNA [135]. The T4CPs are DNA-dependent ATPases that link the T4SS system with its nucleoprotein substrate [136]. TcpA was postulated to be the DNA translocase of the pCW3 conjugation system based on the presence of an FtsK-like domain similar to that present in the FtsK/SpoIIIE family of double-stranded DNA translocases [124]. Homologs of TcpA have been identified in other systems from the MPF_{FA} class, which all lack the classic VirD4-like T4CP, supporting the hypothesis that these systems have acquired a dsDNA translocase to drive DNA transfer [135]. TcpA is essential for conjugative transfer of pCW3, with the ATP-binding motifs in the FtsK-like domain essential for TcpA function, indicative of potential ATPase activity [124]. The FtsK domain was also important for TcpA self-interaction, as well as interactions with components of the T4SS, specifically TcpC, TcpG and TcpH [126]. Two N-terminal TMDs were necessary for wild-type TcpA function since their deletion resulted in a reduced transfer frequency that may be explained by a loss of TcpA oligomerisation and an inability to interact with TcpC and TcpG [124, 126].

Conjugative plasmids are transferred as a nucleoprotein complex from the donor cell to a recipient cell [137]. Prior to transfer, a strand of the plasmid at the *oriT* site is cleaved and subsequently bound by a relaxase protein, a family of site- and strand-specific transferases that possess at least one nucleophilic tyrosine residue [138]. No relaxase-encoding gene or *oriT* site has been identified on pCW3 or any of the other conjugative toxin plasmids [129, 138]. However, the first gene in the *tcp* operon is a potential tyrosine recombinase, IntP, which is postulated to act as an atypical relaxase in the pCW3 conjugation system [122].

3.2 Plasmid compatibility in *C. perfringens*

It is well established that many *C. perfringens* isolates carry more than one toxin plasmid and that these plasmids are very closely related (see earlier), sharing up to 40 kb of almost identical sequences [122]. These shared regions include the *tcp* conjugation locus and genes involved in plasmid replication and maintenance. For example, two separate studies have shown that individual NetB toxin-producing *C. perfringens* type A strains can carry at least three separate, closely related conjugative plasmids, with one plasmid encoding NetB toxin, another encoding CPB2-toxin and the third a tetracycline resistance determinant [61, 62]. Other studies have shown that *C. perfringens* type B, C and D isolates can also carry multiple toxin plasmids that are closely related as already discussed [40, 41, 69].

How are such closely related plasmids stably maintained in the same cell? Examination of the plasmid replication and maintenance region of the tetracycline resistance plasmid pCW3 [129] revealed the presence of a *parMRC* locus that appears to encode a classical type II actin-like plasmid partitioning system [139, 140]. Subsequent studies have shown that the

sequence of this locus in different *C. perfringens* plasmids varies subtly, with individual plasmids that are in the same isolate having slightly different *parMRC* sequences [61, 62]. Based on these observations and bioinformatic analysis, it was further proposed that these differences could account for the coexistence of these plasmids in the same cell and it was postulated that the toxin and resistance plasmids could be divided into four separate *parMRC* incompatibility groups (now designated as $ParMRC_{A\ to\ D}$; J. Rood, V. Adams & J. Prescott, unpublished), with no more than one member of an incompatibility group being found in any one strain [62, 122, 141]. Subsequent studies have revealed the presence of a fifth *C. perfringens* incompatibility group, $ParMRC_E$ [141]. Further experimental studies are required to prove that the observed variation at this locus is responsible for the coexistence of more than one toxin or resistance plasmid in the same *C. perfringens* strain.

3.3 Plasmid replication of *C. perfringens* plasmids

Plasmid replication generally involves Rep proteins that bind to specific plasmid-specific DNA sequences and assist in initiation of plasmid replication [142]. However, initial bioinformatic analyses of *C. perfringens* plasmid-encoded proteins, including their amino acid identity and predicted domain architecture, failed to identify a Rep protein homologue [129]. In order to identify a Rep protein for these plasmids, portions of pCW3 were subcloned and assessed for their ability to replicate independently. This analysis revealed an ~4 kb fragment that afforded a plasmid the ability to independently replicate in *C. perfringens*. Transposon mutagenesis of this region was later performed, revealing that insertions mapping to a specific gene abrogated the ability of that plasmid to replicate in *C. perfringens* [129]. This 831 bp ORF (now called *rep)* encodes a Rep protein that is present on 95–100% of all characterized *C. perfringens* toxin and resistance plasmids, indicating that the mechanism of replication of these *C. perfringens* plasmids is likely to be identical [122, 129]. This Rep protein has a predicted pI of 10, consistent with this being a DNA-binding protein [122, 129, 142]. The Rep protein of *C. perfringens* plasmids does not share similarity, motifs or domains with Rep proteins of other species, which probably explains why the *C. perfringens* plasmids have not been observed in other clostridial species or bacterial genera [143, 144].

3.4 Evolution and diversity of *C. perfringens* toxin plasmids

As mentioned above, all *C. perfringens* toxin plasmids share sequences with pCP13 [72]. Most of these plasmids also contain the *tcp* locus that mediates conjugative transfer between two isolates, as well as a common *dam-rep* region that is required for plasmid replication [36, 41, 43, 44, 69, 70, 121]. Given the nature of plasmid carriage between *C. perfringens* types and strains, a number of events apparently led to the evolution and diversity of the toxin plasmids characterized to this date.

Of likely significance for *C. perfringens* toxin plasmid evolution is the close association between most toxin genes and insertion sequences, as described in Section 2 of this Review. While insertion sequence-directed movement of toxin genes between plasmids has not yet been formally demonstrated, this possibility is supported by the detection, using PCR-based approaches, of several excised circular intermediates carrying toxin genes [39, 41, 43, 69, 122]. As mentioned earlier, circular intermediates have been detected that carry the *cpe* gene

in type A strains [36, 121], *cpb-tpeL* genes in type B strains [69], *cpb* and *cpe* genes in type C strains [39], *cpe* and *etx* genes in type D strains [41], and the *iap/ibp* genes in type E strains [43]. These circular intermediates may represent transposon intermediates capable of integration into *C. perfringens* DNA, particularly into plasmid backbones. Many plasmid-borne toxin genes are present adjacent to the *dcm* gene, which may represent a preferential location on plasmids for insertion of mobile genetic elements carrying toxin genes [36, 41, 43, 44, 69, 70, 121]. If so, this would help to explain why the plasmid-encoded toxin genes are not commonly found on the chromosome.

Based upon the characterization and sequencing of several *C. perfringens* plasmids, a model for evolution of these plasmids can be proposed (Fig. 3). This model entails a common precursor plasmid resembling pCP13 that gave rise to a variety of *C. perfringens* plasmids, including both the characterized toxin and antibiotic resistance plasmids. By homologous recombination or another mechanism, pCP13-like precursor plasmids acquired either the *becAB* locus or the *tcp* locus (Fig. 3A) [122]. At least once, the *tcp*-carrying plasmid then acquired the *netB* gene adjacent to the *dam-rep* region, producing plasmid pJIR3535 [61]. A similar type of event may have occurred to create pCW3, which is related to pJIR3535, but instead of *netB* possesses a *tet*(P) operon that encodes tetracycline resistance (Figs. 1 and 3B) [129].

To form the pCPF5603 family of toxin plasmids, the *tcp*-carrying, pCP13-related precursor plasmid likely obtained the gene containing *cpb2* gene and a metabolic gene cluster via homologous recombination or another mechanism. Those acquisitions formed a pCPF5603 precursor that, via a transposition intermediate, then gained the *cpe* gene to create pCPF5603 [36, 69]. A pCPF5603 plasmid may later have obtained the *iap/ibp* genes via transposition to form the pCPF5603-like plasmid of type E strains [36, 43, 44]. This genetic element apparently inserted into the promoter region of the *cpe* gene, silencing that gene [43]. Alternatively, another pCPF5603 precursor plasmid appears to have obtained *etx* via a transposition intermediate containing an IS*1511*-like insertion sequence to form pCP8533etx (Fig. 3B) [43, 69].

To create the pCPF4969 plasmid family, the *tcp*-carrying, pCP13-related precursor plasmid may have initially gained loci encoding a peptide bacteriocin or a lantibiotic-like bacteriocin gene cluster, as well as a VirS/VirR-like two-component regulatory system. Via a IS*1470*-like transposition intermediate, this pCPF4969 precursor then acquired a *cpe* gene to form pCPF4969 [36]. At least once, this pCPF4969 plasmid picked-up a functional *iap/ibp* gene locus, to form pCPPB-1, which possesses both functional *cpe* and *iap/ibp* genes (Fig. 3B) [36, 44].

Summarizing, many toxin plasmids of *C. perfringens*, including those belonging to the pCPF4969, pCPF5603, or pCW3 plasmid families, are hypothesized to have evolved from a common pCP13-like precursor plasmid [36, 41, 43, 44, 69, 70, 121, 129]. This model offers potential understanding of the origin and evolution of these mobile genetic elements carrying the toxins that impart virulence plasticity to *C. perfringens* types and strains.

4. Conclusions

Toxin-encoding plasmids often play an essential virulence role when *C. perfringens* causes the intestinal infections that are major problems in humans and livestock. Toxin plasmids characterized to date fall within four families, i.e., the non *tcp*-carrying pCP13-like BEC plasmid, pCW3-like plasmids, pCPF4969-like plasmids and pCPF5603-like plasmids. Except for the BEC-encoding plasmid, all of the toxin plasmids share a conserved region carrying, in part, the *tcp* locus that mediates conjugative transfer. Conjugative transfer likely explains the presence of toxin genes on different plasmids amongst genetically-variable strains of this bacterium. It also favors accumulation of toxin plasmids, as evident from identification of *C. perfringens* strains carrying three distinct toxin plasmids [122].

The association of toxin genes with conjugative plasmids may also directly contribute to disease. *C. perfringens* is a common component of the normal microbiota in the intestines. Those normal flora strains are generally type A strains that do not produce toxins with a proven involvement in intestinal disease, but are presumably proficient at intestinal colonization. Therefore, when strains carrying toxin genes important for intestinal disease are introduced into the intestines, the presence of these toxin genes on conjugative plasmids capable of high frequency transfer could result in conversion of the colonization-proficient normal microbiota strains into virulent strains capable of causing gastrointestinal disease.

Plasmid-borne toxin genes are also often closely associated with insertion sequences, which can excise these toxin genes from plasmid backbones. This close association between many toxin genes and insertion sequences likely contributes to the virulence flexibility of *C. perfringens*. However, incompatibility issues place some limitation on the total number of toxin plasmids that can be accumulated by one strain. Again, the common association of toxin plasmids with insertion sequences may provide a potential mechanism to overcome this barrier as it could explain why single plasmids carrying four different toxin genes have been observed.

Further study of *C. perfringens* toxin plasmids is essential. For example, additional toxin plasmids exist that do not fall into the four known toxin plasmid families and those plasmids should be characterized. A greater understanding of plasmid incompatibility mechanisms is needed. Lastly, attempts should be made to demonstrate the insertion into plasmids of mobile genetic elements carrying insertion sequences and toxin genes.

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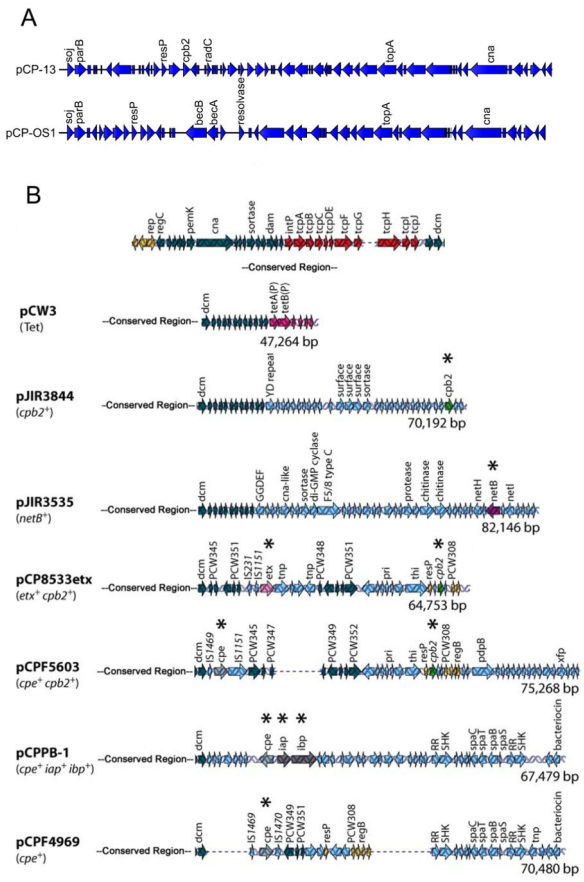

Figure 1. Comparison of *C. perfringens* plasmid diversity and organization

The sequenced plasmids of *C. perfringens* are represented graphically. Panel A shows an aligned comparison of *tcp*-negative plasmids pCP13 (AP003515.1 [72]) and BEC-encoding plasmid pCP-OS1 (AP013033 [71]) demonstrating significant homology between these two plasmids. Panel B shows plasmids with a pCP13-like backbone that harbors the *tcp* locus. Depicted are plasmids: pCW3 (DQ366035 [129]); pJIR3844 (JN689217 [61]); pJIR3535 (JN689219 [61]); pCP8533etx (AB444205 [70]); pCPF5603 (AB236337 [36])]; pCPPB-1 (AB604032 [44]); pCPF4969 (AB236337 [36]). The conserved region of these *C. perfringens* plasmids is shown at the top of Panel B, with variable regions displayed graphically below. Arrows represent ORFs, and are colored as follows in Panel B: red arrows – *tcp* conjugation locus; dark blue arrows – conserved ORFs; yellow arrows – plasmid replication region; light blue arrows – ORFs unique to each plasmid; fuchsia arrows – tetracycline resistance genes; green arrows – *cpb2;* purple arrow – *netB;* pink arrow – *etx;* gray arrows – *cpe;* dark gray arrows – *iap/ibp*. Asterisks designate toxin genes. Modified with permission from [122].

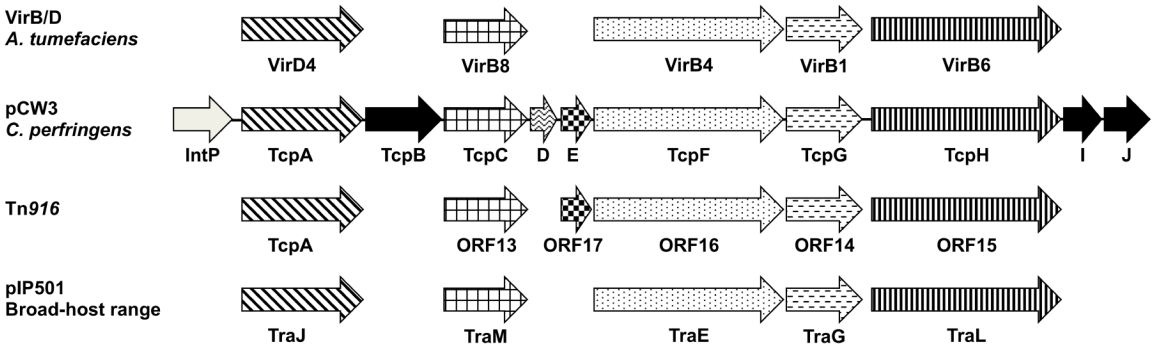

Figure 2. The genetic organization of the pCW3 *tcp* locus

Proteins encoded by the various genes are stated below the arrows. The patterned arrows indicate Tcp proteins involved in pCW3 conjugative transfer, black arrows depict non-essential Tcp proteins and the grey arrow the IntP protein, which is currently uncharacterized. Proteins with sequence, functional or structural similarity from the paradigm VirB/D system from the Ti plasmid from the Gram-negative *Agrobacterium tumefaciciens*, the conjugation region from Tn*916* and the broad-host range plasmid pIP501 from the Gram-positive *Streptococcus agalactiae* are represented by arrows with similar patterns. Each of these conjugation regions has homologs of the putative coupling proteins, VirD4 and TcpA, VirB8-like proteins, VirB4-like ATPases, VirB1-like lytic transglycolases and VirB6-like proteins. Based on data from [128, 129, 135].

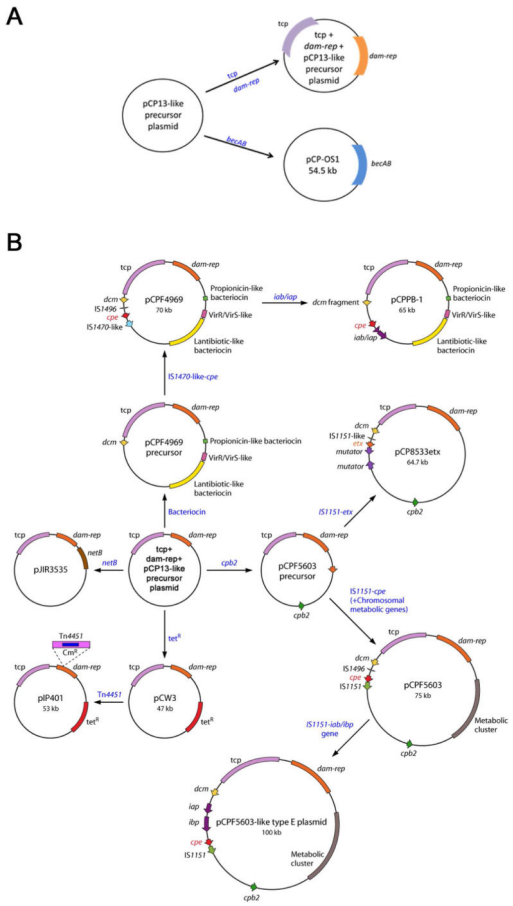

Figure 3. Model for evolution of the *C. perfringens* toxin plasmids

A model of evolution for sequenced and characterized *C. perfringens* toxin plasmids is shown. A) A pCP13-like plasmid acquires the *becAB* locus (pCP-OS1 and pCP-TS1 [71]) or the *tcp* locus (pCP13 [72]). B) The further evolution of pCW3 [129], pJIR3535 [61], pCP8533etx [70], pCPF5603 [36], pCPPB-1 [44], and pCPF4969 [36] are diagrammed. See section 3.3 for a discussion of evolution of these plasmids. Note that important plasmid regions are color coded. Modified with permission from [122].

Table 1

Classification of *Clostridium perfringens* based on the production of the four major typing toxins

Type	Typing toxin produced:			
	Alpha	Beta	Epsilon	Iota
A	+	−	−	−
B	+	+	+	−
C	+	+	−	−
D	+	−	+	−
E	+	−	−	+

Table 2

C. perfringens toxinotypes, plasmid-encoded toxins, and associated diseases

Type	Toxin(s)	Human Disease(s)	Animal Disease(s)
A	CPE*	Human food poisoning; non-food-borne GI diseases	Possible enteritis in dogs, pigs, horses, and goats.
	NetB	Not reported	Necrotizing enteritis in chickens
	CPB2	Not reported	Possible enteritis in pigs; possible enterocolitis in horses
	BEC	Possible human food poisoning	Not reported
B	Beta toxin, Epsilon toxin	Not reported	Necrotizing enteritis and enterotoxemia in sheep, cattle, and horses. Rare focal symmetrical encephalomalacia in sheep.
C	Beta toxin, CPE	Human enteritis necroticans	Necrotizing enteritis and enterotoxemia in pigs, sheep, cattle, horse, and other spp. (usually neonatal)
D	Epsilon toxin	Not reported	Enterotoxemia in sheep and goats; occasionally cattle and other species
E	Iota toxin	Not reported	Possible enteritis in rabbits, sheep and cattle

Modified with permission from [122] and incorporating newly discovered BEC toxin [71]

* CPE is usually chromosomally-encoded in food poisoning strains but plasmid-encoded in non-foodborne GI disease strains (see text)

Table 3

Size and diversity of *C. perfringens* toxin-encoding plasmids

Type	*cpb*	*etx*	*iap/ibp*	*cpe*	*tpeL*	*cpb2*	*netB*	*becAB*
A	-	-	-	70 seq** 75 seq***	ND	- 75***	82 seq*	54.5 seq****
B	65 90	65 seq***	-	-	65 90	65 seq***	-	-
C	65/90 110 75/85/110	-	-	75/85/90/110	65/90 65	75 65/75/90	-	-
D	-	48/75 65*** 75/85/110	-	75/85/110	-	65*** 45/75/85	-	-
E	-	-	97/135 65**	97/135 65**	-	70/85/90/97	-	-

Shared colors other than black indicate a similar/identical plasmid. Modified with permission from[122].

"Seq" indicates a sequenced plasmid; numbers are size in kb

* indicates plasmid from pCW3-like family

** indicates plasmid from pCPF4969 family

*** indicates plasmid from pCPF5603 family

**** indicates plasmid from pCP13-like family