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Virulence Plasmids of the Pathogenic Clostridia

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ABSTRACT The clostridia cause a spectrum of diseases in humans and animals ranging from life-threatening tetanus and botulism, uterine infections, histotoxic infections and enteric diseases, including antibiotic-associated diarrhea, and food poisoning. The symptoms of all these diseases are the result of potent protein toxins produced by these organisms. These toxins are diverse, ranging from a multitude of pore-forming toxins to phospholipases, metalloproteases, ADP-ribosyltransferases and large glycosyltransferases. The location of the toxin genes is the unifying theme of this review because with one or two exceptions they are all located on plasmids or on bacteriophage that replicate using a plasmid-like intermediate. Some of these plasmids are distantly related whilst others share little or no similarity. Many of these toxin plasmids have been shown to be conjugative. The mobile nature of these toxin genes gives a ready explanation of how clostridial toxin genes have been so widely disseminated both within the clostridial genera as well as in the wider bacterial community.

Many clostridial species are ubiquitous in the environment and in the intestinal tracts of birds, fish, and mammals. Commensal species are often carried asymptomatically within a host. However, if the immune status of the host is compromised, due to either age, illness or a change in diet, disease can result from toxigenic strains. Alternatively, some clostridial species or strains don't require predisposing factors. They can cause disease simply if they gain entry into the host either through damage to the skin or through the gastrointestinal tract, often *via* poorly prepared or incorrectly stored food. These clostridia then overgrow and cause cell and tissue damage. Diseases mediated by the clostridial species discussed in this review are predominantly mediated by potent protein toxins, many of which are located extrachromosomally. These toxins have diverse mechanisms of action and include pore-forming cytotoxins, phospholipases, metalloproteases, ADP-ribosyltransferases and large glycosyltransferases. This review focuses on these toxins and the elements that carry the toxin structural genes. For ease of discussion it has been structured on a bacterial species-specific basis.

PAENICLOSTRIDIUM (CLOSTRIDIUM) SORDELLII

Virulence Properties of *P. sordellii*

Paeniclostridium (formerly *Clostridium*) *sordellii* causes several severe diseases in both humans and animals. Most documented animal infections caused by *P. sordellii* are edemic or enterotoxic diseases of livestock

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and poultry, but they also include rare myonecrotic infections of other animals (1). Human infections are infrequent, but severe in nature, with mortality rates approaching 70% overall (2). The majority of *P. sordellii* human infections are soft tissue infections that occur after trauma or surgery (2). Sporadic, yet severe cases of post-partum and post-medical abortion uterine *P. sordellii* infections have occurred over the last 20 years, with almost every case resulting in the death of the patient (1, 2).

Due to the rare nature of *P. sordellii* infections, the pathogenesis of disease is still poorly understood. The major virulence factors involved in *P. sordellii* infection, however, are the production of two large clostridial toxins (LCTs): TcsH (hemorrhagic toxin) and TcsL (lethal toxin) (1). The LCTs enter mammalian host cells and glycosylate small GTPases such as Ras, leading to an altered cytoskeletal arrangement, cellular rounding and eventual death of the cell (3). Interestingly, while being highly potent, both *P. sordellii* LCTs are produced by only ~5- to 13% of isolates tested (4–6), with the majority of toxigenic isolates producing only TcsL and containing a truncated *tcsH* gene (6). Despite these findings, TcsL has been shown to be the major virulence factor responsible for the production of uterine-infection related toxic shock using an animal model of infection (7). In *P. sordellii*, the LCTs are encoded within a region called the pathogenicity locus (PaLoc), alongside genes that are likely to be required for their regulation and release (8). A recent phylogenetic analysis found that in all toxigenic isolates the PaLoc is carried on a group of related plasmids called the pCS1 family (6), which is the major focus of this section. Other virulence factors of *P. sordellii* include a pore-forming phospholipase SDL, the sialidase NanS, and the production of unique endospores (1, 9–11).

Bioinformatic Characterization of Plasmids in *P. sordellii*

The pCS1 family is a group of large plasmids carried by both toxigenic and non-toxigenic *P. sordellii* isolates. Seven such plasmids have been identified to date (12). The pCS1 plasmids encode a putative replication initiation (Rep) protein (Fig. 1), which, based on conserved amino acid motifs, falls within the RepA family that was first identified from the theta-replicating *Escherichia coli* plasmid P1 (6, 13). However, neither the function of this protein nor the location of an origin of replication (*oriV*) region has been experimentally determined. All members of the pCS1 family also encode a number of predicted surface exposed proteins that may behave as adhesion molecules (6). A number of these predicted surface proteins contain an LPxTG-like sortase motif (6, 13). Sortases mediate the attachment of specific proteins via the LPxTG motif to the peptidoglycan of the cell wall (14). A type B sortase enzyme is encoded (*srtB*, Fig. 1) in the vicinity of the majority of these surface protein genes and may therefore facilitate their attachment to the *P. sordellii* cell wall (6, 12), however, the role of these surface proteins is yet to be explored.

While there is a large degree of nucleotide sequence identity between the characterized plasmids (Fig. 1), significant differences also have been observed, both in plasmid size and in the presence of unique open reading frames (ORFs) (6, 12). The paradigm element is pCS1-1, which is encoded by the *P. sordellii* type strain ATCC 9714. This plasmid is 103 kb in size and carries the PaLoc with a truncated version of the *tcsH* gene, just upstream of the hypothetical *rep* gene (6). The pCS1-2 and pCS1-5 plasmids carry a PaLoc almost identical to that of pCS1-1 (6, 12). pCS1-2 is the largest member of the family at 112 kb and while their PaLocs are identical, pCS1-2 is missing a number of genes, including genes encoding a transcriptional regulator and a cold shock protein (6). A large insertion that carries a putative lantibiotic synthesis cluster is present on pCS1-2 (6). On the other end of the spectrum is the smallest plasmid member, pCS1-5, at 92 kb (12). pCS1-5 displays regions of high identity to the other pCS1 members, but also contains two small unique regions (12). The plasmids that carry a full-length *tcsH* gene, pCS1-3, -6 and -7, are very closely related (Fig. 1), displaying 99% identity across their ~106-kb sequences (12). Major differences between these plasmids and the other pCS1 members include an absence of the genes encoding subunits of an anaerobic sulfite reductase and the presence of a number of putative transposases (6). Non-toxigenic isolates of *P. sordellii* also carry pCS1 family plasmids, such as pCS1-4 from strain UMC2 (6). This plasmid is 100 kb in size and in place of the PaLoc is a string of ORFS with unknown functions, with the exception of a single putative transposase-encoding gene (6).

A second type of extrachromosomal element, named pCS2, has been identified in *P. sordellii* (6). This ~37-kb circular element has not been as thoroughly characterized as the pCS1 plasmids and appears to encode a number of bacteriophage-related genes (6). Putative plasmid-related genes are present, however, in the form of partitioning and replication initiation genes (6). Related elements appear to be present in another four strains of *P. sordellii*, two of which also carry a pCS1-like plasmid (6). Whether pCS2-like elements represent

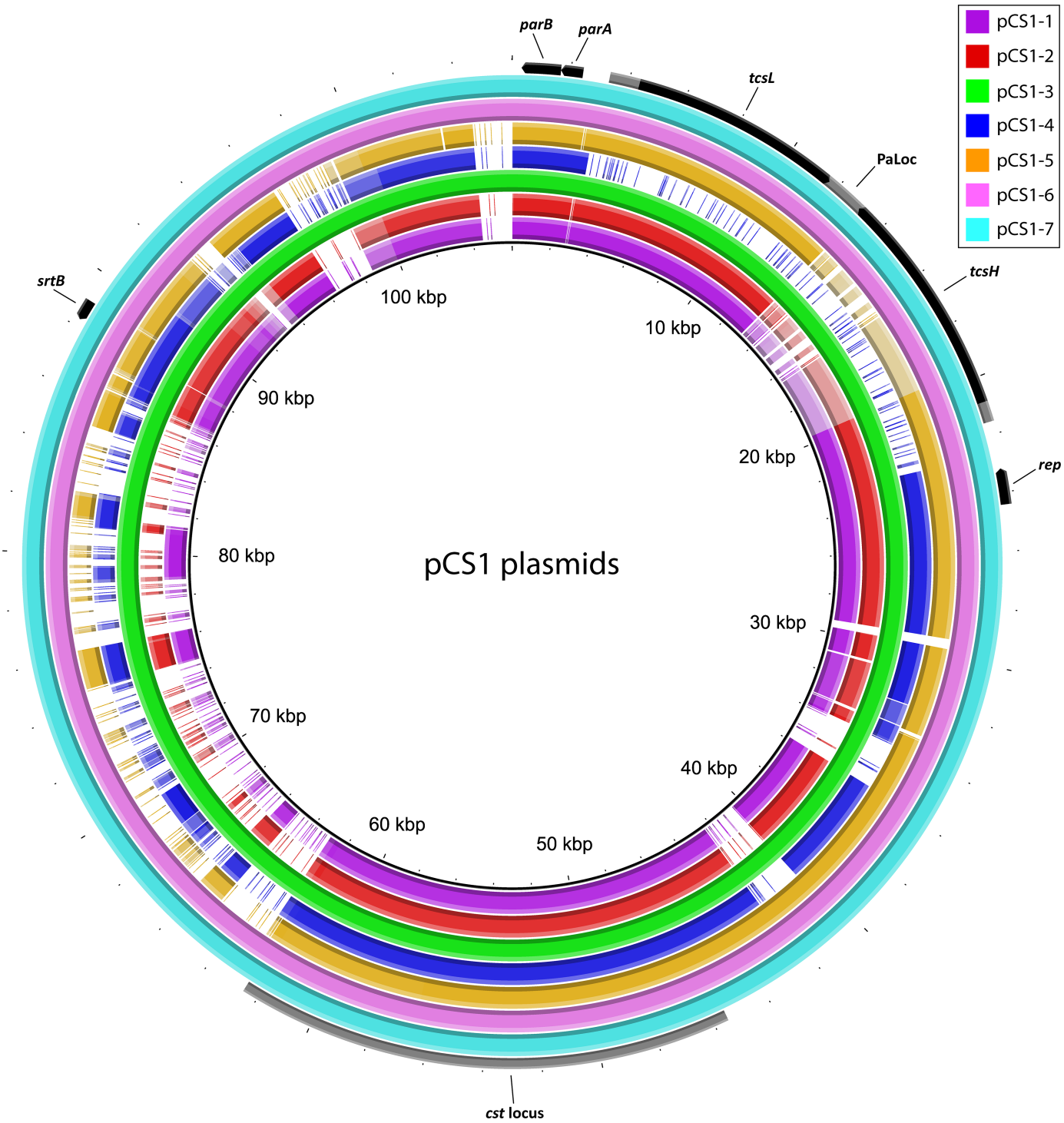


FIGURE 1 The pCS1 family plasmids of *P. sordellii*. Shown is a visual representation of a blastn analysis comparing each of the seven sequenced pCS1 plasmids to the reference sequence of pCS1-3 from strain JGS6382. The third ring from the center (green) and the coordinates (in kb) correspond to pCS1-3. Plasmids displaying 70 to 100% identity to pCS1-3 at a particular locus are shown with a solid block of colour on their respective ring. Identity to pCS1-3 between 50 and 70% is represented as a pale block of colour and if the identity is lower than 50% it is represented as a gap in the corresponding ring. Conserved loci on pCS1-3 are indicated as a gray arc on the outermost ring and labeled. Genes of interest are annotated as black arrows on the outermost ring and also labeled. Sequences analyzed and accession numbers: pCS1-1 (LN679999), pCS1-2 (LN681232), pCS1-3 (LN681235), pCS1-4 (LN681233), pCS1-5 (MG205643), pCS1-6 (MG205642), pCS1-7 (MG205641). Produced using BRIG (224).

plasmids, cryptic plasmids, or phage is currently unclear, but no virulence genes have been identified within this plasmid family to date.

Stability and Conjugative Transfer of the pCS1 Family of Plasmids

A recent study has functionally characterized conserved genes of the pCS1 family plasmids, using pCS1-1 as a model (12). All pCS1 plasmids carry *parA* and *parB* genes, which are putative members of a ParABS plasmid partitioning system (6). Such systems are utilized by low-copy-number plasmids to ensure stable inheritance of the plasmid in each daughter cell upon cellular division (15). Stability assays conducted on a marked version of pCS1-1 in different strain backgrounds showed that the plasmid is stable across ~120 generations (12). Independent insertional inactivation mutants of the pCS1-1 *parB* gene displayed gradual loss of the plasmid during continual subculture, providing evidence that ParA and ParB represent a true plasmid partitioning system (12).

Bioinformatic analysis of pCS1-1 also leads to the identification of an ~17.3-kb region, subsequently named the *cst* (for *C. sordellii* transfer) locus (Fig. 1), that encoded proteins with similarity to components of a plasmid conjugation system (12). The conjugative transfer of a marked derivative of pCS1-1, from type strain ATCC 9714 to a *P. sordellii* isolate from a distant clade, was subsequently demonstrated (12). The *cst* locus is conserved between all members of the pCS1 family so far characterized (Fig. 1) (12). Encoded within the *cst* region is a putative relaxase protein belonging to the MobMG family of relaxases, along with a cognate origin of transfer (*oriT*) located upstream of the relaxase gene (12). A number of genes encoding components of a type IV secretion system are also located within the *cst* locus. Two of these, the putative coupling protein, CstD4, and the putative ATPase, CstB4, were demonstrated experimentally to be required for conjugative transfer, confirming the *cst* region to be a true conjugation locus (12). Regions with significant similarity to the *cst* locus also have been identified in plasmids of *Clostridium perfringens* and *Clostridium botulinum*, and within plasmid or conjugative transposon-like regions of the genomes of *Clostridioides* (previously *Clostridium*) *difficile* isolates, indicating that *cst* represents a variant of a common clostridial conjugation locus (12).

CLOSTRIDIUM PERFRINGENS

C. perfringens is ubiquitously distributed among animals, humans and the environment (16, 17), but is also the causative agent of numerous opportunistic infections (16). The diseases caused by *C. perfringens* range from typically mild, self-limiting human food poisoning, to the more severe pathologies of human gas gangrene, and enterotoxemia and necrotic enteritis in domestic livestock (18). It is this broad spectrum of disease that makes this bacterium of particular interest to both veterinary and human medicine. Enteric disease caused by *C. perfringens* is estimated to cost the poultry industry in excess of $6 billion globally as well as $466 million in human health service costs in the United States alone (19, 20). What makes *C. perfringens* such a successful pathogen is its ability to encode and produce up to 20 toxins, many of which are associated with conjugative plasmids (21, 22).

The *C. perfringens* Typing Toxins

Strains differ in their carriage and expression of toxins, and this forms the basis of the *C. perfringens* toxinotype classification scheme (23). This scheme previously categorized *C. perfringens* strains into one of five toxinotypes based on the expression of four lethal typing toxins: alpha, beta, epsilon and iota (23). However, this scheme recently was expanded to include two new typing toxins, *C. perfringens* enterotoxin (CPE) and NetB toxin (Table 1) (24). This toxinotype classification is unique because particular "types" are often associated with specific diseases in humans and animals, for example; type A strains typically cause gas-gangrene, whereas type D strains are typically associated with enterotoxemia in sheep and goats (25).

Alpha toxin is encoded by all strains of *C. perfringens* and is the major toxin involved in the pathogenesis of gas gangrene infections (26). In all *C. perfringens* strains, the alpha toxin structural gene, *plc*, is chromosomally encoded with basal levels of *plc* mRNA always transcribed (27). The relative amount of toxin produced varies based on the strain background, with type A strains producing the highest levels of alpha toxin *in vitro* (28, 29). This toxinotype-specific control of toxin expression is said to be dependent on strain-specific global control elements, such as the two-component regulatory system VirR/VirS, as well as the structure of the promoter sequences preceding the *plc* gene (27, 28, 30, 31). Alpha toxin is a zinc-metalloenzyme that possesses both phospholipase C and sphingomyelinase activity, and functions by cleaving the phosphatidylcholine and ceramide head groups from the components of membrane phospholipid bilayers (32, 33). It is this targeted membrane disruption that leads to the severe disease often seen in gas gangrene infections (32, 34, 35).

TABLE 1 The *C. perfringens* toxinotype classification scheme

	Toxins Produced[a]						
type	Alpha	Beta	Epsilon	Iota	CPE	NetB	Associated Diseases
A	+	–	–	–	–	–	Gas gangrene in humans and animals Canine and equine enteric disease
B	+	+	+	–	–	–	Lamb dysentery
C	+	+	–	–	+/–	–	Necrotic enteritis in humans, sheep, horse, cattle and pigs
D	+	–	+	–	+/–	–	Enterotoxemia of sheep and goats
E	+	–	–	+	+/–	–	Enterotoxemias of rabbits, canines and cattle
F	+	–	–	–	+	–	Food poisoning and nonfoodborne gastrointestinal disease in humans
G	+	–	–	–	–	+	Chicken necrotic enteritis

[a]+, Produced; –, not produced; +/–, as produced by some strains.

Beta toxin, like many of the *C. perfringens* toxins, is a pore-forming toxin and is produced by both type B and C strains of *C. perfringens* (36). Beta toxin is responsible for disease development in *C. perfringens* type C infections; with beta toxin null mutant strains displaying avirulent phenotypes in animal models of enterotoxemia and necrotizing enteritis (37–39). The precise mechanism of action of beta toxin has not been fully elucidated, but, the hypothesis is that the toxin forms pores in the membranes of susceptible cells leading to cell lysis (40–42).

Beta toxin is encoded by the *cpb* gene, which is located on large conjugative plasmids (43–45). In type B stains, *cpb* is encoded on one of two plasmids: either ~65 kb or ~90 kb in size (44). More commonly the *cpb* gene is found on the larger ~90-kb plasmid (44). Preceding the *cpb* gene is the insertion sequence (IS) element, IS*1151* (44, 45). The presence of IS elements co-located in the toxin gene region may facilitate mobilization of these gene regions and may explain the presence of this toxin gene on multiple different plasmids (44, 45). The potential for mobilization is further supported by the detection of toxin-encoding circular intermediates in these strains (44, 45). In type B strains of *C. perfringens* there appears to be a strong association between carriage of *cpb* and the gene encoding the LCT, TpeL, which has no demonstrated role in disease (44). This study found that in all type B strains examined, the *tpeL* gene was always encoded ~3 kb after the *cpb* gene. The *cpb* plasmids of type B strains co-exist with at least one other virulence plasmid, an epsilon toxin (*etx*)-encoding plasmid, and sometimes another accessory toxin plasmid, encoding lambda toxin (*lam*) and a putative urease operon (44). In these strains the size of the *etx*-encoding plasmid and other accessory plasmids is consistent, with the only variable plasmid being the *cpb*-encoding plasmid (44). The limited variation of these plasmid combinations may be due to incompatibility issues with resident plasmids present in type B isolates. No type B plasmids have been identified that encode both the *cpb* and *etx* genes (44).

In contrast, the *cpb*-encoding plasmids of type C strains demonstrate much greater size variation (45). In these strains, the beta toxin gene is encoded on plasmids ranging in size from 65 kb to 110 kb (45). Restriction analysis suggests that some type C isolates may carry the same *cpb* plasmids found in type B strains (90- and 65-kb plasmids) (45). However, in addition to size differences, the *cpb*-encoding plasmids of type C strains also differ in their carriage of accessory toxin genes (45). As in type B strains, *cpb* is often associated with *tpeL* (45), although *tpeL* and *cpb* are not always encoded on the same plasmid (45). This result suggests potential movement of either of these IS-associated toxin genes (45). In addition to *tpeL*, *cpb*-plasmids can also encode other toxin genes, such as the CPE structural gene, *cpe* (45).

Epsilon toxin is the most potent of all toxins produced by *C. perfringens* (40, 46). It is the third most potent bacterial toxin, after tetanus and botulinum toxins (47). During infection, epsilon toxin is secreted as a pro-toxin from type B and D *C. perfringens* strains and is proteolytically cleaved to produce the active toxin (48, 49). Activation of epsilon toxin can be facilitated by proteolytic cleavage catalyzed by the *C. perfringens* protease lambda toxin (*lam*), but has been shown to be more commonly activated by chymotrypsin, trypsin or other carboxypeptidases encountered within the gut of the host organism (49–52). Once active, epsilon toxin primarily targets endothelial cells near the intestinal border, forming a prepore complex before inserting itself into cell membrane regions following binding to specific cellular receptors (53, 54). The toxin oligomerizes, forming a pore in the cell wall and inducing cellular damage (55). These damaged endothelial cells then allow epsilon toxin to be absorbed, where it systemically

targets organs such as the lungs, and kidneys and in the brain, where it induces the release of the excitatory neurotransmitter, glutamate (47, 56, 57). Excessive glutamate release and subsequent overstimulation of neurons results in altered neurological capabilities, which are commonly observed in animals suffering from epsilon toxin-induced enterotoxemia (47, 57, 58).

Epsilon toxin is encoded by the *etx* gene (57). The *etx* gene is harbored on conjugative plasmids found in both type B and D strains of *C. perfringens* (44, 59, 60). The *etx* plasmids of type B strains again appear to have a limited degree of plasmid diversity, with the *etx* gene only encoded on an ~65-kb plasmid (44). In contrast, the *etx* plasmids of type D strains are more variable in size, ranging from ~45 to ~110 kb (60). In addition to the size variability observed for type D *etx* plasmids, they are also more diverse in their toxin gene carriage (60). Some of the larger *etx* plasmids have been found to encode numerous other toxin genes such as *cpe*, *lam*, and *cpb2*, which encodes beta2 toxin (60). Whether the toxin genotype of a type D strain is simple (carrying only *plc* and *etx*) or more complex (possessing other toxin genes such as *cpe* or *cpb2*) appears to delineate the size of the plasmid on which the *etx* gene is located (44). 'Simple' strains tend to encode *etx* on plasmids ranging in size from ~45 to ~75 kb. Conversely, more 'complex' strains tend to encode *etx* on larger plasmids of roughly 75 to 110kb (44).

Iota toxin is a binary toxin encoded by type E strains of *C. perfringens*, which infrequently cause enterotoxemia in rabbits, lambs and calves (61). It comprises two protein components: Ia -(the enzymatic component) and Ib -(the binding component) (62, 63). Each component alone is non-toxic, but when combined the intact toxin produces rapid cytotoxic effects that often result in death (64, 65).

Iota toxin is encoded by two genes, *iap* and *ibp*, which form an operon (62). These genes are located on large virulence plasmid that range in size from 65 kb to ~135 kb (66, 67). Like many other *C. perfringens* toxins, the genes encoding iota toxin can be associated with the insertion sequence element, IS*1151* (66), with a complete element upstream of *iap* and *ibp* and a partial element downstream (66). The association of the toxin genes with IS*1151* may explain the existence of a highly conserved silent *cpe* gene upstream of the iota toxin operon in some strains (68). Current theories suggest that an insertion event on a *cpe*-encoding plasmid with the IS*1151*-associated iota toxin operon resulted in the formation of the type E iota toxin plasmids (66, 68). However, surveys of environmental strains and isolates from healthy humans has indicated that some type E strains derived from these sources encode an intact, but variant *cpe* gene that is not associated with IS elements (67).

Many type E strains carry additional genes, such as those encoding for urease and lambda toxin, on their iota plasmid (66). The components of iota toxin, like epsilon toxin, are activated by proteolytic processing (64), and the lambda toxin protease is known to process iota toxin into an active form (64, 69). The carriage of both toxin genes on a single plasmid would be considered advantageous because it would be beneficial for cells expressing iota toxin to also encode its activator, lambda toxin (66). In all strains characterized both *lam* and *iap/ibp* were encoded on the same large conjugative plasmid (66).

CPE is responsible for *C. perfringens*-mediated food poisoning in humans (70, 71) and is produced by the newly designated *C. perfringens* type F strains during sporulation in the small intestine, following the ingestion of a large number of *C. perfringens* cells from contaminated food (24, 72). Once released, CPE binds to enterocytes and forms a range of complexes on the cell surface (70). These complexes then form pores in the cell membrane, activating cell death pathways that result in cellular destruction (71). Like epsilon and iota toxins, CPE requires proteolytic cleavage for activation (73). It is encoded by the *cpe* gene, which can be located both chromosomally and on conjugative plasmids (74, 75), which appears to play a role in the epidemiology of disease (75, 76). *C. perfringens* type F food poisoning isolates generally possess chromosomally-encoded *cpe* genes, whereas isolates from nonfood borne gastrointestinal infections, such as antibiotic-associated diarrhea, have plasmid-encoded *cpe* genes (75). In fact, the *cpe* encoding plasmid from strain CPF4969, pCPF4969, was the first toxin plasmid of *C. perfringens* demonstrated to be conjugative (74). It is thought that the carriage of *cpe* on conjugative plasmids is a key factor in the disease phenotype observed during nonfood born gastrointestinal infections (40). In this syndrome, unlike *C. perfringens*-food poisoning, only a small number of *cpe*-positive *C. perfringens* cells are required to be ingested for disease establishment, which is postulated to be facilitated by the conjugative transfer of the plasmid to commensal *C. perfringens* strains in the gastrointestinal tract (40, 74).

The *cpe* gene is encoded on numerous plasmids that range in size from ~70 kb up to ~110 kb (45, 60, 66, 77). Plasmids encoding *cpe* have been found in type C, D, E and F strains, but not in any type B isolates (45, 60,

66, 77). In the newly designated type F strains (24), whether chromosomal or plasmid-borne, there is an IS*1469* element directly upstream of the *cpe* gene (78). However, the sequence downstream of *cpe* differs greatly, depending on the different genetic locations (78). Chromosomal *cpe* loci possess flanking IS*1470* sequences, which are proposed to comprise a larger mobile element (74, 77, 78). Plasmid-encoded *cpe* genes can have either a downstream IS*1151* or IS*1470*-like element, which forms the basis of the two *cpe* plasmid families: pCPF4969-like (IS*1470*-like) and pCPF5603-like (IS*1151* and *cpb2*) (77). The *cpe* plasmids show significant diversity and can differ in their carriage of other toxin genes (45, 60). In some type C strains *cpe* can either be carried on the same plasmid as *cpb* or on a separate plasmid (45). The larger *etx*-encoding plasmids characterized in type D strains tend also to carry *cpe* genes (60).

NetB is a β-pore forming toxin which is the causative agent of necrotic enteritis in chickens and forms the basis for the new toxinotype G. NetB was discovered after *C. perfringens* strains isolated from necrotic lesions were found to still induce disease after inactivation of the *plc* gene, which previously was thought to be the primary toxin involved in this syndrome (79, 80). Sequencing of these strains identified a putative toxin which was denoted NetB, Necrotic Enteritis Toxin B-like (80). Deletion of *netB* resulted in the formation of a strain that was unable to produce necrotic lesions in chickens (80). Complementation with the *netB* gene *in trans* restored the strain to wild-type virulence (80). NetB is produced by type G strains of *C. perfringens* and is encoded on large plasmids ranging in size from 82 to 95 kb (81), which like pCPF4969, have been shown to be conjugative (81, 82). Strains harboring *netB* plasmids can also contain two other closely related conjugative plasmids, encoding *cpb2* and tetracycline resistance (80–82).

Other Plasmid-Encoded *C. perfringens* Toxins

C. perfringens strains can harbor and express numerous other toxins and extracellular enzymes. The toxins of *C. perfringens* are functionally classified into four broad categories: pore-forming toxins, intracellular toxins, membrane damaging enzymes and hydrolytic enzymes (36). With some exceptions, most of the toxins and hydrolytic enzymes produced by *C. perfringens* are encoded on large conjugative plasmids (36, 83).

The beta2 toxin gene, *cpb2*, is possibly the most promiscuous of all toxin genes, because it can be found on plasmids from all *C. perfringens* strain types (44, 45, 60, 66). Despite its name, beta2 toxin has little amino acid sequence identity (less than 15%) to beta toxin (84). The involvement of beta2 toxin in disease is often debated because there is no direct evidence of beta2 toxin-induced virulence (85). However, there is a correlation between the prevalence of *C. perfringens* strains expressing beta2 toxin among animals with enteritis, particularly piglets, suggesting that it may play a role in the pathogenesis of this disease, but no genetic studies have been carried out (85).

The *cpb2* gene is carried by numerous plasmids ranging in size from 45 to 90 kb (44, 45, 60, 81, 82). These plasmids can carry numerous other toxin genes such as *cpe* and *etx*, with one plasmid characterized in a type D strain carrying all three toxin genes (60). In many strains *cpb2* is carried on a conjugative plasmid distinct from other toxin plasmids, as is seen in many type B to G strains (44, 45, 66, 81, 82).

Delta toxin is another β-pore forming toxin produced by *C. perfringens* (86, 87). Although it has not been shown to be involved in disease, it is thought that there may be a synergistic effect between delta and beta toxins, because they are often produced by the same strains (88, 89). Delta toxin is produced primarily by type C strains and possibly some type B strains (89, 90). Delta toxin is encoded by the *cpd* gene, which has so far only been characterized from two type C strains, CP24-03 and NCTC8131 (88). Not much information regarding the genetics of delta toxin is currently available, however, Southern blot analysis has shown that a *cpd* probe hybridizes to both total DNA and plasmid DNA preparations, suggesting that the *cpd* gene is likely carried on a plasmid (88). Further sequencing determined that *cpd* was encoded within a region flanked by ORFs that could make up a mobile genetic element, as is seen for other *C. perfringens* toxins (88).

NetE, NetF and NetG are putative β-pore forming toxins recently discovered in *C. perfringens* type A strains from dogs and foals presenting with enteric disease (91). Sequencing of the strains identified three genes—*netE, netF*, and *netG*—that were predicted to encode pore forming toxins with sequence similarity to NetB (91). In the first *netE/F/G*-positive type A strain characterized, both *netE* and *netF* were found to be encoded on the same large conjugative plasmid (92, 93), whereas *netG* was located on a second large conjugative plasmid that also carried the *cpe* gene (91). Sequencing of other *netF*-positive strains showed that co-carriage of *netE* and *netF* on the same plasmid remained consistent; however, carriage of the *netG* plasmid was variable among these isolates (92, 93).

NetF is cytolytic for equine ovary cells (91). There is also a strong association of *netF*-positive strains isolated from diseased dogs and foals which, along with the cytotoxicity assays, suggests that NetF is involved in these diseases (91). Further genetic characterization of *netF*-positive strains has demonstrated that *netE/netF* and *netG* are encoded on two distinct and relatively large pathogenicity loci on their respective plasmids (92, 93).

TpeL is a large glycosylating toxin, closely related to the LCTs, toxin A and toxin B from *C. difficile*, as well as TcsH and TcsL from *P. sordellii*. A role for TpeL in *C. perfringens*-associated disease has not yet been established (94). However, due to the presence of TpeL in some hypervirulent type G avian necrotic enteritis strains it has been suggested that TpeL may play a synergistic role with other toxins such as NetB (95). TpeL is encoded by the *tpeL* gene, which is located on a series of large conjugative plasmids from type B, C and G strains (44, 45, 96, 97). To date, *tpeL* has not been found in any type D or type E strains (96). In type B strains, *tpeL* is consistently encoded on plasmids in a location downstream of the *cpb* gene (44). In contrast, in type C strains that also carry the *cpb* and *tpeL* genes plasmid carriage is more diverse, and TpeL can be encoded on plasmids with *cpb*, or *cpb2* or on entirely separate plasmids (45).

Lambda toxin is a thermolysin-like metalloprotease produced by type B, D and E strains of *C. perfringens* (69). Lambda toxin possesses casein-hydrolyzing activity and has been demonstrated to degrade biological substances such as fibrinogen, collagen and complement component C3 (69). Purified lambda toxin increases vascular permeability, resulting in edema in an *in vivo* model, suggesting that the toxin is potentially involved in pathogenesis of *C. perfringens* infections (69). Lambda toxin is encoded by the *lam* gene, which is located on large conjugative plasmids of varying size (44, 69). In many type B strains it is encoded with the urease genes on a plasmid of approximately 80 kb (44). The carriage of lambda toxin is not as prevalent in type D strains, even though it has been shown experimentally that lambda toxin can activate epsilon toxin, however, host proteases also can activate epsilon toxin (60). The majority of type E strains carry lambda toxin on the same plasmid as the iota toxin and urease genes (66).

Numerous type B, D and E strains possess the ability to produce urease, which is encoded by a plasmid-encoded *ureABC* operon (98). These plasmids also may carry toxin genes, such as *cpe*, *lam*, *iap*, *ibp*, and *etx* (44, 60, 66, 98).

Toxin Association with Insertion Sequences

A common theme among the toxin genes of *C. perfringens* is the strong association with insertion sequence elements even though none of these genes have been shown to be located on a genetically confirmed transposon (60, 66). Frequently, toxin genes are located in close proximity to elements such as IS*1151*, IS*1470*, IS*1469*, and IS*406* (44, 45, 60, 68). The presence of these elements near toxin genes raises the possibility that toxin genes of *C. perfringens* can be easily mobilized and indeed circular DNA molecules that encompass toxin genes have been detected (44, 60, 66, 74). This theory could explain why several toxin genes, such as *cpb*, are found on numerous diverse plasmids and why toxin genes such as *cpe* are encoded both chromosomally and on plasmids (45, 74, 76).

Other *C. perfringens* Plasmids

Plasmids were first described in *C. perfringens* in 1973 (99) with the identification of a small bacteriocin-encoding plasmid, pIP404, in a type A strain (100). This plasmid forms the basis for most of the current *C. perfringens* cloning vectors (101). Other studies then focused on the identification and characterization of plasmids encoding antibiotic resistance determinants and bacteriocins (102–105). The first conjugative plasmid to be identified in *C. perfringens* was the tetracycline and chloramphenicol resistance plasmid pIP401 (102), in which the chloramphenicol resistance gene was later shown to be encoded on the integrative mobilizable element Tn*4451* (106, 107). Early studies also led to the identification and characterization of the conjugative tetracycline resistance plasmid pCW3 (108) and a series of plasmids that are closely related to pCW3 and pIP401 (109–112). Toxins were first associated with plasmids after a beta toxin-producing strain lost the ability to produce toxin after the loss of an uncharacterized plasmid (113).

Many of the antibiotic resistance and toxin plasmids of *C. perfringens* were initially characterised by methods such as pulsed-field gel electrophoresis, restriction analysis and Southern hybridization (44, 45, 60, 66, 105, 109–111, 114). It was through this analysis that the great diversity of toxin plasmids harbored by *C. perfringens* strains was demonstrated. The first known conjugative *C. perfringens* antibiotic resistance plasmid to be sequenced was the 47.3 kb plasmid pCW3 (115). Comparative analysis of this sequence revealed that there was a region that was common to many large *C. perfringens* plasmids (115). The first toxin plasmids to be sequenced were the *cpe*-encoding plasmids,

pCPF4969 and pCPF5603, which were derived from two type F food poisoning isolates (77). Sequence analysis showed that the *cpe* plasmids had ~35 kb of conserved sequence, indicating that these two plasmids may have been derived from a single common ancestor (77). Subsequently, several other toxin and antibiotic resistance plasmids of *C. perfringens* have been sequenced and characterized (74, 81, 82, 91, 97, 115, 116). As was seen for the *cpe*-encoding plasmids, approximately 35 to 40 kb of plasmid sequence remained highly conserved, despite these plasmids arising from different strain types. This analysis demonstrated that there was a significant similarity between the toxin plasmids and the tetracycline resistance plasmid, pCW3 (83, 115) and this plasmid has become the paradigm for the genetic analysis of this family of toxin and antibiotic resistance plasmids (Fig. 2).

Sequencing of pCW3 identified novel replication and conjugation regions within the 35- to 40-kb conserved *C. perfringens* plasmid backbone (115). Within this conserved backbone there were regions identified with sequence similarity to another *C. perfringens* plasmid, pCP13 (115, 117). pCP13 is a resident plasmid of *C. perfringens* strain 13 and carries a *cpb2* gene (117). Sequence analysis showed that these two plasmids are unrelated, apart from nine similar genes (115). Until recently pCP13 was not considered important to toxin gene carriage, because the *cpb2* gene located on this plasmid was defective, due to the presence of a premature stop codon (117). However, it is now apparent that pCP13-like plasmids can encode other toxin genes, including a new *C. perfringens* binary toxin, BEC (also called CPILE), which appears to be involved in non-CPE mediated human food poisoning in Japan (118, 119). Now, *C. perfringens* toxin plasmids can be broadly categorized into two families: the well characterized pCW3-like plasmids and the less well-characterized pCP13-like plasmids. All the toxin plasmids mentioned so far, other than the BEC plasmids, belong to the pCW3-like plasmid family.

Comparative Analysis of pCW3-Like Antibiotic Resistance and Toxin Plasmids

Two key genetic loci were identified on the conserved backbone of pCW3 following sequence analysis: the plasmid replication and maintenance region (81, 82, 115, 120) and the putative conjugation locus, denoted as the transfer clostridial plasmid, or *tcp* locus (115). The *tcp* locus encodes a novel transfer mechanism that is common to pCW3-like plasmids of *C. perfringens* (Fig. 2) (115, 121–127). To date, six large toxin plasmids (*cpe, netB, cpb2, etx, net G*, and the plasmid carrying the *netE, and netF* genes) and many antibiotic resistance plasmids have been shown experimentally to be conjugative (59, 74, 82, 91, 97, 106, 108, 109, 111). Furthermore, sequencing, pulsed-field gel electrophoresis, and subsequent Southern hybridization have demonstrated that virtually all large toxin plasmids carry a *tcp* locus that is closely related to that of pCW3, albeit with some minor variations in genetic organization and sequence, meaning that these plasmids are highly likely to be conjugative (44, 59, 60, 66, 74, 77, 82, 116).

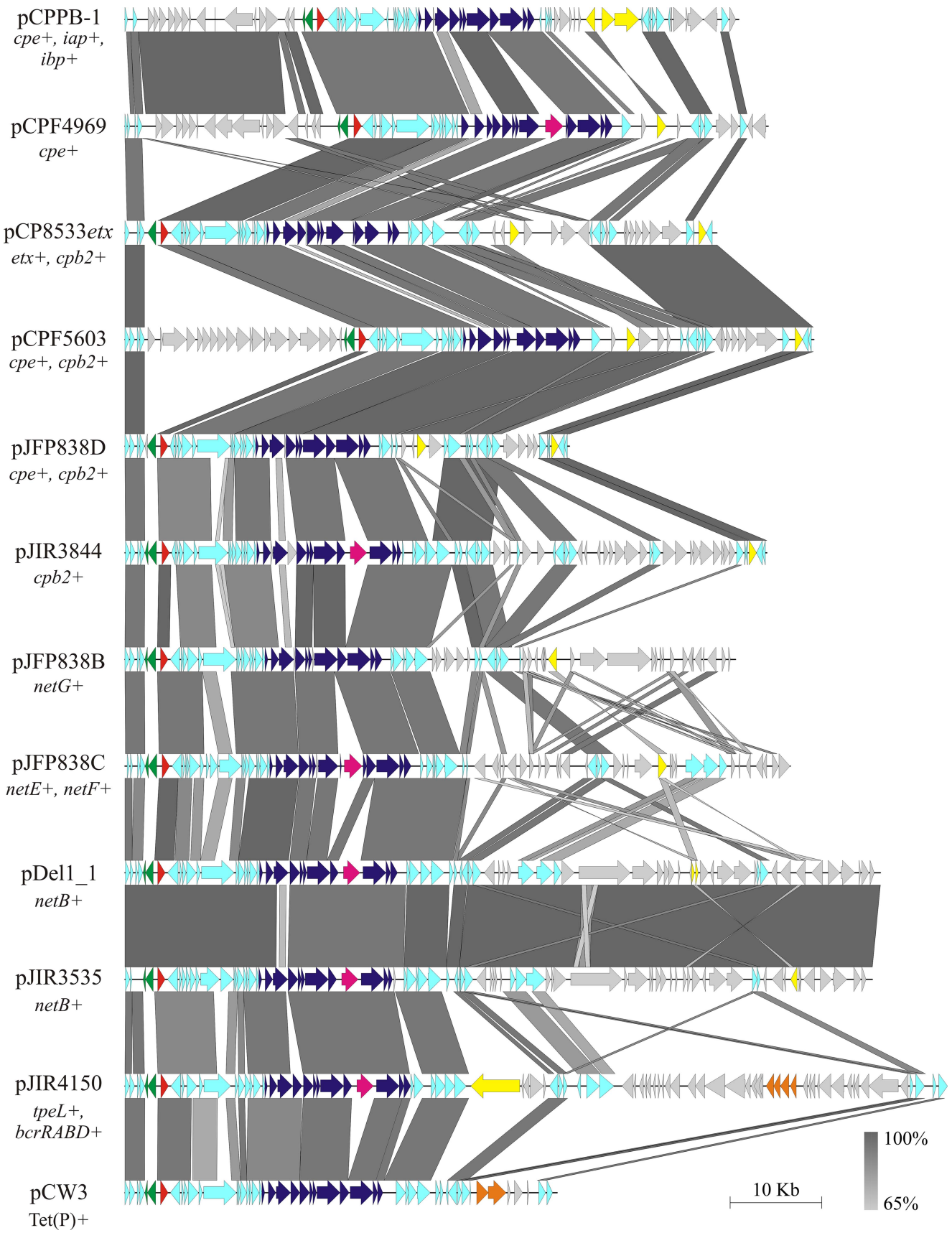
The biological importance of the toxins being encoded on conjugative plasmids is the potential of *C. perfringens* strains, particularly non-toxigenic commensals, to be converted to virulent isolates following the acquisition of a toxin plasmid(s) (59, 74, 128). Recently, this concept was validated *in vivo* in the gastrointestinal tracts of chickens, whereby non-pathogenic strains of *C. perfringens* were converted to disease causing isolates, following the horizontal transfer of a NetB toxin-encoding plasmid (129).

Current Model for Tcp-Mediated Conjugation

pCW3 is the smallest sequenced conjugative plasmid in *C. perfringens*, it contains a complete *tcp* locus and its transfer can easily be tracked by monitoring the acquisition of tetracycline resistance. Therefore, it has become the archetypal plasmid for conjugation studies in this bacterium (115, 128). The *tcp* locus of pCW3 encodes 11 genes, *tcpM* and *tcpA* to *tcpJ* (115). Many of the genes within the *tcp* locus were predicted to encode functional or structural components of the mating pair formation or transferosome complex. By contrast, no relaxase or other relaxosome components were identified from these initial investigations. Functional mutagenesis and complementation studies on each of the *tcp* encoded genes showed that most of these genes were required for efficient pCW3 conjugation (115, 121–127).

The pCW3 relaxosome

Prior to transport into the recipient cell pCW3 DNA must first be processed by the relaxosome. Typically, the relaxosome complex comprises a relaxase enzyme, which nicks DNA at the origin of transfer (*oriT*) of the plasmid to be transferred; in most cases, accessory proteins are also required (130). Initial analysis of the pCW3 *tcp* locus did not identify a typical relaxase gene, nor were any sequences identified with similarity to known *oriT* sites (115). However, the first gene encoded in the putative *tcp* operon, *tcpM*, encodes a protein



showing limited similarity to tyrosine recombinase enzymes and was hypothesized to function as an atypical relaxase (83, 115, 127). Mutagenesis and complementation studies have shown that TcpM is necessary for efficient transfer (127). Subsequent complementation with site-directed mutants revealed that a single C-terminal tyrosine (Y259) residue was essential for TcpM function, in contrast to typical tyrosine recombinases, which require seven conserved arginine, histidine and tyrosine residues for catalytic function (127, 131). Therefore, it was concluded that TcpM was a unique relaxase enzyme with Y259 postulated to be responsible for the essential nucleophilic attack on the sugar-phosphate backbone at the *oriT* site of pCW3 (127).

In many conjugation systems, the *oriT* site is located adjacent to genes involved in the relaxosome complex (132). It was postulated that the pCW3 *oriT* was located within a 391-bp intergenic region located upstream of *tcpM* (127). Mobilization assays using the 391-bp region cloned into a non-conjugative shuttle-vector confirmed that the *oriT* sequence was located within this region and defined the minimal *oriT* site (127).

The pCW3 transferosome

Transferosomes are large multi-protein membrane-associated complexes, or type 4 secretion systems, that physically transfer the relaxase-plasmid DNA complex into a recipient cell using ATPase activity to power the process (133). Initial bioinformatic analysis of the pCW3 *tcp* locus identified several putative proteins predicted to assemble as part of the transferosome complex (115). TcpF is a putative hexameric ATPase (115); ATPases are common components of conjugation systems, and are required to power DNA translocation and to assist in the formation of the transferosome complex (134, 135). TcpH is a putative structural protein with eight putative transmembrane domains; it is predicted to comprise a large proportion of the transmembrane channel that spans from the donor cell into the recipient (115). TcpF and TcpH were the first proteins tested for their involvement in pCW3 conjugation. Mutation by allelic exchange of the genes encoding these proteins and subsequent complementation and conjugation analysis demonstrated that the TcpF and TcpH proteins are essential for the conjugative transfer of pCW3 (115). In a later study, functional domains of TcpH were determined by deletion and site-directed mutagenesis. It was concluded that a region located between amino acids 514 and 581 was essential for TcpH function *in vivo* (125). Despite being essential for pCW3 transfer, TcpF remains to be functionally characterized.

tcpG and *tcpI* are both predicted to encode peptidoglycan hydrolase enzymes, which are predicted to function by hydrolyzing local regions of the thick peptidoglycan layers of the donor and recipient cells to allow for the formation of the transferosome. Mutation and complementation analysis determined that TcpG, but not TcpI, was required for efficient pCW3 transfer (121). Further analysis confirmed that TcpG had peptidoglycan hydrolase activity (121).

tcpC, *tcpD*, *tcpE*, and *tcpJ* all encode hypothetical proteins of unknown function (115, 123, 126). With the exception of *tcpJ*, they all are required for maximal pCW3 transfer. Analysis of the crystal structure of the C-terminal portion of TcpC (123) showed that although it had no conserved domains or similarity to other protein sequences, TcpC had a structure similar to VirB8, a conserved structural protein of the *Agrobacterium tumefaciens* type 4 secretion system (123). The structural similarities between VirB8 and TcpC suggest that TcpC plays a structural role in the pCW3 transferosome (123). The small transmembrane proteins TcpD and TcpE are essential for pCW3 transfer, because plasmids with mutations in either of these genes are non-conjugative (126). Their function remains to be elucidated, but immunofluorescence imaging showed that these proteins localize to the cellular poles, such as TcpF and TcpH (125, 126). It is highly likely that TcpD and TcpE also compose part of the transferosome.

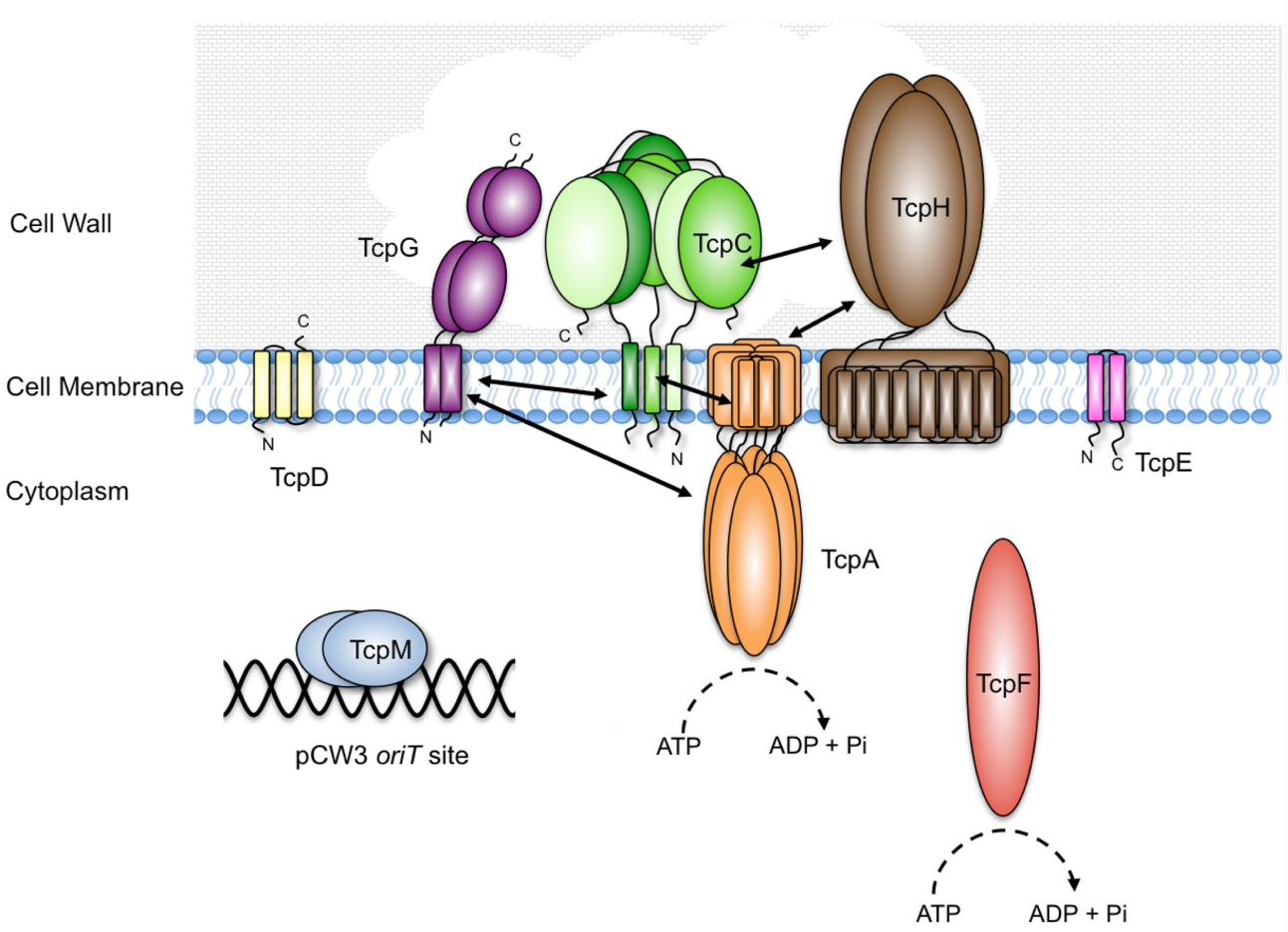
The second gene in the *tcp* operon, *tcpA*, encodes a putative integral membrane protein, with a conserved FtsK/SpoIIIE domain (122). Proteins within the FtsK/SpoIIIE family bind DNA in cellular processes such as chromosomal segregation and the transfer of genetic material into the forespore during sporulation (136). Therefore, TcpA was postulated to function as

FIGURE 2 Nucleotide alignment of pCW3-like plasmids from *C. perfringens*. Full plasmid sequences were aligned using the blastn algorithm (225) and Easyfig for visualization (226). The plasmid names are noted on the left along with the toxin or antibiotic resistance determinants encoded within each plasmid sequence. Predicted (ORFs) are indicated by arrows with the following color code: conserved ORFs (light blue), *tcp* conjugation genes, dark blue; toxin genes, yellow; *parMR* partitioning genes, green; *rep* red; group II introns, pink; antibiotic resistance genes (orange) less-conserved ORFs, gray. The scale bar and key for nucleotide identity are shown.

a coupling protein in pCW3 conjugative transfer (122). Subsequent mutation and complementation analysis confirmed that TcpA was essential for pCW3 transfer (122). As the coupling protein, it was assumed that TcpA mediated a range of protein-protein interactions with other Tcp proteins in both the relaxosome and transferosome complexes (124). Through chemical cross-linking and bacterial two-hybrid analysis, TcpA was found to self-associate, as well as to interact with the transferosome components TcpC, TcpG and TcpH (124). At the time of this study no relaxosome components had been identified, and thus, interactions with pCW3 relaxosome components have not yet been assessed (124). Based on these studies and other protein-protein interaction studies, including structural studies (121–125) a model for the conjugative apparatus utilized by pCW3 has been constructed (Fig. 3) (128).

Although a large body of work has been conducted on the *tcp* locus of pCW3, there are still large gaps in our knowledge and understanding of the structure and function of the conjugation apparatus. To date, it is still not certain whether pCW3 DNA transport occurs in a

FIGURE 3 Model of the pCW3 conjugation apparatus. The arrangement of the proteins within the model is based on protein localization studies and bioinformatics analysis. Black arrows indicate confirmed protein interactions between Tcp proteins and only Tcp proteins required for wild-type transfer of pCW3 are shown. Indicated within the membrane are the integral membrane proteins TcpH (brown), the peptidoglycan hydrolase TcpG (purple), the assembly factor TcpC (green; monomers as different shades), the proteins of unknown function, TcpD (yellow) and TcpE (pink) and the putative coupling protein TcpA (orange). Within the cytoplasm are a putative ATPase TcpF (red) and the novel relaxase TcpM (blue) in complex with the double stranded pCW3 *oriT* site. Dotted arrows indicate putative ATPase activity. Reproduced with permission from Wisniewski and Rood (2017).



single-stranded or double-stranded manner. Additionally, the precise processing reactions that are catalyzed by the relaxosome remain uncharacterized and the protein composition of this structure remains to be elucidated. Finally, the mechanism of direct cell-to-cell contact between *C. perfringens* cells remains elusive. These genetic and functional studies of pCW3 are important because they serve as a model system for the analysis of the pCW3-like toxin plasmids.

Multiple pCW3-Like Toxin Plasmids Are Often Present in the Same Strain

Plasmid incompatibility is defined as the inability of two plasmids to coexist in the same cell without the application of external selection (137). Plasmid incompatibility usually arises when two plasmids encode similar essential plasmid encoded factors such as replication initiation factors, replication control mechanisms or partitioning machineries.

All plasmids must replicate and maintain a stable copy number relative to the host chromosome to be faithfully inherited by the daughter cells at cell division (138). The copy number of a plasmid is related to the properties of the replication machinery and other factors. Initial attempts to identify the pCW3 replication gene could not identify an ORF with significant similarity to any known plasmid replication proteins (115). Therefore, a functional genetics approach was employed to determine which plasmid encoded regions were involved in plasmid replication. A combination of deletion and transposon mutagenesis studies led to the identification of the *rep* gene, which was essential for the replication of pCW3 (115). The intergenic region upstream of the *rep* gene had five pairs of inverted repeats and a series of conserved 17-bp direct repeats that were postulated to act as iterons or as a centromeric binding site for partitioning proteins (115). Comparative bioinformatic analysis of the available *C. perfringens* conjugative plasmid sequences revealed that the pCW3-like replication protein is highly conserved (120).

As already mentioned, many strains of *C. perfringens* carry more than one pCW3-like toxin or antibiotic resistance plasmid, each of which encodes a similar replication protein (82, 115, 120). These plasmids are stably maintained in the same cell, which is unexpected given the conventional relationship between plasmid incompatibility and plasmid replication functions (137). The best studied example of this phenomenon is the avian necrotic enteritis type G isolate, EHE-NE18. This strain stably harbours three highly similar pCW3-like conjugative plasmids: a NetB plasmid, a beta2-toxin plasmid and a tetracycline resistance plasmid. These plasmids each encode an almost identical replication protein (≥ 98% amino acid sequence identity) (82, 120). The apparent stability of this plasmid combination, and other toxin plasmid combinations in strains from other toxinotypes, suggested that factors other than the replication machinery were involved in the determination of plasmid incompatibility in *C. perfringens*.

pCW3 is a low-copy number plasmid (~5 plasmid copies/chromosome) (T. Stent, X. Han, R. Moore, V. Adams and J. Rood, personal communication) and therefore most likely requires an active maintenance system to ensure that it is inherited correctly (139). Closer inspection of the pCW3 sequence revealed two genes that were adjacent to, but divergently transcribed from, the *rep* gene. These genes encoded putative partitioning system homologues, ParM and ParR, and a putative *parC* centromere site (115).

Partitioning systems act as positioning systems to ensure that sister plasmids are positioned at the cell poles thus ensuring that the plasmid is maintained in daughter cells when cell division occurs (139). Type II or ParMRC-like plasmid partitioning systems encode three components: ParM, an actin-like ATPase that polymerizes to form filaments, ParR, a DNA-binding adaptor protein, and *parC*, a centromeric ParR binding region that usually comprises a series of direct repeats located upstream of the *parM* gene (140). ParMRC systems segregate plasmids via a pushing mechanism in which two sister plasmids are linked through a bundle of ParM filaments that recognize and interact with ParR adaptor proteins bound to the *parC* centromere sites (140). *C. perfringens* ParMRC-like plasmid partitioning systems have not been thoroughly investigated, although a *parMRC*$_{B}$ family partitioning system from the *C. perfringens* isolate JGS1987 has been demonstrated to stabilize a mini-replicon in *E. coli* (141).

An extensive survey of sequenced pCW3-like plasmids showed that there were at least 10 distinct families of *parMRC*-like partitioning systems (*parMRC*$_{A\text{-}J}$) present in *C. perfringens* (81, 82, 115, 120, 142). Comparative analysis showed that ParM homologues had upwards of 90% amino acid sequence identity within a family, but only 15- to 54% identity between the various groups (120). ParR homologues clustered into the same phylogenetic groups as the ParM homologues, but showed more amino acid sequence variation within a family (120). The *parC* centromere sites also clustered with their cognate ParM and ParR family groups, although the *parC* sites are very AT-rich and have a lot more sequence variability between the 10 groups than

the ParM and ParR components (120). It was observed that *C. perfringens* strains that house multiple pCW3-like plasmids generally do not have plasmids that encode the same *parMRC* allele (120). This observation suggested that *parMRC* partitioning families were responsible for maintenance within the same strain of multiple pCW3-like plasmids that encode a highly similar replication region.

There now is experimental evidence to support this hypothesis (143). In this study different combinations of genetically marked, *parMRC*-encoding, pCW3-like plasmids were introduced into the same *C. perfringens* strain. These strains were constructed so that they housed either two plasmids with the same *parMRC* locus, or two plasmids with *parMRC* loci from different families. Analysis of these isolates showed that plasmids with the same *parMRC* families were unable to stably coexist in the same cell, whereas plasmids with different *parMRC* families could stably coexist even though they encoded similar Rep proteins (143).

pCP13-Like Plasmids

Most studies of *C. perfringens* plasmids have focused on the pCW3-like family of toxin and antimicrobial resistance plasmids, but another class of toxin plasmids is beginning to garner more interest in the field (144). Genome mapping and whole genome sequencing of the *C. perfringens* type A isolate, strain 13, revealed the presence of a 54.3 kb plasmid called pCP13 (117, 145). pCP13 encodes 63 putative ORFs, many of which encode proteins of unknown function. Several ORFs could be assigned a predicted function including ORFs encoding a defective, consensus beta2-toxin, a predicted collagen adhesin called CnaB (146), and plasmid partitioning homologues, ParA (Soj) and ParB (117). Both the consensus and atypical beta2-toxins have been implicated as accessory virulence factors becuase they are found in various *C. perfringens* disease isolates (81, 82, 84). However, beta2-toxin has no confirmed association with disease. Cna proteins are known to act as adhesins and virulence factors in other Gram positive pathogens such as *Staphylococcus aureus* (147–149). A Cna homologue, CnaC, encoded on pCW3 and recently CnaA, which is produced by several avian necrotic enteritis isolates of *C. perfringens*, was shown to be important for gastrointestinal colonization and subsequent infection in chickens (146, 150).

pCP13-like BEC (CPILE)-encoding plasmids

Recently, three *cpe*-negative *C. perfringens* strains, OS1, TS1 and W5052, were isolated from three separate food poisoning outbreaks in Japan (118, 119). The supernatants of two of these isolates showed enterotoxic activity in a rabbit ileal loop model, even though no CPE was produced (119). Sequence analysis led to the discovery of two 54.5 kb plasmids, pCP-OS1 and pCP-TS1, each of which had 38 kb sequence identity with pCP13 (Fig. 4). Sequencing of these pCP13-like plasmids led to the identification of a novel clostridial binary toxin designated BEC (or CPILE, see reference 119 for a discussion of the terminology). BEC comprises two components, BECa and BECb, which show 43% and 44% amino acid identity to iota-toxin components Ia and Ib, respectively (118, 119). The BECb component is responsible for some of the enterotoxic effects, however its activity is enhanced by the BECa component, which has been shown to have ADP-ribosyltransferase activity on purified actin (119). In addition, both BECa and BECb components are required to cause Vero cell rounding *in vitro* (119). X-ray crystal structures of BECa have been solved, revealing a fold and substrate recognition mechanism similar to other ADP-ribosyltransferase toxins (151, 152).

Comparative analysis of pCP13 plasmids

The BEC plasmids, pCP-TS1 and pCP-OS1, were isolated from different strains, but are almost identical at the nucleotide sequence level (99% identity) (Fig. 4), which suggests that a recent horizontal gene transfer event occurred (119). The original sequencing of pCP13 did not initially reveal any ORFs with homology to known conjugation proteins (117). However, more recent comparative analysis of clostridial plasmid sequences has shown that pCP13 has several genes that encode proteins with amino acid sequence identity to conjugation proteins from the conjugative toxin plasmids pCS1-1 (12) and pCLL (153) from *P. sordellii* and *C. botulinum*, respectively. These proteins include a putative coupling protein with VirB4 domains, a putative essential ATPase with predicted VirD4 domains and a potential membrane scaffold protein with predicted VirB6-like domains (12). The presence of these conjugation machinery homologues on pCP13 together with the identification of almost identical BEC plasmids pCP-TS1 and pCP-OS1 in two distinct strains suggested that pCP13-like plasmids may be conjugative. We now have experimental evidence that pCP13 is indeed conjugative (T. Watts, C. Vidor, M. Awad, D. Lyras, J Rood and V. Adams, unpublished results). These studies highlighted the importance of the pCP13 plasmid family to the toxin armory of *C. perfringens* and to the dissemination of the toxin genes.

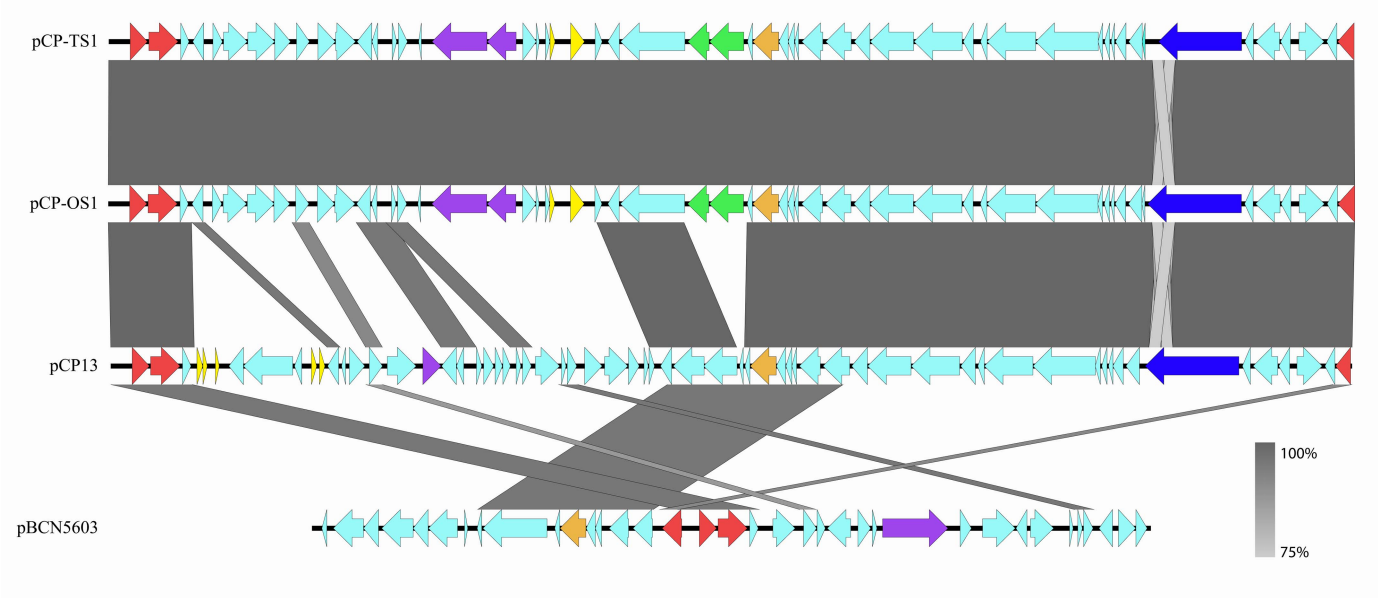


FIGURE 4 Sequence alignment of pCP13-like plasmids from *C. perfringens*. The plasmid sequences of pCP-TS1, pCP-OS1, pCP13 and pBCNF5603 were aligned using the Blastn algorithm using Easyfig (226). The percentage identity is indicated by the scale bar at the bottom right and each sequence is compared separately to the sequences above and below. ORFs are indicated by arrows and ORFs of particular interest are colored as follows: green, restriction modification systems; red, replication and maintenance; purple, toxin genes; yellow, transposase genes; dark blue, putative collagen adhesins; orange, putative relaxase enzymes.

The most recent addition to the pCP13-like family is the bacteriocin-encoding plasmid pBCNF5603, a plasmid found to coexist with a pCW3-like, CPE-plasmid in the type F isolate F5603 (144). Bacteriocins are antibacterial factors produced by bacteria to inhibit the growth of similar bacterial strains (154). *C. perfringens* strains often produce bacteriocins (155), some of which have been implicated in strain competition in the gastrointestinal tracts of broiler chickens (129, 144, 156, 157). pBCNF5603 was recently sequenced and found to have genes with a high level of similarity to homologues on pCP13 and on the small bacteriocin plasmid pIP404, suggesting that pBCNF5603 arose from a recombination event between these two *C. perfringens* plasmids (144) (Fig. 4). Sequencing of this plasmid has provided insight into the replication functions of pCP13-like plasmids by identifying two potential replication regions (144). The first replication region showed homology to the *rep* and *cop* genes of pIP404, whereas the second region displayed similarity to the *parA* and *parB* gene regions of pCP13, pCP-TS1 and pCP-OS1. The 5.5 kb gene cluster with similarity to pCP13 supported replication of a recombinant plasmid in *C. perfringens*, whereas the replication region with similarity to pIP404 did not (144). Analysis of subsequent deletion derivatives showed that the homologue to PCP63 may be the pBCNF5603 Rep protein (144). The region upstream of the putative *rep* gene was AT-rich, with a series of inverted repeats that are likely to be important for replication initiation.

THE TOXIN PLASMID OF *C. TETANI*

The neurotoxic clostridia comprise the *C. botulinum* complex, the causative agents of botulism, and *C. tetani*, which causes tetanus in both humans and animals. Both the clostridial species that comprise the *C. botulinum* complex and *C. tetani* are renowned for their ability to produce potent neurotoxins, botulinum toxin (BoNT) and tetanus toxin (TeNT), respectively (158). The various BoNT structural genes can be encoded on the chromosome, on a temperate bacteriophage or on a plasmid, whereas the TeNT structural gene is plasmid encoded (159).

TeNT is a zinc metalloprotease that acts on the SNARE protein VAMP/synaptobrevin at the synaptic junction of the nerve relaxation pathway in the spinal cord. Cleavage of VAMP/synaptobrevin means that it is unable to form a complex with other SNARE proteins, preventing fusion of the synaptic vesicle with the cellular

membrane at the synaptic nerve junction and preventing release of the neurotransmitter from the inhibitory interneurons to the motor neurons in the spinal cord. The net effect is inhibition of the muscle relaxation pathway and a rigid muscular paralysis that may be fatal (158, 160).

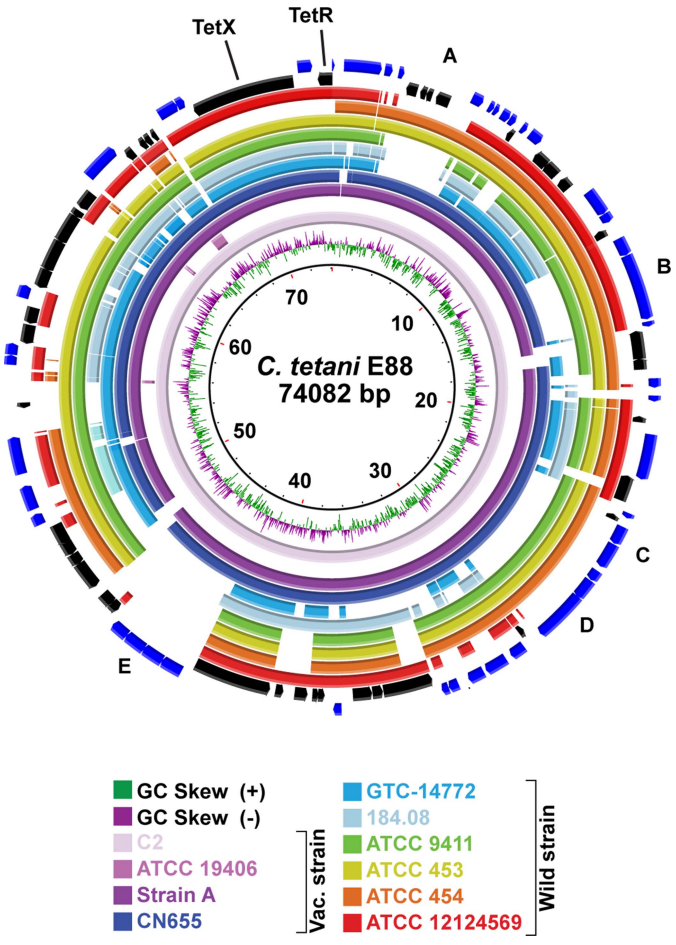
The first *C. tetani* strain that was sequenced, the Harvard vaccine strain E88, carries a 74,082 bp plasmid, pE88, that encodes 61 putative genes (159). These genes include the TeNT structural gene, *tetX*, and the *tetR* gene, which encodes an alternative sigma factor that is required for *tetX* expression and consequently for TeNT production (161). TetR belongs to a family of alternative sigma factors together with BotR from *C. botulinum*, TcdR from *C. difficile*, TcsR from *P. sordellii*, and UviA and TpeR from *C. perfringens* and (162, 163). Little is known about the function of other genes encoded on the TeNT plasmid, although there is another potential virulence factor gene, the *colT* collagenase gene, and genes encoding five distinct ABC transporter complexes (159). The regulatory proteins encoded by pE88 include TetR, two other putative alternative sigma factors, a putative two-component signal transduction system (CTP21/22) and a homologue of the bacteriocin plasmid pIP404-encoded UviB protein from *C. perfringens*. pE88 encodes two IS*200*-family transposases and an IS*605*-like transposase. It has *parMR*-like plasmid partitioning genes and a duplicated replication gene, the product of which is related to the replication protein encoded by pIP404. The structure of the actin-like ParM protein, Alp12, has been determined and shown to be compose of a four-stranded filament that is in a different conformation than other actin-like proteins (164, 165). Note that there is no genetic or bioinformatic evidence that pE88 or its homologs are conjugative or mobilizable.

A transcriptomics study that included plasmid-encoded genes has been carried out on strain E88 grown in fermentation bioreactors (166). The results showed that most of the pE88 genes have a transcriptional profile similar to the *tetX* and *tetR* genes, with gene expression increasing over time. No significant expression of the *colT* collagenase gene is observed under these conditions.

Since the publication of the genome sequence of strain E88 (159), the sequences of 10 other *C. tetani* strains have been reported (167–169). The chromosomes of these strains are highly conserved, with variations occurring at putative bacteriophage insertion sites, and CRISPR loci and within a flagellar glycosylation genomic island. Therefore, the *C. tetani* pan-genome can be considered closed, supporting the concept that *C. tetani* has only limited genetic exchange with other bacteria (170).

All of the sequenced strains, with the exception of the non-toxigenic isolate ATCC 19406, carry a pE88-like plasmid (168, 169) (Fig. 5). Another non-toxigenic strain, ATCC 454, carries a plasmid that has >95% identity to

FIGURE 5 Blast analysis of the tetanus toxin plasmid family. Plasmid sequences derived from *C. tetani* were compared to the 74 kb pE88 plasmid (GenBank accession number NC 004565). The two outermost rings show predicted (ORFs) for both the (+) and (-) DNA strands. The position of the tetanus toxin gene (*tetX*) and the regulator (*tetR*) are shown. DNA regions with less than 80% sequence identity are indicated by gaps. Strain ATCC 454 does not encode either the *tetX* or *tetR* genes. The regions denoted A through E indicate deletions within plasmids found in wildtype strains and include *uviB* and DNA directed RNA polymerase sigma-70 factor (A), *ftsX* and an ABC anti-microbial transporter system (B), an *ompR*/*baeS* two-component system (C), *ftsX* and an ABC-type lipoprotein export system/permease complex (D), and another ABC multidrug-resistance transporter/permease complex (E). Reproduced with permission from Cohen *et al* (2017).



pE88, but has a 20 kb deletion that encompasses both the *tetX* and *tetR* genes (Fig. 5). The remaining plasmids have considerable conservation as well as specific pE88 regions that are not present (Fig. 5). The *tetX* genes are highly conserved (99.3 to 99.4% identity) and the *tetR* genes are all identical (169). The lack of genetic tools for the analysis of *C. tetani*, combined with the inherent difficulties in working with this anaerobic, very motile and highly toxigenic pathogen, have clearly limited the genetic and functional studies that can be carried out on these important plasmids.

BOTULINUM TOXIN-ENCODING MOBILE GENETIC ELEMENTS

The production of BoNT neurotoxin is one of the defining features of the clostridial species *C. botulinum*. However, unlike TeNT-producing *C. tetani*, *C. botulinum* constitutes a highly heterologous group of organisms (171–175). This collection of strains consists of six groups (I- to VI), with groups I- to III generally designated *C. botulinum* (171, 172, 175). Groups IV, V, and VI consist of BoNT-producing strains of *C. argentinense*, *C. baratii*, and *C. butyricum*, respectively (175). This highly divergent spread of related toxin genes suggested early on that mobile genetic elements may be involved in toxin gene dissemination and subsequent studies have demonstrated that multiple types of elements are involved (171, 173).

All of these different species are able to produce BoNT, which is related to TeNT in both sequence, structure and function (176, 177). Both BoNT and TeNT are highly specific zinc metalloproteases. However, unlike TeNT, BoNT associates with non-toxigenic proteins to form large complexes (178), which is related to the different routes of intoxication between TeNT and BoNT (wound versus intestinal absorption). The non-toxic components are involved in the protection and transport of the active proteolytic component of BoNT from the gut to its site of action within the synaptic vesicles of excitatory neurones, where it blocks the release of neurotransmitters at the neuromuscular junction (179). The resultant flaccid paralysis can be fatal without appropriate supportive treatment (180). Many species, including humans, livestock, aquatic birds and fish, have been demonstrated to be susceptible to botulism (171).

The BoNT protein sequences have conserved functional domains, but are still highly variable (between 29 and 64% amino acid sequence identity), and historically have been separated into seven serologically distinct types (A- to G) (161, 179, 181). Many of the BoNT types have been further segregated into subtypes, designated by a number (179, 182), for example, BoNT/F6. An eighth serotype designated H, was reported in 2014, but there is controversy about whether this serotype constitutes a chimeric protein between serotypes BoNT/F5 and BoNT/A or a distinct group (172, 183, 184). Subsequently, another BoNT type has been identified and named BoNT/X. This neurotoxin appears not to be a mosaic enzyme and constitutes a new class of BoNTs (185). A ninth class of BoNT, BoNT/En, has recently been identified after genome sequencing of an *Enterococcus faecium* isolate derived from a manure sample in South Carolina and appears most closely related to BoNT/X (38.7%) (181). The heterogeneity exhibited by BoNTs is intriguing because it has resulted in differences in the complexes that are formed prior to cellular uptake and in the mechanisms of transportation. Most importantly, they have different host targets, catalyzing the proteolytic cleavage of different SNARE proteins, which facilitate neurotransmitter vesicle fusion and therefore neurotransmitter release and propagation of the electrical signal from the nerve cell to the muscle cell (186). In spite of this diversity, the result is the same: inhibition of neurotransmitter vesicle fusion, leading to flaccid paralysis (186).

The BoNT structural (*bont*) genes are encoded within two distinct genetic regions, the *orfX+* (function unknown) and *ha+* (hemoglutinin) loci (Fig. 6) (173). Both loci contain conserved ORF's (*orfs*), including *bont*, found downstream, and in the same orientation, as the conserved *ntnh* (non-toxigenic, non-hemagglutinin) gene as well as the *botR* gene mentioned above (161, 178, 187). All BotR-related proteins are responsible for positively regulating the expression of toxin genes in their native hosts. Likewise, BotR has been shown to regulate transcription of the *bont* gene (161, 162, 188).

The variable regions of the *bont* loci encode either the *orfX1-3* genes (*orfX+* loci) or the *ha17*, *-33*, and *-70* genes (*ha+* loci; Fig. 6, Table 2). The organization of these two loci is largely conserved between different strains, the surrounding nucleotide sequences having co-evolved with the variable *bont* gene (173). In addition to the nine serological BoNT types, hybrid *bont* genes have also been identified, such as serotype C/D and D/C derivatives (189). Certain BoNT positive strains encode multiple *bont* loci (173, 190), suggesting an easy mechanism for genetic exchange and explaining how recombination between *bont* loci could readily occur. It has been shown that when multiple *bont* loci are present within the same strain, one particular BoNT toxin is

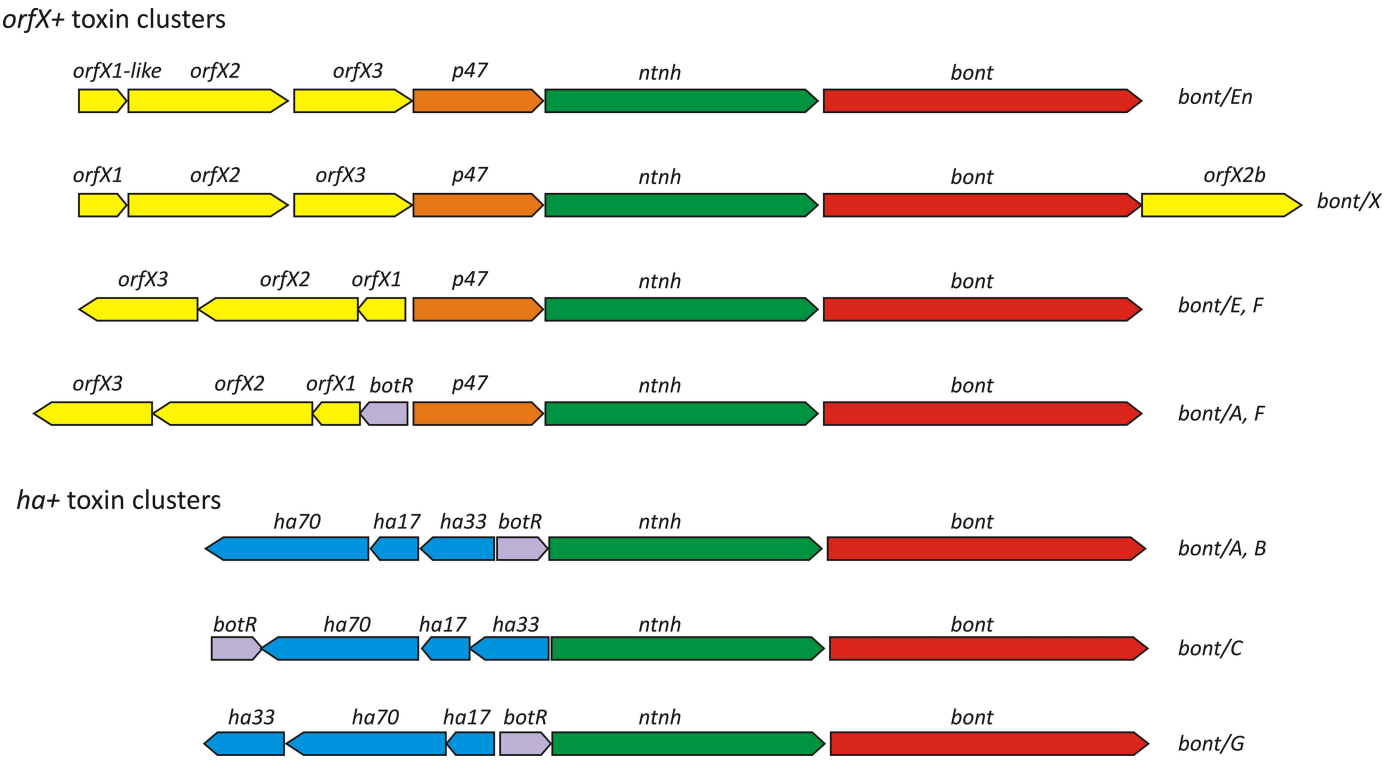


FIGURE 6 Gene arrangement for *bont* toxin loci. The two basic loci types, *orfX+* and *ha+*, are indicated. Similar genes are designated with the same color and the toxin gene serotype designation is given on the right.

expressed to a higher degree than the other(s) and is thereby designated with uppercase (indicating the more abundant toxin type) and lower case toxin types, for example BoNT/Ba (191).

Group I: — Proteolytic *C. botulinum*

Bacterial species belonging to this clade or group include *C. botulinum* as well as the non-neurotoxigenic, *Clostridium sporogenes* (173, 175). The toxin serotypes that have been identified within this clade include BoNT/A, /B, /F, /H (F5/A) and /X (172, 173, 184, 185) (Table 2). The serotype B *bont* loci (*ha+* operon) has a chromosomal location within the *oppA/brnQ* site (172). Alternatively, some B subtypes such as *bont/B1*, */B2*, and */B5* are found within the "B" site of large plasmids (172, 191). Very similar plasmids have been shown to harbor *orfX+* operons encoding either *bont/A2-4* or */F2* toxin genes integrated at another site known as the "A/F" site (172). Bivalent strains often carry one or even two plasmid encoded *bont* loci, where both the "B" site and the "A/F" sites are occupied (such as pCLJ from the bivalent strain 657 Ba) (191), for example, the *bont/F5* locus from the trivalent strain, Af84, also was found to be plasmid located (190, 192).

All clade I plasmids identified appear to be related and range in size from 149- to270 kb (173, 191, 193–195). A significant number of genes are conserved, including multiple genes involved in DNA replication (173, 194). In addition, there appear to be many genes show similarity to bacterial type II and IV secretion systems and two clade I plasmids, pBotCDC-A3 and pCLJ, are conjugative, with transfer demonstrated between plasmid-carrying donors and two different clade I recipients (153). The plasmids derived from clade I strains exhibit different degrees of stability, impacting the stability of toxin production as a result (194, 196). It is clear that the *bont* toxin genes of serotypes A, B, and F are commonly carried by highly related clade I conjugative plasmids capable of transfer between *C. botulinum* strains, but also to non-toxigenic *C. sporogenes* (197). Given the ability of the toxin loci to both recombine and move in the context of mobile genetic elements, there is significant capacity for the further spread either to other strains or to other locations within the same strain.

Group II: – Non-proteolytic *C. botulinum*

Group II strains produce BoNT serotypes B4, F6 and E (multiple subtypes; Table 2) (198, 199). The *bont/F6* loci

TABLE 2 Summary of BoNT-producing species and toxin gene locations

Bacterial clade or species[a]	I					II		III	IV	V	VI	*Enterococcus faecium*
BoNT type[b]	A(8)	B(8)	F(7)	H	X	B(8)	E(12)	F(7)	C	D	G	F(7)	E(12)	En
bont locus[c]	*orfX* *ha*	*ha*	*orfX*	*orfX*	*orfX*	*ha*	*orfX*	*orfX*	*ha*	*ha*	*ha*	*ha*	*orfX*	*orfX*
Chromosome[d]	*arsC* (*orfX*) *oppA/brnQ* (*ha*)	*oppA/ brnQ*	*arsCpulC* (*F4*)	*IS110* flanking *bont/H*	Cys. biosyn. Pathway		*rarA*(MGE)	*topB* (MGE) (*F6*)			Strain CDC2741	*IS1182* flanks *bont/F7* locus Compound Transposon?	*rarA* (MGE) (*E4, E5*)	
Plasmid	150-270 kb A/F site E (*orfX*)	150-270 kb B site	150-270 kb A/F site			47-63 kb (*B4*)	133-144 kb Helicase (*E1, E3, E10*)		Yes	Yes	140 kb Strain 89G			207 kb Putative Conjugative Plasmid
Bacteriophage encoded Species[e]	Humans Chickens	Humans Horses Cattle	Humans	Human (Bivalent B2h)	Human (Bivalent B2x)	Human Horses Cattle	Human Fish Birds	Human	Birds Cattle Horses	Birds Cattle Human	Soil Humans?	Humans	Human	Cow Manure

[a]Bacterial clade designation: I-III *C. botulinum*, IV *C. argentinense*, V *C. baratii*, VI *C. butyricum*.
[b]Designation of serologically distinct BoNT proteins, the number of toxin subtypes are in parentheses.
[c]Genes encoding BoNT toxins are found associated with one of two distinct genetic loci; *orfX+* or the *ha+* loci (see Fig. 6).
[d]Chromosomal location of *bont* locus, often associated with a mobile genetic element (MGE). F6 is the *bont* subtype.
[e]The vertebrate species in which each toxin type is most frequently demonstrated to cause Botulism. Clade IV strains have not been definitively associated with botulism but have been associated with multiple sudden deaths (199).

are chromosomally located and found within what is thought to constitute a mobile genetic element that carries a recombinase gene, *topB* (172, 200). The *bont/B4* loci found within this clade are believed to be exclusively plasmid-borne (193, 198, 199). The first group II plasmid sequenced is derived from strain Eklund 17B and carries the *bont/B4* locus on the 48-kb plasmid, pCLL (173, 174). Subsequent studies demonstrated that *bont/B4* loci are carried by plasmids 47 to 63 kb in size that are unrelated to group I plasmids (198). These plasmids form two distinct plasmid groups, perhaps sharing a common ancestor (198). One group (class 1) is related to pCLL and its members are of similar size, whereas class 2 plasmids only have about 33% similarity to class 1 plasmids; this similarity is limited to the large neurotoxin gene cluster (198). Class 2 plasmids range in size from 58 to 63 kb (198). A third plasmid appears to be a hybrid of the two other plasmid classes (198). All plasmid types encoding *bont/B4* loci were found only in group II strains and related plasmids that were nontoxigenic have subsequently been found by analysis of genome sequence data from other group II strains (199).

Serotype E strains belonging to group II had previously been reported to encode only chromosomal *bont/E* loci, but recent pulsed-field gel electrophoresis studies showed that a proportion of type E strains (6-10%) carry *bont/E* loci on large plasmids in group II strains (199, 201). These plasmids range in size from 134 to 144 kb and are closely related (199). Functional CRISPR systems were identified on plasmids carrying *bont/E1* and *bont/E10* loci, but not on plasmids carrying the *bont/E3* loci (199). The type E toxigenic plasmids appear to be very low copy number as judged from sequencing read abundance, approximately one copy per chromosome (199). The *bont/E* plasmid loci are very similar to the chromosomal *bont/E* toxin clusters that have integrated into a chromosomal copy of the resolvase gene, *rarA*. The toxin loci in both cases consists of a 24-kb cassette that include genes outside of the normal toxin cluster. This region includes a second, related *rarA* gene that remains intact (199). The plasmid-borne type E toxin cassette is completely conserved, but the integration site is different. The plasmid gene that has been interrupted is a helicase gene and the insertion site appears to be a 6-bp sequence that is conserved between the chromosomal *rarA* gene and the plasmid-encoded helicase gene (199).

All of the toxin plasmids so far identified from group II organisms (class1 and 2 *bont/B4* and *bont/E* plasmids) have a significant number of ORFs whose predicted function involves conjugative transfer (198,

199). The smallest plasmid characterized so far, pCLL, which contains the least putative conjugation genes (198), is the only group II plasmid to have a demonstrated ability to transfer, remarkably, into a group I recipient (153). No group II- like plasmids have been detected in any other related strain and they show little conservation between themselves or plasmids from other groups, suggesting that these plasmids have a narrow host range (199). pCLL has a low level of similarity to pCS1-family plasmids from *P. sordellii* and pCP13-like plasmids from *C. perfringens* (12). The plasmid location of *bont* genes in this bacterial group explains how the *bont* genes have been so widely disseminated.

Group III: – *Clostridium novyi sensu lato*?

The clade designated type III includes a number of clostridial species and recently it was proposed that it be re-named from *C. botulinum* to *C. novyi sensu lato* (189). This designation includes three species depending on the toxins that each strain produces (189): *C novyi, Clostridium haemolyticum* and *C. botulinum*. *C. novyi* produces the lethal alpha toxin, which is related to the alpha toxin produced by *Clostridium septicum*. In *C. novyi*, the alpha toxin gene is located on a bacteriophage (202) and any particular *C. novyi* strain may also produce one of two phospholipases (beta and gamma) (189). *C. haemolyticum* produces a phospholipase serologically indistinguishable from the beta phospholipase of *C. novyi* (189). Group III *C. botulinum* strains are associated with the expression of BoNT/C and BoNT/D and the chimeric derivatives, C/D and D/C (203) (Table 2). The location of the *bont* locus within group III strains of *C. botulinum* has been known for many decades to be within temperate bacteriophage, called pseudolysogens, that could be readily cured from toxigenic strains (203). The curing mechanism was shown to be due to the unusual nature of the lysogenic state — that of a replicating plasmid (203). For example, the *bont*-encoding bacteriophage from strain C-ST has a large (186-kb) linear genome with 404-bp terminal direct repeats when packaged within the phage particle (203), but the genome is found as a circular, self-replicating molecule within the bacterial cell (189, 203). Many other plasmid and phage-like molecules can be found not just in *C. botulinum* group III, but in all *C. novyi sensu lato* strains, indicating that this group is highly dependent on the accessory genome to determine the virulence of any particular strain (189, 202–205).

Group IV: – *Clostridium argentinense*

This group was renamed *C. argentinense* because it had very little similarity to other *C. botulinum* strains other than the production of BoNT (171). Only BoNT/G has been detected in *C. argentinense* and the *bont/G* toxin gene was demonstrated to be located on large plasmids in at least two strains (206) (Table 2). The first type G strain sequenced, CDC 2741, remains a draft assembly, but analysis of the sequence data indicated that the *bont/G* cluster was located on a large 1.1-Mb contig and was thought to be chromosomally located (172, 207). Recently, another completed genome sequence for a BoNT/G-expressing strain was released, strain 89G (208), and a circular plasmid sequence was obtained and shown to carry the *bont/G* locus. This plasmid, pRSJ17_1, is 140 kb in size and, although no further analysis of this sequence has been published (208), it appears to share little or no similarity to plasmids of group I or II *C. botulinum* strains. However, the entire pRSJ17-1 sequence appears to be located within the 1.1 Mb contig assembled from the genome sequence of strain CDC 2741 so it is possible that either the *bont/G* plasmid has become integrated into the chromosome in this strain or that the CDC 2741 draft assembly is erroneous and CDC 2741 contains a 140-kb plasmid almost identical to pRSJ17-1. Further analysis is required to confirm the toxin cluster location in strain CDC 2741.

Group V: – Neurotoxigenic *Clostridium baratii*

Type V BoNT-expressing strains are not designated as *C. botulinum* but are members of the non-neurotoxigenic species, *C. baratii* (172, 173). Again only one serotype of BoNT has been detected from this species: BoNT/F7, and the location of this gene is chromosomal, where it is flanked by directly repeated IS*1182* elements (207) (Table 2). The context of the *bont*/F7 locus in this strain suggests that it has been acquired by horizontal gene transfer (207).

Group VI: – Neurotoxigenic *Clostridium butyricum*

Neurotoxigenic *C. butyricum* strains have only been demonstrated to carry *bont/E* genes within *orfX*+ loci (173) (Table 2). The neurotoxin gene cluster was originally thought to be plasmid determined rather than chromosomal (209). However, more recent data have clearly indicated that the *bont/E* genes in *C. butyricum* are chromosomally located (173, 210, 211). The *rarA* gene is the position of insertion for the *bont/E* locus in both group II *C. botulinum* strains that carry *bont/E* as well as neurotoxigenic *C. butyricum* strains (172, 173, 199), which suggests that the *bont/E* locus has most likely been inserted *via* the same mechanism in both organisms, possibly via the recombinase RarA (172, 173).

CLOSTRIDIOIDES (*CLOSTRIDIUM*) *DIFFICILE* VIRULENCE PLASMIDS

C. difficile is a genomically diverse species, that consists of eight phylogenomic clades (212), and it contains many integrated and extrachromosomal genetic elements (213). The presence of numerous potentially mobile genetic elements suggests that horizontal gene transfer has played an important role in the evolution of *C. difficile*. Although *C. difficile* pathogenesis involves multiple factors (214), disease resulting from infection with this microbe is primarily toxin-mediated, and strains can encode up to three toxins. These toxins include toxin A (TcdA) and toxin B (TcdB), which are both encoded within the Pathogenicity Locus (PaLoc), and the *C. difficile* transferase or binary toxin (CDT), encoded within the CDT locus (CdtLoc) (215). Both the PaLoc (216) and CdtLoc (217, 218) were found in chromosomal locations when first identified. However, the chromosomal PaLoc is mobilizable, and can be transferred to a non-toxigenic recipient thereby converting it to a toxigenic strain, but neither a defined mobilizable or conjugative PaLoc element nor the mechanism of transfer have been determined (219).

Few studies have investigated plasmids in *C. difficile* and only a small number of native plasmids have been described or characterized to date. Unlike many other clostridial species, in which toxin genes are often encoded on extrachromosomal elements, as discussed earlier in this review, *C. difficile* toxins appeared to be encoded exclusively on the host chromosome (219, 220). However, a recent study using single-molecule real-time sequencing technology showed that the genome of a bacteriophage, designated phiSemix9P1, carries a complete functional binary toxin locus within its genome (221). The location of the CDT genes on this 56.6 kb bacteriophage suggests that a transduction-mediated mechanism may be involved in transferring these toxin genes across different *C. difficile* lineages. Of relevance to this review, the genome of phiSemix9P1 was found to encode a putative ParA-like protein and a gene for a protein that might play a role in the initiation of plasmid replication in addition to typical bacteriophage-encoded genes involved in DNA packaging, head and tail morphogenesis or host cell lysis, suggesting that this element may represent a bacteriophage-plasmid hybrid (221). Such hybrid elements have recently been identified in *C. difficile* (221), and the presence of these elements can sometimes modulate the virulence of strains by regulating the expression of the chromosomally located toxin genes (222). Genome sequencing studies are continuing to identify new and novel hybrids (223) which may impact phenotypes that influence strain virulence or lateral gene transfer processes, and therefore the evolution and dissemination of *C. difficile* variant strains. The ubiquity of mobile elements in *C. difficile* suggests that horizontal gene transfer plays an important role in the movement of DNA between strains, and the identification of plasmid-bacteriophage fusions may suggest that intra- and inter-species DNA exchange has occurred, probably within the diverse bacterial populations found in the gastrointestinal environment (213).

CONCLUSIONS

The importance of mobile genetic elements to toxin dissemination within the clostridia is clearly evident. These genera produce a large variety of protein toxins, including the three most potent toxins known to man: botulinum, tetanus and epsilon toxins. Many of these toxin genes are not only located on extrachromosomal elements, but also may be associated with mobile genetic elements that may facilitate their spread from one plasmid or bacteriophage to another. The carriage of these toxins on extrachromosomal elements greatly facilitates the spread of toxin genes either within a species or between species. No doubt the extent of the spread detected to date is limited only by our survey capabilities and the advent of next generation sequencing will facilitate the identification of an even greater range of elements that can facilitate the movement of the genes encoding these important, and sometimes devastating, toxins.

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