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Virulence Plasmids of Spore-Forming Bacteria

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ABSTRACT Plasmid-encoded virulence factors are important in the pathogenesis of diseases caused by spore-forming bacteria. Unlike many other bacteria, the most common virulence factors encoded by plasmids in *Clostridium* and *Bacillus* species are protein toxins. *Clostridium perfringens* causes several histotoxic and enterotoxin diseases in both humans and animals and produces a broad range of toxins, including many pore-forming toxins such as *C. perfringens* enterotoxin, epsilon-toxin, beta-toxin, and NetB. Genetic studies have led to the determination of the role of these toxins in disease pathogenesis. The genes for these toxins are generally carried on large conjugative plasmids that have common core replication, maintenance, and conjugation regions. There is considerable functional information available about the unique *tcp* conjugation locus carried by these plasmids, but less is known about plasmid maintenance. The latter is intriguing because many *C. perfringens* isolates stably maintain up to four different, but closely related, toxin plasmids. Toxin genes may also be plasmid-encoded in the neurotoxic clostridia. The tetanus toxin gene is located on a plasmid in *Clostridium tetani*, but the botulinum toxin genes may be chromosomal, plasmid-determined, or located on bacteriophages in *Clostridium botulinum*. In *Bacillus anthracis* it is well established that virulence is plasmid determined, with anthrax toxin genes located on pXO1 and capsule genes on a separate plasmid, pXO2. Orthologs of these plasmids are also found in other members of the *Bacillus cereus* group such as *B. cereus* and *Bacillus thuringiensis*. In *B. thuringiensis* these plasmids may carry genes encoding one or more insecticidal toxins.

INTRODUCTION

Spore-forming bacteria cause some of the most significant diseases of both humans and animals, including tetanus, botulism, gas gangrene, anthrax, and many different enteric or gastroenteritis syndromes. The pathogenesis of most of these diseases involves the production of potent protein toxins, including tetanus and botulinum toxins, anthrax toxin, and alpha-toxin, epsilon-toxin (ETX), and enterotoxin (CPE) from *Clostridium perfringens*. The genes for many of these toxins, as well as other virulence factors such as the capsule biosynthesis genes of *Bacillus anthracis*, are located on plasmids, with examples including the tetanus toxin plasmid, the conjugative toxin plasmids of *C. perfringens*, and the pXO1 and pXO2 virulence plasmids from *B. anthracis* (1). In this chapter

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we will review our knowledge of these plasmids, the virulence factors that they encode, and their role in disease.

TOXIN PLASMIDS OF *C. PERFRINGENS*

C. perfringens causes many histotoxic and gastrointestinal diseases in both humans and domestic livestock, and strains are divided into five toxinotypes (A to E) based on their ability to produce one or more of four typing toxins: alpha-toxin, beta-toxin (CPB), ETX, and iota-toxin (ITX) (2). This classification system is based functionally on the presence or absence of toxin plasmids, specifically plasmids encoding CPB (*cpb* gene), ETX (*etx*), or ITX (*iap* and *ibp*) (3). Most of these plasmids are very closely related, sharing core plasmid replication, maintenance, and conjugation regions (Fig. 1) (4). These regions are also shared by a group of almost identical conjugative tetracycline resistance plasmids typified by pCW3 (5). Accordingly, the nature of these common regions will be described before the various toxin plasmids are discussed.

Replication and Maintenance of Toxin Plasmids of *C. perfringens*

Determination of the first complete sequences of *C. perfringens* toxin plasmids, those of the CPE (*cpe* gene) encoding plasmids pCPF5603 and pCPF4969, did not reveal any obvious plasmid replication genes (6). However, functional studies of the sequenced conjugative tetracycline resistance plasmid, pCW3, led to the identification of the plasmid replication protein, Rep (5). The Rep protein is not related to any other plasmid replication proteins and, although disruption of the *rep* gene abolishes plasmid replication, the precise mode of action of Rep remains unclear (5). The *rep* gene subsequently has been shown to be conserved (at least 97% Rep amino acid sequence identity) in all plasmids related to pCW3, including pCPF5603 and pCPF4969, that have been sequenced to date (Fig. 1). Therefore, it is postulated that all members of this plasmid family replicate by the same Rep-dependent mechanism.

In all of these plasmids the gene immediately upstream of the *rep* gene has similarity to *parM* genes. The *parM* gene encodes an actin-like protein that, in conjunction with a downstream *parR* gene and an upstream *parC* centromere-like site, constitutes the plasmid partitioning system of several large, low-copy-number plasmids from Gram-negative bacteria (7). The ParMR system relies on the ability of the ParM protein to form

long ATP-dependent filaments, the ends of which bind to ParR adaptor proteins that are in turn bound to the centromere-like *parC* region located upstream of the *parM* gene (8). Filament extension subsequently results in the separation of similar plasmid molecules with the same *parCMR* locus to opposite ends of a ParM filament, and therefore to opposite ends of the cell, prior to cell division, resulting in stable plasmid inheritance (8). Potential *parR* genes have been annotated on several of the sequenced toxin plasmids, and on the other plasmids an unannotated *parR* gene can be identified downstream of *parM*. These data suggest that a ParMRC-like partitioning system is utilized by pCW3, the toxin plasmids, and other members of this plasmid family (5, 9, 10).

The other factor relevant to plasmid maintenance is the ability of many spore-forming bacteria to carry multiple plasmids within the same cell. In *C. perfringens*, many of these multiplasmid strains contain several plasmids that are closely related and encode the same replication and conjugation functions (4). For these very similar plasmids to be stably inherited, distinct plasmid maintenance systems need to be employed. The detailed study of one such multiplasmid strain, EHE-NE18, has demonstrated that three very closely related plasmids are able to coexist in this strain and that they are capable of independent transfer (9). It was postulated that allele differences that can be identified between *parC* and the ParM and ParR proteins encoded by these different toxin and resistance plasmids determine plasmid incompatibility. Therefore, plasmids with different *parCMR* alleles can be stably maintained in the same cell; plasmids with the same alleles are predicted to be incompatible (9, 10).

Subsequent analysis of published sequence data has led to the assignment of ParM sequences into one of four distinct groups (10), which have now been designated as the ParMRC clades A to D (J. Rood, V. Adams, and J. Prescott, unpublished). A dendrogram of the ParM proteins from all available *C. perfringens* plasmid sequences clearly indicates the distinct ParM_A, ParM_B, ParM_C, and ParM_D clades and leads to the identification of a fifth *C. perfringens*-derived group, designated here as the ParM_E clade (Fig. 2). Recently, another clostridial toxin plasmid has been shown to utilize a ParMR-like system for plasmid maintenance, the pE88 plasmid from *Clostridium tetani*, which encodes the tetanus toxin structural gene (11). This ParM protein is only distantly related to the *C. perfringens* ParM proteins and serves to anchor the phylogenetic tree (Fig. 2). There is also a ParM homologue present in *Clostridium botulinum* Eklund strain 17B, which contains a plasmid-encoded

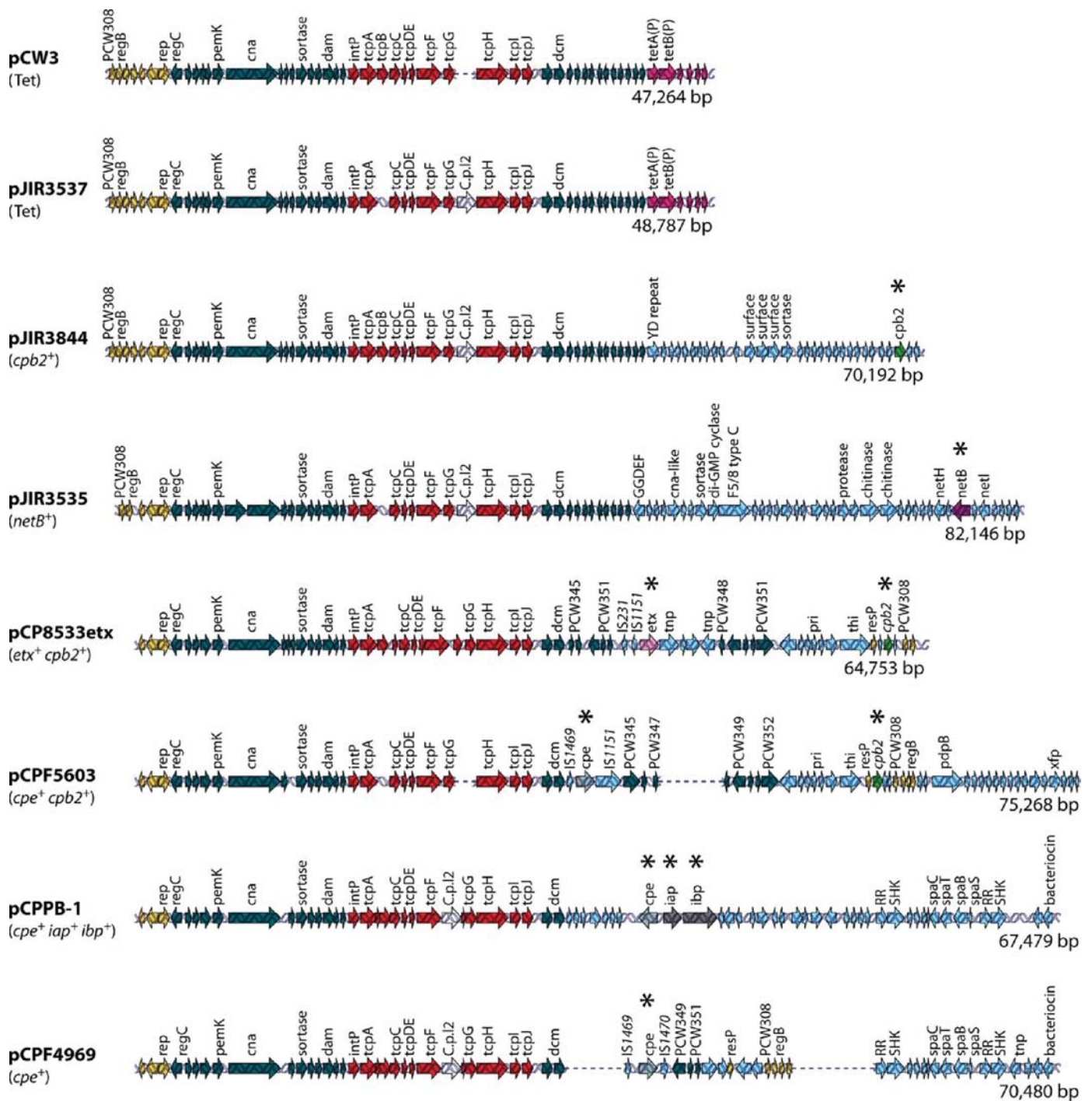


FIGURE 1 Comparative alignment of *C. perfringens* plasmids. Open reading frames (ORFs) are indicated by arrows as follows: red, the *tcp* locus; dark blue, other shared ORFs; light purple, tetracycline resistance genes; green, the *cpb2* toxin gene; purple, the *netB* toxin gene; pink, the *etx* gene; gray, the *cpe* gene; dark gray, the iota-toxin gene; yellow, plasmid replication region; light blue, regions unique to each plasmid. Asterisks denote a toxin gene. Reproduced with permission from reference 4. [doi:10.1128/microbiolspec.PLAS-0024-2014.f1](https://doi.org/10.1128/microbiolspec.PLAS-0024-2014.f1)

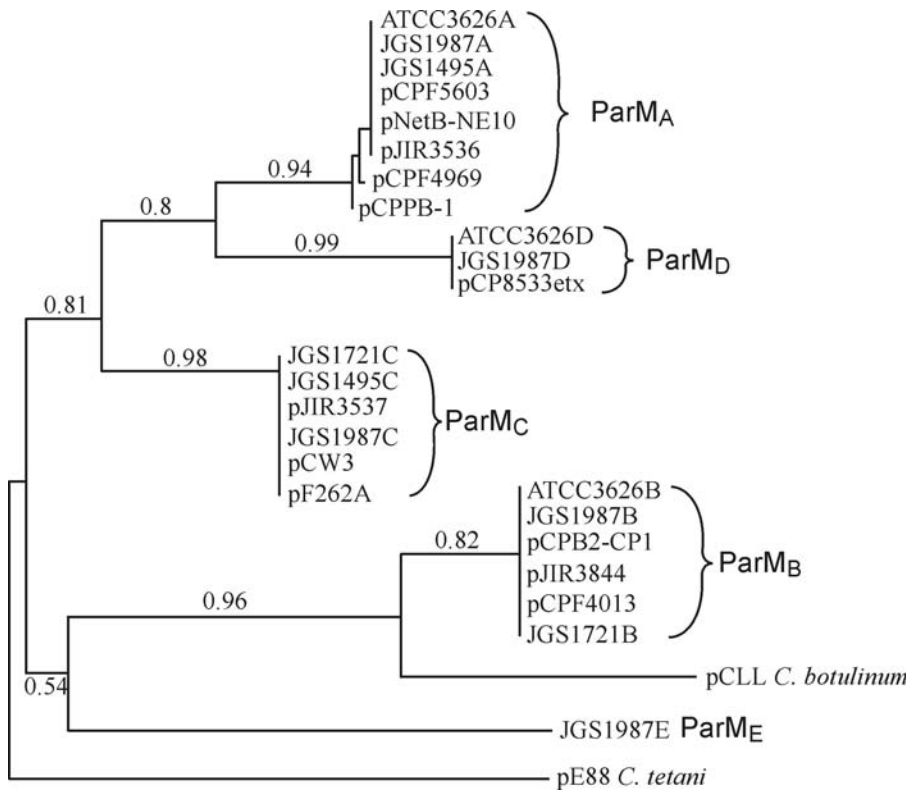


FIGURE 2 Phylogenetic analysis of ParM variants. The phylogenetic tree was constructed using the amino acid sequences of ParM proteins identified using BlastP searches of the nonredundant NCBI protein database. The phylogenetic tree was constructed using the phylogeny analysis software: http://www.phylogeny.fr/version2_cgi/index.cgi (171, 172). The JGS1495, JGS1987, and ATCC3626 sequences are from genome sequencing projects and yielded multiple ParM homologues from putative plasmid sequences; each ParM homologue was named according to its ParM group. [doi:10.1128/microbiolspec.PLAS-0024-2014.f2](https://doi.org/10.1128/microbiolspec.PLAS-0024-2014.f2)

neurotoxin locus (Fig. 3). This putative ParM protein has a much closer relationship to the ParM_B family of *C. perfringens* ParM proteins (Fig. 2). Overall, the data suggest that ParMR systems represent a highly conserved mechanism of plasmid maintenance, not just in the clostridia, but in many other bacterial genera (12).

Mechanism of Conjugative Transfer of Toxin Plasmids of *C. perfringens*

Conjugative transfer in *C. perfringens* was recognized first in experiments that involved the transfer of closely related tetracycline resistance plasmids (13, 14). Subsequently, the first toxin plasmid shown to transfer independently was a marked clinically derived CPE plasmid, pMRS4969 (15). Transfer of pMRS4969 resulted in transconjugants that were able to act as plasmid donors, highly suggestive that transfer was mediated by a conjugation-like mechanism encoded on the plasmid. Conjugative transfer has now been demonstrated for several toxin plasmids, including plasmids encoding ETX, NetB toxin, and CPB2, as well as a lincomycin resistance plasmid (9, 16, 17).

Early analysis of the conjugative plasmids from *C. perfringens* suggested that they shared large regions of similarity (15, 18, 19). Ten toxin and resistance plasmids

from *C. perfringens* have been completely sequenced, including known conjugative plasmids; the data confirmed that these plasmids share a highly conserved 35-kb region that includes the transfer of the clostridial plasmid (*tcp*) conjugation locus (Fig. 1) (4–6, 9, 10, 20, 21).

The *tcp* locus has been shown to mediate conjugative transfer of the 47-kb tetracycline resistance plasmid pCW3 (5). The locus originally was identified based on low levels of amino acid identity between several of the encoded Tcp proteins and conjugation proteins from the conjugative transposon Tn916. Significant genetic rearrangements between the conjugation regions from Tn916 and pCW3, as well as limited primary amino acid sequence similarity to Gram-negative systems, suggested that the mechanism of conjugation in *C. perfringens* was unique. Since this locus is conserved in most of the toxin plasmids, is present in all known conjugative *C. perfringens* plasmids, and appears to be responsible for the conjugative transfer of toxin genes, it is important to review what is currently known about the function of this conjugation system.

The *tcp* locus comprises an 11-gene operon (*intP*, *tcpA-tcpJ*), and mutagenesis studies have shown that several of the Tcp proteins encoded by this locus are involved in pCW3 transfer (5, 22–24). The genes in the

tcp locus are conserved in the toxin plasmids, except that in some plasmids the nonessential *tcpB* gene is missing and/or there may be insertions of small hypothetical open reading frames (ORFs) and group II introns (5, 6, 9, 16, 17). A model for the *C. perfringens* conjugation apparatus has been proposed based on functional studies of the Tcp proteins involved in the conjugative transfer of pCW3 (4, 25). Analysis of these Tcp proteins has identified some functional similarity to protein families that form the Gram-negative inner membrane secretion channel (26).

Coupling proteins are an essential component of conjugation systems and are involved in substrate recognition and translocation (27). Two proteins encoded on the *tcp* locus, TcpA and TcpB, were identified as possessing FtsK-like domains belonging to the FtsK superfamily of DNA translocases (23). TcpA was shown to be essential for transfer, while TcpB was not required, a result consistent with the absence of the *tcpB* gene in some of the toxin plasmids. Bioinformatic analysis of the TcpA protein identified two N-terminal transmembrane domains (TMDs) and three ATP-binding and hydrolysis motifs in the C-terminal cytoplasmic domain. Supporting the hypothesis that TcpA is an ATPase-dependent DNA translocase, the Walker A, Walker B, and RAAG ATP-binding motifs were shown to be essential for TcpA function (23). Similar to other coupling proteins, the TMDs of TcpA were shown to be required for wild-type TcpA function and to be involved in TcpA homo-oligomerization as well as protein-protein interactions between TcpA and TcpC, and TcpH and TcpG (25). Deletion of 61 amino acids from the C-terminus of TcpA reduced TcpA-TcpC interactions and transfer efficiency, providing evidence that this interaction was important for the transfer process (25). Although TcpA was not identified as a classical coupling protein, these studies provide convincing evidence that TcpA is the DNA translocase of the *C. perfringens* conjugation system.

One of the first proteins shown to be essential for conjugation was TcpH, with transfer not detected in *tcpH* mutants (5). Predicted to be an integral membrane protein, this 832-amino acid protein contains 8 TMDs and a large cytoplasmic domain. TcpH has a VirB6 domain between TMD5-TMD8 and was therefore proposed to have a similar role as the VirB6 protein, which forms part of the central channel in the inner membrane of the Gram-negative conjugation apparatus (28). In agreement with a proposed role for TcpH as the major component of the conjugation apparatus in the donor cell wall, TcpH was shown to localize to the cell membrane fraction at the poles of *C. perfringens* cells (29).

The first 581 amino acids of TcpH, which include the TMDs and VirB6 domain, comprised the minimal TcpH derivative required to complement a *tcpH* mutant. This region was also essential for TcpH homo-oligomerization and interactions with TcpA and TcpC (25, 29). TcpH interactions were not dependent on the VirB6 domain or the conserved $_{242}VQQPW_{246}$ motif, also identified as being essential for TcpH function (29). The unique C-terminal cytoplasmic domain was required for wild-type TcpH function but was not involved in TcpH protein-protein interactions. This cytoplasmic domain is not present in other VirB6-like conjugation proteins, demonstrating the functional complexity of TcpH and the unique nature of the *C. perfringens* conjugation system.

TcpC was originally identified as a bitopic membrane protein with 24% amino acid identity to ORF13 from Tn916 (5). A very significant reduction in transfer efficiency was observed in a *tcpC* mutant, indicating that TcpC plays a key role in conjugative transfer (24). Determination of the crystal structure of the soluble TcpC₉₉₋₃₅₉ derivative to 1.8 Å identified two domains that each unexpectedly shared a similar fold to members of the nuclear transport factor-2 superfamily, which includes VirB8 proteins that form part of the transfer apparatus at the inner membrane in Gram-negative conjugation systems. VirB8-like proteins act as scaffolding proteins promoting transfer complex assembly and stabilization; TcpC is therefore proposed to have a similar role in *C. perfringens*. Consistent with this role, TcpC was shown to localize independently to the cell wall of *C. perfringens* cells as well as interacting with itself, TcpA, TcpH, and TcpG, with all of these interactions requiring the presence of the essential N-terminal TMD. TcpC forms a trimeric structure with the internal central domain shown to be involved in TcpC self-interaction (24). The essential C-terminal domain forms the major exterior surface of the trimer and is required for TcpC interactions with TcpA, TcpH, and TcpG. It is through these protein-protein interactions that TcpC is postulated to direct the formation of the transfer apparatus.

TcpF was identified as a putative ATPase with distant similarity to the VirB4 ATPase of Gram-negative conjugation proteins (29). Like ATPases from other conjugation systems, TcpF was shown to be essential for conjugative transfer (5). Although TcpF was demonstrated to colocalize with TcpH at the poles of *C. perfringens* donor cells, it remains unclear how TcpF interacts with the conjugation apparatus, with no protein-protein interactions identified between TcpF and the other Tcp proteins (29).

Peptidoglycan hydrolases are another family of proteins commonly present in conjugation systems, playing a role in the assembly of the conjugation apparatus in the cell wall (22). Two genes encoding potential peptidoglycan hydrolases were identified in the *tcp* locus, *tcpG* and *tcpI* (5). Genetic studies showed that the *tcpI* gene product was not required for conjugative transfer (22). Mutagenesis of the *tcpG* gene resulted in a reduced transfer frequency, consistent with results for peptidoglycan hydrolases from other conjugation systems. The role of TcpG as a peptidoglycan hydrolase was confirmed by the demonstration that purified TcpG had hydrolase-like activity on cognate *C. perfringens* peptidoglycan (22).

In the model of the *C. perfringens* conjugation apparatus (4, 25) the integral membrane protein TcpH, with the atypical VirB8-like protein TcpC acting as a scaffold, forms the core channel of the conjugation apparatus. As well as stabilizing the complex, TcpC is proposed to be involved in recruiting TcpA, TcpH, and TcpG (24). The putative motor ATPases TcpA and TcpF are predicted to energize the system, although it is currently unknown how TcpF interacts with the other components (25, 29). Similarly, the early steps in plasmid transfer are not clear since neither a classical relaxase protein gene nor an origin of transfer (*oriT*) have been identified on these plasmids (5). The relaxase protein and *oriT* site are usually essential components that are involved in plasmid recognition and nicking prior to transfer (30). The first gene in the *tcp* locus, *intP*, which is conserved in the toxin plasmids, encodes a putative tyrosine recombinase that is postulated to be a potentially novel relaxase (5). Genetic and functional studies are required to determine whether IntP, as well as the remaining highly conserved proteins TcpD, TcpE, and TcpJ, are involved in *C. perfringens* conjugative transfer. These studies will further define our understanding of the unique conjugation process in

C. perfringens, a process that is central to the transfer of both antibiotic resistance and toxin genes.

Enterotoxin (CPE) Plasmids of *C. perfringens*

C. perfringens type A strains that produce *C. perfringens* CPE are causative agents of human food poisoning and non-food-borne diarrhea (31). CPE is encoded by the *cpe* gene and is only produced during sporulation, where its synthesis is controlled by Spo0A and the alternative sigma factors SigF, SigE, and SigK (32–34). The toxin then accumulates intracellularly until it is released when the mother cell lyses to free the mature endospore at the completion of sporulation. CPE then binds to claudin receptors present in the gastrointestinal tract, which induces toxin oligomerization and pore formation in enterocytes. The resultant Ca²⁺ influx causes cytotoxicity that leads to villus blunting, necrosis, and epithelial desquamation in all sections of the small intestines, along with luminal fluid accumulation (31).

CPE, a 35-kDa polypeptide, has a highly conserved amino acid sequence in all CPE-producing *C. perfringens* strains, with the exception of type E strains. Structurally, this toxin belongs to the aerolysin family of pore-forming toxins, which also includes ETX (35, 36). The CPE protein consists of a C-terminal domain that confers binding to claudin receptors and an N-terminal domain that mediates both the oligomerization of toxin monomers and membrane insertion during pore formation (37–39).

While some *C. perfringens* type A, C, D, and E isolates produce CPE, no CPE-producing type B strains have been identified (20, 21, 40–44). In the 1 to 5% of global type A strains that are *cpe*-positive (45), the *cpe* gene is located either on the chromosome or on large plasmids that range in size from ~50 to ~75 kb (6). Virulence studies testing molecular Koch's postulates have confirmed the importance of CPE expression for the gastrointestinal virulence of both chromosomal and plasmid *cpe* type A strains (46).

FIGURE 3 Plasmid maps of toxin plasmids from spore-forming bacteria. The circular maps of the *B. anthracis* toxin plasmids, pXO1 (AF065404) and pXO2 (AF188935), the *C. tetani* neurotoxin plasmid pE88 (AF528077), and the conjugative group I (pCLJ, CP001081) and group II (pCLL, CP001057) *C. botulinum* neurotoxin plasmids are shown. Predicted ORFs are depicted as black arrows or bars along the circular maps. Regions of interest are indicated inside the plasmid circles, such as the pathogenicity islands present on pXO1 and pXO2. Genes of interest are indicated on the outside of the plasmid maps. Gene names are italicized, while ORFs with similarity to known proteins (such as IS elements) are not italicized. The two neurotoxin loci encoded on the pCLJ plasmid are enlarged showing an example of an *orfX* neurotoxin locus (*bontA* region, top) and a *ha* neurotoxin locus (*bontB* region, bottom); see text. [doi:10.1128/microbiolspec.PLAS-0024-2014.f3](https://doi.org/10.1128/microbiolspec.PLAS-0024-2014.f3)

Type A strains with a chromosomal *cpe* gene are estimated to cause ~75 to 80% of all *C. perfringens* type A food poisoning cases (31). This illness is now considered the second most common bacterial food-borne disease in the United States, where it involves one million cases per year (31, 47). The remaining cases of *C. perfringens* type A food poisoning are caused by strains carrying a *cpe* plasmid. Type A strains with a *cpe* plasmid are also responsible for 5 to 10% of all cases of human non-food-borne gastrointestinal diseases, which include both antibiotic-associated and sporadic diarrheas (31, 48). These diseases occur more frequently in the elderly and are more severe and long-lasting than typical type A food poisoning (49). Type A strains carrying a *cpe* plasmid also reportedly cause enteric disease in domestic animals, particularly dogs (50).

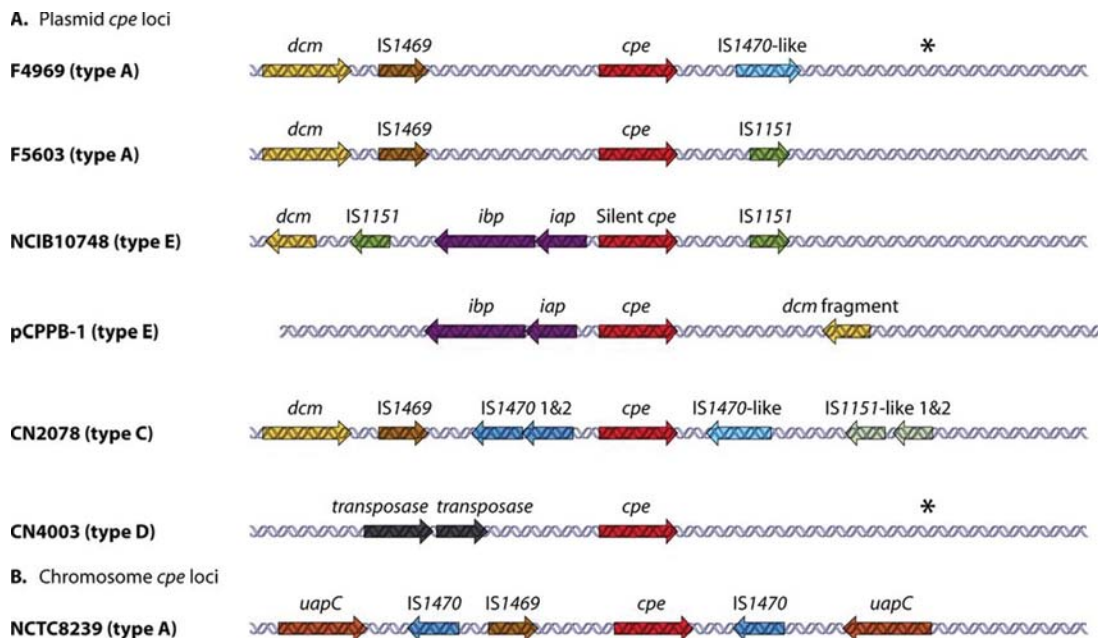
Based upon results from sequencing, pulse-field Southern blot analyses, and overlapping PCR assays, it has been shown that there are two major families of *cpe* plasmids in type A strains (4). The pCPF5603 *cpe* plasmid family includes plasmids that are usually ~75 kb in size and carry genes encoding both CPE and CPB2 (encoded by the *cpb2* gene) (6) (Fig. 1). In contrast, the ~70-kb pCPF4969 *cpe* plasmid family has the *cpe* gene, but not the *cpb2* gene. Both *cpe* plasmid families share an ~35-kb conserved region (6) that contains the *tcp* region, which mediates the

conjugative transfer of several *C. perfringens* plasmids (5). The presence of this *tcp* region explains the conjugative transfer of a tagged F4969 plasmid derivative (15). The variable region of pCPF5603-like plasmids contains a cluster of metabolic genes in addition to a functional *cpb2* gene. In contrast, the pCPF4969 variable region includes two putative bacteriocin genes and genes encoding a putative two-component regulator with similarity to VirS and VirR from *C. perfringens* (6).

Whether chromosomal or plasmid-borne, the *cpe* gene is closely associated with insertion sequences (IS). However, there is considerable variation among type A strains regarding the specific IS elements present in the *cpe* locus, as well as their arrangement relative to the *cpe* gene (Fig. 4). In the pCPF5603-like plasmids, the *cpe* gene is bounded by an upstream IS1469 sequence and a downstream IS1151 sequence, while in pCPF4969-like plasmids; the *cpe* gene is upstream of an IS1470-like sequence rather than IS1151 (6). By comparison, the chromosomal *cpe* locus has an IS1469 immediately upstream of the *cpe* gene (51). This chromosomal IS1469-*cpe* region is then flanked by IS1470 sequences, suggesting it could be part of an integrated transposon (51).

C. perfringens type C Darmbrand strains produce CPE, as well as CPB, and are genetically related to type A food poisoning strains carrying a chromosomal *cpe* gene

FIGURE 4 Genetic organization of the *cpe* gene regions. Organization of plasmid-borne (A) and (B) chromosomal *cpe* loci from *C. perfringens*. *cpe* genes are indicated by red arrows, *iap* and *ibp* genes by purple arrows, and *dcm* genes by yellow arrows. Related IS elements are indicated by identical colors. Reproduced with permission from reference 4. doi:10.1128/microbiolspec.PLAS-0024-2014.f4



(52). However, these type C strains carry a plasmid-borne *cpe* gene, as well as a plasmid-borne *cpb* gene located on ~75- or ~85-kb plasmids. In other Darmbrand strains the *cpe* gene is present on an ~110-kb plasmid that does not carry the *cpb* gene. The *cpe* plasmids in all studied CPE-positive type C strains have the *tcp* region, suggesting that they are conjugative. Interestingly, the plasmid *cpe* locus in type C strains is similar to the chromosomal *cpe* locus of type A isolates, except that an IS1469 sequence is located upstream of IS1470, while an IS1151-like sequence is located downstream of the *cpe* gene (52) (Fig. 4). Experiments have indicated that IS mobile genetic elements can excise the *cpe* gene as circular DNA molecules that could be transposition intermediates (52). Those results suggest, but do not yet prove, that the chromosomal *cpe* locus in type A strains may be derived from insertion of a *cpe*-carrying transposon originating from a *cpe* plasmid similar to the *cpe* plasmid found in type C Darmbrand strains.

Many type D isolates also carry a *cpe* gene on large plasmids that range in size from 75 to 110 kb (40). In some of these strains, the *cpe* gene is located on the same plasmid as the *etx* gene, but other CPE-positive type D strains carry separate *cpe* and *etx* plasmids (40). Like *cpe* plasmids in type A and C strains, most *cpe* plasmids of type D isolates have a *tcp* region, suggesting they are conjugative (53). However, in *cpe*-positive type D strains, the *cpe* locus has a unique genetic organization differing from the *cpe* locus of *cpe*-positive type A or type C strains (53). While the region downstream of the *cpe* gene in type D strains is similar to the sequences downstream of the pCPF4969 *cpe* gene, except for the absence of an IS1470-like sequence, two copies of an ORF sharing 67% identity with a Tn1456-like transposase gene are located upstream of the *cpe* gene in the type D strains that have been studied (Fig. 4). All type E isolates studied to date were shown to carry *cpe* sequences on large plasmids that also encode ITX (21, 54), which will be discussed in the section describing the ITX plasmids.

Beta Toxin (CPB) Plasmids of *C. perfringens*

CPB is encoded on large plasmids in both toxinotype B and C isolates (41, 52, 55). The CPB-encoding toxinotypes are responsible for a range of highly significant and often fatal animal diseases. In addition, type C isolates are the only non-type A strains known to cause disease in humans.

Type C-mediated human disease affects undernourished individuals existing on a protein-deficient diet and

is often accentuated by the consumption of foods rich in trypsin inhibitors (56). It is known as Darmbrand in Germany (52) and pig bel in Papua New Guinea (56). Symptoms include severe upper abdominal pain, bloody diarrhea, and vomiting (57). As the disease progresses, diarrhea ceases and the bowel becomes obstructed due to damage in the small intestine (56, 57). Enterotoxemia and necrotizing enteritis of livestock are also caused by CPB-producing strains of *C. perfringens* (58). Type C disease occurs mostly in neonates of several animal species. This age predisposition is believed to be related to the presence of colostrum, which inhibits trypsin activity and in turn allows CPB to remain active in the intestinal lumen. Animal diseases attributed to type B isolates resemble both type C- and/or type D-mediated enteric and/or neurologic syndromes (58). Like type C diseases of livestock, type B infections can involve necrotic enteritis or sudden death, but animals with type B infections may display neurological signs similar to type D disease (59).

Mutational studies combined with the use of a rabbit small intestinal loop model have shown that CPB is essential for the intestinal pathology observed in type C-mediated disease (60). In addition, these type C mutants were used to identify a key role for CPB in lethal enterotoxemia in a mouse model (61), which was confirmed in a large animal model (goat) more recently (62). When cultures of a wild-type strain, a *cpb* null mutant, and a genetically reversed mutant, were introduced into the goat small intestine, disease progression for the wild type and the complemented mutant occurred in a manner indistinguishable from the natural disease. By contrast, goats inoculated with the *cpb* mutant showed no sign of disease (62). These studies (60, 62) provide compelling evidence that plasmid-encoded CPB is the most important toxin in type C-mediated disease.

The pathology of type B infection is complicated, as type B isolates typically express the largest number of different lethal toxins: alpha-toxin, CPB, and ETX (41, 58, 59). The only virulence study of type B isolates to date (59) correlated toxin levels in culture supernatants with mouse intravenous lethality. The results indicated that (i) CPB is a major contributor to lethality, (ii) ETX is also implicated, and (iii) other toxins may also be involved. The complexity of disease progression when caused by type B isolates is likely dependent on a number of factors including the protease status of the host and the toxin expression profile of the infecting isolate (59). Such studies are complicated by the fact that while CPB is highly sensitive to trypsin, ETX requires trypsin for activation.

CPB is a 35-kDa pore-forming toxin with about 22 to 28% amino acid sequence similarity to several pore-forming toxins of *Staphylococcus aureus* (63), 38% amino acid sequence identity to *C. perfringens* NetB toxin (64), and 43% amino acid sequence identity with delta-toxin of *C. perfringens* (65). CPB forms an oligomer after binding to an unidentified receptor on sensitive cells, which results in pore formation that produces an ~12-Å channel that is selective for monovalent cations (66). This toxin is very sensitive to degradation by trypsin or chymotrypsin (67), and intestinal trypsin is therefore the main natural defense against type C infections.

All type B and C isolates produce CPB toxin during late log-phase growth. Beta toxin production by type C strains is controlled by the chromosomal two-component VirS/VirR regulatory system (68). CPB production is also regulated by a chromosomal Agr-like quorum sensing (QS) system in both the type C strain CN3685 and the type B strains CN1793 and CN1795 (69, 70). CPB production increases when the bacteria closely contact enterocyte-like Caco-2 cells, and this effect requires both the VirS/VirR and Agr-like QS systems (68, 69). Furthermore, both the VirS/VirR and Agr-like QS systems are essential for CN3685 to cause either necrotic enteritis or lethal enterotoxemia in animal models because these regulatory systems are required for *in vivo* CPB production (68, 69).

In all type B and C strains studied to date, the *cpb* genes localized on large plasmids (41, 55). Southern hybridization of pulsed-field gels showed that the *cpb* gene in most type B strains was located on ~90-kb plasmids, although a few type B strains carried an ~65-kb *cpb* plasmid (41). These studies also showed that the *cpb* plasmid in these type B strains is distinct from their *etx* plasmid, although a few type B strains possess *cpb* and *etx* plasmids of the same (65 kb) size, just to make the picture more confusing (41). While type B strains carry only 65-kb or 90-kb *cpb* plasmids, the *cpb* plasmids in type C strains display more diversity, ranging in size from ~65 to ~110 kb. The ~65-kb and 90-kb *cpb* plasmids present in these type C strains are very similar to the plasmids of matching size found in type B isolates (55). Sequencing and overlapping PCR analysis revealed that in these 65-kb and 90-kb *cpb* plasmids, a *tpeL* gene is located ~3 kb downstream from their *cpb* gene. This gene encodes a large clostridial glycosylating toxin, TpeL, that is related to the large glucosylating toxins from *Clostridium difficile* (71). This *cpb*- and *tpeL*-containing locus is associated with IS1151 sequences that can excise to form circular molecules that may be transposition intermediates (41, 55).

Type C strains can carry other potential virulence plasmids (55). Type C strains carry either plasmid-borne *cpe* or *tpeL* genes, but never both genes. Some type C strains have ~65- to ~90-kb plasmids that carry *cpb2* genes (72), but these *cpb2* plasmids never carry the *cpb* gene (55).

Almost without exception, all CPB-encoding plasmids appear to encode the *tcp* conjugation locus (41, 55). Since there have been no reports of a *tcp* locus-containing plasmid that is nonconjugative, it is highly likely that all *cpb* plasmids are conjugative. It is postulated that type B strains most likely are derived from a conjugative transfer event from a type C to a type D strain (or vice versa) (55), but more detailed genomic studies are required to validate this hypothesis.

Epsilon-Toxin (ETX) Plasmids of *C. perfringens*

ETX is produced by *C. perfringens* type B and type D strains and is expressed as an inactive prototoxin that is activated by cleavage with proteases such as trypsin (73). Livestock diseases caused by *C. perfringens* type B isolates include both enterotoxemias with neurological clinical signs and lesions and necrotic enteritis (59). *C. perfringens* type D strains are the most common cause of enterotoxemia in sheep and goats (74), and genetic studies recently have shown that this syndrome is mediated by ETX (75). The disease has been reported, albeit rarely, in cattle. Type D disease in sheep, goats, and cattle is characterized by neurological and respiratory clinical signs and lesions. Enterocolitis with severe diarrhea also occurs in goats.

ETX is a neurotoxin that is able to be absorbed from the gastrointestinal tract, where it is produced, to spread systemically through the circulatory system, where the toxin mostly affects the heart, lungs, kidneys, and brain (74). It is able to bind to vascular endothelial cells and cause cell lysis, leading to increased vascular permeability that causes brain and pulmonary edema. The latter eventually leads to degeneration and necrosis of surrounding tissues. In addition, ETX is also able to cross the blood-brain barrier and target neuronal cells directly. The neurological signs of type B and D disease are believed to be a consequence of the brain damage produced by ETX acting on the vasculature and on neurons (74, 75).

Regulation of ETX production in a type D isolate is controlled by an *agr*-like QS system (76) and the global virulence regulator CodY (77). How these two regulatory systems interact is not known, but CodY is also responsible for the upregulation of adherence to Caco2 cells *in vitro* as well as regulating spore production

under nutrient-rich conditions. By contrast, regulation studies in two type B isolates have indicated that ETX is not regulated by the *agr*-like QS system in these strains, unlike other toxins (70). The role of CodY in type B strains is not known.

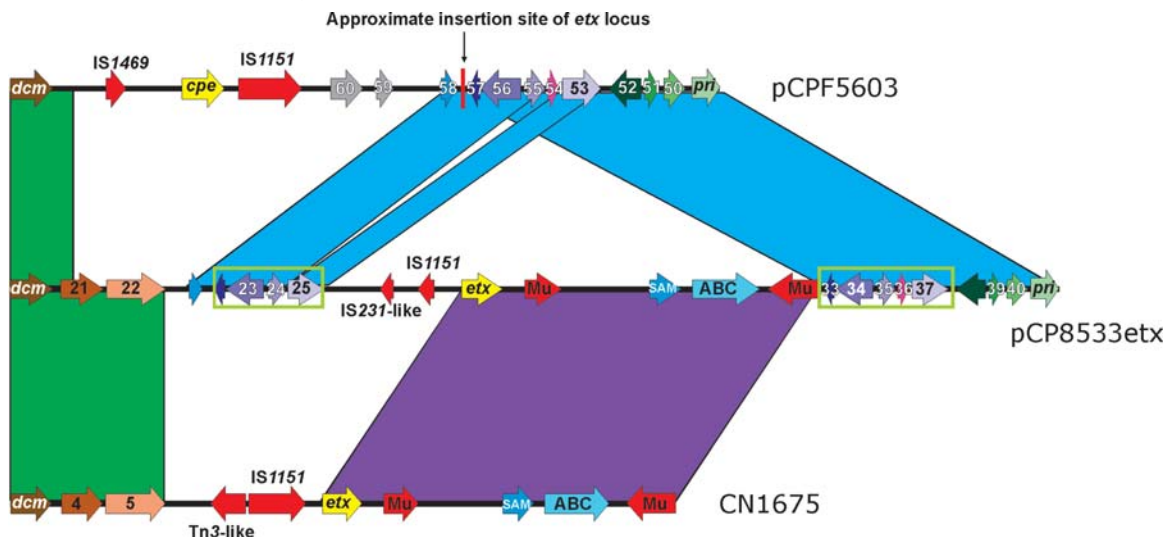
In all strains examined to date the *etx* gene is located on a large plasmid (4, 40, 41). In type B strains the *cpb* and *etx* plasmids show limited plasmid variation, and no strains have been identified to date that encode both toxins on the same plasmid (20, 41). The *cpb* gene, as discussed earlier, is encoded on either a 65-kb plasmid or, more commonly, on a 90-kb plasmid in these strains (41). There is no discernable variation in the 65-kb *etx* plasmids of type B isolates; these plasmids also carry the *cpb2* gene (20).

The only complete sequence available for an *etx* plasmid is from the type B isolate NCTC8533. This plasmid, pCP8533etx (64,753 bp), is closely related at the sequence level to the pCPF5603 family of CPE-encoding plasmids, although it lacks the *cpe* locus and another large region that encodes putative metabolic genes that are more commonly found on the chromosome (20). Instead of the *cpe* locus, pCP8533etx carries an *etx* locus that includes a Mu-like element down-

stream of the *etx* gene and IS1151- and IS231-like elements directly upstream of the *etx* gene (Fig. 5). This *etx* locus is largely conserved in all type B plasmids and some type D *etx* plasmids (20). The *etx* gene is located approximately 9 kb downstream of the *dcm* gene in these isolates, and there appears to have been an IS element-mediated duplication of several genes located downstream of the *cpe* locus in pCPF5603. These genes now flank the *etx* locus in pCP8533etx (Fig. 5). Another variant of the *etx* locus, represented by a plasmid in strain CN1675, has been identified in type D isolates that do not carry the *cpe* or *cpb2* genes. These isolates share the same downstream sequences as the pCP8533etx-like loci, but the upstream sequence is significantly different, encoding an IS1151 sequence and a Tn3 family transposase upstream of the *etx* gene. In addition, the *etx* locus is located closer to the *dcm* gene in these strains (20) (Fig. 5).

Type D *etx* plasmids show a marked degree of plasmid diversity, in direct contrast to the situation in type B isolates (20, 40). It is clear that a minority of surveyed type D strains contain a type B-like 65-kb *etx* plasmid that also carries the *cpb2* gene (20). Type D isolates that lack the *cpe* and *cpb2* genes mostly contain ~48-kb

FIGURE 5 Comparative alignment of *etx* loci. The two *etx* (yellow arrows) loci from plasmid pCP8533etx and strain CN1675 are compared to the location of the *cpe* (yellow arrow) gene and surrounding sequences in plasmid pCPF5603. The aligned region begins with the *dcm* gene (left side) found downstream of the *tcp* locus in all sequenced plasmids. Genes (or DNA sequences) with greater than 90% nucleotide identity are colored alike (except for the *cpe* and *etx* genes; both are in yellow but are not related). The genes inside the green boxes appear to have been duplicated and flank the *etx* locus in plasmid pCP8533etx. Numbers are the CDS designations from the respective sequences: pCPF5603 (accession number: NC_007773), pCP8533etx (accession number: NC_011412), and CN1675 (accession number: EU852100). Arrows without numbers represent pseudogenes. doi:10.1128/microbiolspec.PLAS-0024-2014.f5



plasmids (although a few contain 75-kb plasmids) that are likely to be conjugative since they appear to carry a *tcp* locus (40). Most of these strains contain a CN1675-like *etx* locus (20). Type D isolates that encode the *etx*, *cpe*, and/or *cpb2* gene(s) are larger, ranging in size from ~65 to 110 kb. In one type D isolate, carrying all three of these toxin genes, each toxin gene is located on a separate plasmid, while other isolates encode all three genes on the one plasmid (40).

Conjugative transfer of *etx* plasmids has been demonstrated in two type D isolates, CN1020 and CN3718 (16); these strains do not encode *cpe* or *cpb2* genes and contain CN1675-like *etx* loci (20, 40). Transfer was initially demonstrated using marked plasmids, where the *etx* gene was deleted and replaced with a chloramphenicol resistance gene (16). Transfer frequencies from both primary matings (a mating between two unrelated strains) and secondary matings (a mating between two genetically distinct but isogenic strains) were very high, ranging from 1.5×10^{-1} transconjugants/donor cell. Due to the very high frequency of transfer, a wild-type *etx* plasmid was subsequently transferred to a *C. perfringens* type A laboratory strain without any genetic selection (16). Plasmid transfer was detected at a frequency of 0.8% and effectively converted a toxinotype A strain to a toxinotype D strain. These results demonstrated for the first time that one of the toxins that is used to determine the toxinotype of an isolate was mobile and therefore that strains that do not contain the *etx* gene may be able to acquire it laterally (and at high frequency) from a coresident type D isolate perhaps encountered within the milieu of the gastrointestinal tract.

Iota-Toxin (ITX) Plasmids of *C. perfringens*

C. perfringens type E isolates produce ITX and have been associated with enteric disease in rabbits, sheep, and cattle, although conclusive evidence of the role of type E strains and ITX in the pathogenesis of animal disease is lacking. Type E strains are the only *C. perfringens* isolates that produce ITX, a member of the clostridial binary toxin family (4, 78–80). This toxin family also includes *C. botulinum* C2 toxin, as well as the *Clostridium spiroforme* and *C. difficile* binary toxins. Consistent with its designation as a binary toxin, ITX is comprised of two polypeptides, IA and IB, that are encoded by the *iap* and *ibp* genes, respectively. IA and IB are secreted from *C. perfringens* as proproteins and must be proteolytically activated by removal of their N-terminal regions by either host proteases or *C. perfringens* lambda-toxin (78–80).

IB, the receptor-binding component of ITX (80), interacts with the host lipolysis-stimulated lipoprotein (81). Lipolysis-stimulated lipoprotein is also a receptor for *C. difficile* and *C. spiroforme* binary toxins, although it is not a C2 toxin receptor (82). The IA component interacts with cell-bound IB, promoting the endocytosis of ITX. IA is transported through the endocytic vacuole, presumably via a heptameric pore formed by IB, and once in the cytoplasm it ADP-ribosylates actin at Arg177, which depolymerizes actin and collapses the cytoskeleton of host cells (78–80). The multifunctional mammalian surface protein CD44 also increases ITX binding, perhaps by acting as a second receptor or a coreceptor (83).

The *iap* and *ibp* genes comprise an operon that in “classical” type E strains is located on the largest (~100 to 135 kb) known toxin plasmids found in *C. perfringens* (54, 84). These ITX plasmids generally have a pCPF5603-like backbone, plus additional genes that encode several other potential virulence factors, including lambda-toxin and urease. In these classical type E strains, IS1151 sequences are closely associated with the *iap/ibp* genes, where they may be involved in their movement by a transposition process. These toxin genes can be detected on small circular DNA molecules that could represent transposition intermediates (84). On this basis, it has been suggested that the ITX plasmids in classical type E strains originated from the integration of an IS1151-based, *iap/ibp*-carrying mobile genetic element into an F5603-like *cpe* plasmid in a *C. perfringens* type A strain (54). This putative integration event apparently occurred near the promoter region of a *cpe* gene (Fig. 4) (84) and thereby inactivated *cpe* expression, such that these strains produce ITX but not CPE. Subsequent to this silencing of the *cpe* gene by promoter inactivation, the silent *cpe* genes have accumulated additional frame-shift and nonsense mutations, converting them to pseudogenes (54).

C. perfringens type E strains have traditionally been viewed as the least important of the *C. perfringens* types with respect to causing human or animal disease. However, the disease potential of type E strains may require some reevaluation given a recent study reporting that the standard *C. perfringens* multiplex PCR toxinotyping assay incorrectly classifies some “nonclassical” type E strains as type A due to variations in their *iap/ibp* gene sequences (21). Since those variant ITX genes are expressed, some strains classified by PCR as type A may actually be type E strains.

The ITX-encoding pCPPB-1 plasmid from one nonclassical type E strain has been sequenced (21). The

analysis of this sequence revealed four fundamental differences between pCPPB-1 and the classical ITX plasmids: (i) pCPPB-1 is smaller (~65 kb), (ii) the backbone of pCPPB-1 is similar to that of pCPF4969, in contrast to the pCPF5603-like backbone of classical ITX plasmids, (iii) pCPPB-1 does not encode lambda-toxin or urease, and (iv) an IS1151 element carrying the *iap/ibp* genes has apparently inserted near the most upstream of three *cpe* promoters in pCPPB-1; however, this *cpe* gene is still expressed, presumably from the other two more downstream *cpe* promoters. Interestingly, the CPE encoded by pCPPB-1 is a variant compared to the CPE produced by other CPE-positive strains. A *tcp* locus is present on both the pCPPB-1-like ITX plasmid and the classical ITX plasmids, strongly suggesting that all of these plasmids are conjugative (21, 84). However, conjugative transfer has not yet been experimentally demonstrated.

NetB Plasmids of *C. perfringens*

NetB, a plasmid-encoded 33-kDa β -barrel pore-forming toxin, is a major virulence factor in *C. perfringens*-mediated necrotic enteritis of poultry (85). This disease is manifested in two forms: as a frank clinical disease characterized by a sudden increase in flock mortality and as a milder subclinical form characterized by chronic damage to the intestinal mucosa, leading to poor digestion and nutrient absorption and resulting in a loss of bird performance. The subclinical form is of greatest consequence to the industry because of its wider prevalence and the difficulty of detection and amelioration before economic damage occurs. Treatment and control measures are estimated to cost the global poultry industry \$2 billion per annum (86).

Structural studies of the secreted soluble monomeric form (87) and the heptameric pore form (88) of NetB have revealed that the conformation of the membrane-binding domain of NetB is significantly different from other beta-barrel pore-forming toxins such as the alpha-hemolysin of *S. aureus*, indicating that there are likely to be differences in the way these toxins interact with cell membranes. There are clear differences in the toxic activity of NetB toward different cell types; among a number of cultured cell lines, only chicken LMH cells were susceptible (85), and avian red blood cells were much more susceptible than mammalian red blood cells (87). NetB interacts directly with cholesterol (88), and the pores demonstrate a high single-channel conductance and have a preference for cations (87). Mutagenesis studies have shown that key residues in structural elements, such as those required for oligomerization and membrane binding, reduce toxic activity (87, 88).

Several recent studies have shown that NetB can be effectively used as an antigen to induce a protective immune response in vaccinated birds (89–92).

Strain surveys have shown that *netB* is restricted to poultry-derived isolates of *C. perfringens* (93), apart from a single example of a *netB*-carrying strain isolated from cattle (94). All *C. perfringens* isolates that have been rigorously proven to cause necrotic enteritis in experimental infections carry the *netB* gene (85, 93, 95). Some *C. perfringens* strains isolated from clinical cases of necrotic enteritis lack the *netB* gene; however, none of these strains can reproducibly induce disease in experimental infection models (93, 95). We postulate that these disease-derived strains lacking the *netB* gene may have lost the plasmid carrying the gene during the initial strain isolation from clinical samples. In all isolates for which information is available the *netB* gene is carried on a large plasmid.

In the extensively studied strain, EHE-NE18, the *netB* plasmid pJIR3535 is one of three large conjugative plasmids (9). Each of these plasmids has been sequenced, as have two plasmids from a Canadian necrotic enteritis isolate (10), and all have very similar conjugation loci and plasmid replication and maintenance regions, as already discussed (Figs. 1 and 2). The plasmids are differentiated by the other genes they encode: pJIR3535 carries *netB* and a series of other genes that could potentially have a role in virulence, pJIR3844 carries a *cpb2* gene encoding an atypical form of CPB2 plus genes that encode putative surface proteins of unknown function, and pJIR3537 carries the *tet(P)* tetracycline resistance operon (Fig. 1). All of these plasmids can transfer at high frequency. The extensive region of sequence similarity (~35 kb) made it difficult to assemble whole-genome sequence data and to complete the sequence of each of these plasmids. This problem is common to the analysis of many *C. perfringens* strains, of all toxin types, where the presence of multiple, closely related large plasmids is very common. Sophisticated bioinformatic approaches, such as the construction of de Bruijn graphs (9), were used to assist in determining whole-plasmid sequences, but the clearest and most readily assembled results can be obtained by transferring each of the plasmids to *C. perfringens* strains lacking endogenous plasmids and then sequencing the whole genomes of transconjugant clones. Multiple large plasmid carriage has been found in all necrotic enteritis-derived strains investigated, with some reported to have up to five large plasmids (96).

Two *netB* plasmids (pJIR3535 and pNetB-NE10) have been sequenced and shown to be very similar 82-kb

plasmids despite being derived from geographically distinct *C. perfringens* strains (9, 10). Analysis of the genomes of other strains by microarray hybridization suggests that the *netB* plasmid gene content is largely conserved across strains carrying the plasmid (96). One smaller *netB* plasmid has been reported (in strain CP2 [88]), but the alterations in the plasmid have not been characterized. The *netB* plasmids contain other genes that may have a role in virulence, for example, genes homologous to internalins, chitinases, leukocidins, proteases, and carbohydrate binding proteins. Expression of *netB* is under the control of the VirS/VirR two-component regulatory system (97), and the adjacent *netI* gene also has a predicted VirR binding box in the upstream promoter region and thus may also be under VirS/VirR control (96).

The mobility of virulence factors such as NetB is potentially important in the origin and spread of pathogenic strains. It appears that NetB is absolutely required for the pathogenesis of avian necrotic enteritis, but it is not yet clear what other plasmid- or chromosomally encoded genes may also be required to arm *C. perfringens* with the ability to cause necrotic enteritis. Is it simply enough for a *C. perfringens* strain capable of colonizing chickens to carry a *netB* plasmid, or are there wider requirements? Other genes that are associated to varying degrees with strains isolated from diseased birds have been identified (98), and it will be important to determine the contribution that these genes make toward the pathogenic phenotype. Multi-locus sequence typing has shown that necrotic enteritis-derived *C. perfringens* isolates cluster into two major clonal groups that are associated with carriage of *netB* (99). It will be of interest to determine whether the clonal grouping is related to other properties, besides *netB*, that pathogenic strains may require (e.g., the ability to colonize the appropriate gastrointestinal niche) or whether it indicates some selective ability of these clonal groups to stably maintain the plasmids carrying *netB*.

Beta2-Toxin (CPB2) Plasmids of *C. perfringens*

CPB2 is a putative pore-forming cytolysin, but it is generally regarded as an accessory toxin that is not yet associated with any specific disease. Although there has been much speculation about the role of CPB2 in animal disease, most studies were based on detection of *cpb2*-positive isolates in the intestinal tract. However, the prevalence of such isolates seems to be similar in diseased and healthy individuals of most animal species (with the possible exception of the pig), which renders

this diagnostic criterion unreliable. Most experimental studies have failed to find any role for CPB2 in the pathogenesis of animal disease (100–102).

The *cpb2* gene has been identified in some strains of all five *C. perfringens* toxinotypes and is always located on a large plasmid, although there is considerable variation in plasmid size (45 to 90 kb). In *cpel/cpb2*-positive type A strains the two toxin genes can be carried on separate plasmids that are characterized by *cpe* genes with downstream IS1470-like sequences. In some of these isolates they are on the same plasmid, which is characterized by IS1151 sequences downstream of the *cpe* gene (103, 104).

Several *cpb2*-encoding plasmids have been fully sequenced (6, 9, 10, 105). The *cpb2* gene locus is conserved (Fig. 6) between sequenced plasmids from diverse origins such as the human gas gangrene isolate type A strain 13 (105), the lamb dysentery-derived type B strain NCTC8533 (20), the pig necrotizing enterocolitis type C strain CWC245 (72), two type A human gastrointestinal disease isolates (F5603 and F4013 [6]), a type A strain (F262) from a bovine clostridial abomasitis case (106), and two type A chicken necrotic enteritis strains isolated from two different countries (9, 10).

The location of the *cpb2* gene is also conserved in these plasmids. It is found downstream of a putative resolvase gene, *resP*, and is flanked by the conserved hypothetical gene, *pcw308* (pCW3 nomenclature [5]), that is located downstream of the *resP* gene in pCW3-like *cpb2*-negative plasmids (Fig. 6). pCP13 from strain 13 (107) has a silent *cpb2* gene and is different from the conjugative toxin and antibiotic resistance plasmids described in detail in this chapter; for example, it does not carry the *tcp* conjugation locus and has a very different plasmid replication and maintenance region, but it still has some sequence conservation with this plasmid family. Despite the more distant relationship between pCP13 and other *cpb2* plasmids, the genes located downstream of the *cpb2* gene are still conserved (Fig. 6), suggesting that a *cpb2* cassette, consisting of the *cpb2* gene and the two downstream hypothetical genes, may have been mobile at some stage. The plasmid encoding the *cpb2* gene from the chicken necrotic enteritis strain EHE-NE18 encodes an intact *tcp* locus, and conjugative transfer at a high frequency has been demonstrated both from the original host as well as from secondary hosts, in the absence of the other conjugative plasmids (9). Finally, expression of the *cpb2* gene has been shown to be under the regulatory control of both the VirS/VirR two-component regulatory system (108) and the Agr-like QS system (109).

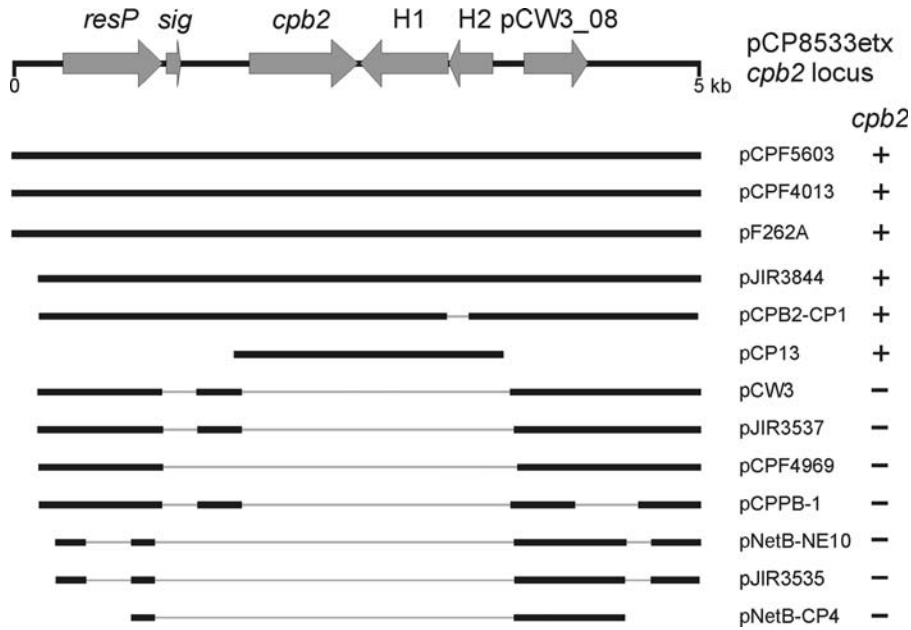


FIGURE 6 Genetic conservation of *cpb2* locus. Labeled arrows indicate the *cpb2* locus from pCP8533etx: *resP* encoding a putative resolvase, *sig* encoding a putative signal peptidase I, the *cpb2* gene, two hypothetical genes (H1 and H2), and a conserved hypothetical (pCW3_08). Below the locus are heavy lines indicating nucleotide sequence homology of 68% (pCP13) or >95% (all others). The gray lines indicate gaps in the sequence alignment where the sequence is absent from that particular plasmid sequence. Whether the *cpb2* gene is present in each plasmid is indicated on the right. [doi:10.1128/microbiolspec.PLAS-0024-2014.f6](https://doi.org/10.1128/microbiolspec.PLAS-0024-2014.f6)

THE NEUROTOXIN PLASMIDS OF *C. BOTULINUM* AND *C. TETANI*

The most potent bacterial toxins are the neurotoxins encoded by *C. botulinum* and *C. tetani*. These bacteria are the causative agents of botulism and tetanus, respectively, and the pathogenesis of these often fatal conditions is mediated by similar neurotoxins: botulinum toxin (BoNT) and tetanus toxin (TeNT). Both BoNT and TeNT are related zinc metalloproteases that cleave either *v*SNARE or *t*SNARE proteins that are associated with neuronal synaptic vesicles. Cleavage of SNARE proteins stops neural signal transmission; for BoNT, the neuromuscular junctions are affected, leading to flaccid paralysis, while TeNT affects the relaxation pathway in the spinal cord, leading to the characteristic spastic paralysis of tetanus (110).

TeNT is encoded by the *tetX* gene, which is highly conserved between strains and is located on a 74-kb plasmid in the sequenced strain E88 (111, 112) (Fig. 3). This plasmid, pE88, encodes 61 ORFs that include a collagenase gene, *colT*, genes encoding a two-component signal transduction system that may be involved in the regulation of TeNT production (113), and several putative alternative sigma factors. Regulation of toxin production is mediated, at least in part, by the *tetR* gene, which is located immediately upstream of the *tetX* gene (Fig. 3). TetR is an alternative sigma factor that is closely related to the BotR protein, which in *C. botulinum* regulates the expression of BoNT and associated proteins. TetR and BotR also are similar to the TcdR and UviA

proteins, which positively regulate the expression of toxins A and B in *C. difficile* and bacteriocin production in *C. perfringens*, respectively. These alternative sigma factors interact with RNA polymerase to facilitate toxin gene expression (114).

In addition, it was recently shown that several two-component signal transduction systems are involved in both positive and negative regulation of the toxin locus in *C. botulinum* (115, 116). *C. botulinum* group I organisms have two *agr*-like quorum sensing systems, one regulating sporulation and the other BoNT production (117). It is likely that the regulation of TeNT production in *C. tetani* is also complex (118).

C. botulinum isolates were historically designated to belong to the species *C. botulinum* based on the single phenotype of BoNT production. However, it now is clear that strains able to produce BoNT are, in some instances, only distantly related at the phylogenetic level (119). As a result, BoNT-producing bacteria are classified into four groups: group I or proteolytic *C. botulinum*, group II or nonproteolytic *C. botulinum*, group III encoding the BoNT/C and BoNT/D serotypes, and group IV (often referred to as *Clostridium argentinense*). In addition, *Clostridium baratii* strains producing BoNT/F and strains of *Clostridium butyricum* producing BoNT/E have been identified (120). Until very recently there were seven serologically distinct BoNT types (BoNT/A to BoNT/G), but an eighth serotype, BoNT/H, recently was identified from a case of infant botulism (121, 122). Most of the serotypes have been further divided into

subtypes (for example A1-A5). The BoNT serotype and the mechanism of toxin carriage often are common among members of the same group.

The BoNT locus is also variable and consists of two polycistronic operons: the *orfX* and HA loci. The *bont* and *ntnh* genes are common to all strains; the *botR* gene is also well conserved, but is not present in serotype E strains, and is often located between the two operons (123). Some bivalent strains have more than one BoNT locus, encoding BoNT proteins of different serotypes. In this situation, one BoNT serotype is generally found to be more abundant than the other, and the toxin designation used is, for example, Bf, where serotype B will demonstrate higher expression levels than toxin serotype F (123). Some chimeric Cd and Dc strains have been identified that have undergone recombination within the *bont* gene (113, 123). The variability of BoNT distribution is most likely due to mobile genetic elements such as plasmids and bacteriophages as well as transposons (120, 124).

BoNT loci can be chromosomally encoded, located on large plasmids, or encoded by lysogenic bacteriophages (123). In group III *C. botulinum* type C and D strains, the BoNT locus is located on large 186- to 203-kb pseudolysogenic bacteriophages (125, 126). These bacteriophage genomes are not integrated into the chromosome but exist as plasmids, although functional virus particles can be purified and used to infect susceptible strains (126). Group III isolates may also encode the C2 toxin, a binary toxin related to the ITX toxin of *C. perfringens* (127). The C2 toxin in these strains is also plasmid encoded, on large plasmids in excess of 100 kb. Several type C and D strains have been shown to contain other plasmids that encode putative toxin genes including a homologue of *C. septicum* alpha-toxin and two orthologues of ETX from *C. perfringens* (125).

For many years it was thought that, apart from the association of type C and D BoNT loci with bacteriophages, most other serotypes encoded chromosomal neurotoxin loci, an assumption borne out by the release of the first type A genome sequence—strain Hall (128). However, it is now clear that the toxin genes in all of the toxin serotypes studied to date may be either chromosomally encoded or plasmid-determined. This variability was first demonstrated in *C. botulinum* type A strains such as Loch Maree and 657Ba (pCLJ; Fig. 3), which encode BoNT/A loci on 267- and 270-kb plasmids, respectively (115, 129, 130). Plasmid-borne toxin loci in type B strains have also been demonstrated. In group I BoNT/B isolates, the plasmids range in size from 140

245 kb, while group II BoNT/B strains encode the toxin locus on 48- to 55-kb plasmids (Fig. 3) (130, 131). Some BoNT/E strains have been shown to harbor BoNT/E plasmids, although most appear to have chromosomally located neurotoxin genes (130, 132).

Notwithstanding the variation in the BoNT loci and their very different locations (chromosome, plasmid, or bacteriophage) the available sequence data indicate that the BoNT gene regions integrate into only one of three chromosomal locations or one of two plasmid insertion points, indicating that this locus, which is flanked by IS elements in many strains, potentially constitutes a mobile genetic element. Transfer of these loci could easily be facilitated by their carriage on elements such as bacteriophages, and recently it has been demonstrated that three type A and B toxin plasmids, two from group I organisms (pCLJ from strain 657Ba and pBotCDC-A3 from strain CDC-A3) and one from group II (pCLL from strain Eklund 17B), are capable of conjugative transfer (133). Transfer rates varied between the three plasmids, but all transferred at a relatively low level; however, plasmid transfer frequencies increased for the group I strains when a different nontoxigenic *C. botulinum* recipient was used. The plasmid genes responsible for the conjugative transfer remain to be identified, although pCLL showed some limited homology to the nonconjugative *C. perfringens* plasmid pCP13 as well as to regions of the conjugative *C. perfringens* plasmids (133). The similarity of several predicted ORFs to components of type II and IV bacterial secretion systems has suggested potential regions of pCLL and pCLJ that may be involved in conjugative transfer (Fig. 3). Conjugation adds yet another mode of potential transfer for these highly potent neurotoxins, which are clearly not restricted to a single clostridial strain.

TOXIN AND CAPSULE PLASMIDS OF *B. ANTHRACIS*

B. anthracis belongs to a family of six closely related pathogenic bacilli that includes *Bacillus cereus* and *Bacillus thuringiensis* and is known as the *B. cereus* group; members of this group are very closely related and are probably derived from a common ancestor (134). *B. anthracis* is the causative agent of anthrax, an important disease of both humans and domestic livestock. In recent years it has had increased attention as a result of its use as a bioterrorism agent. There are two major forms of anthrax: pulmonary anthrax, which is often fatal, and cutaneous anthrax, which is a more localized infection that is often restricted to workers in the wool,

hide, or meat industries. The epidemiology of anthrax involves the acquisition of spores or vegetative cells by inhalation or by contact with contaminated animal material or soil (135). Disease pathogenesis is somewhat unusual, as it involves two major toxin subunits that enter the target host cell by forming heterooligomeric complexes with the third, binding and pore-forming component, protective antigen (PA, encoded by the *pagA* gene). The toxic components are edema factor (EF, *cya* gene), a calmodulin-dependent adenylyl cyclase, and lethal factor (LF, *lef* gene), a zinc metalloprotease that cleaves the N-terminal region of MAPKK. Another important *B. anthracis* virulence factor is its ability under appropriate conditions to produce a poly-D-glutamic acid capsule that protects the invading bacterial cell from phagocytosis (136).

Toxic activity is initiated by the binding of the 83-kDa PA protein to one of two receptors, TEM8 or CMG2, on the host cell surface (137, 138). The PA protein then is proteolytically cleaved by host furins, and the resultant activated PA₆₃ derivative then oligomerizes to form a heptameric prepore. This PA₆₃ complex can bind up to three EF or LF monomers, in any combination. After subsequent endocytosis, acidification of the endosome leads to the formation of a heptameric PA₆₃ pore in the membrane; conformational changes then lead to the active translocation of EF and LF into the cytoplasm, where they have their lethal biological effects (139, 140).

The genes for the three components of anthrax toxin and an operon that encodes capsule production (*capBCADE*), together with genes involved in the regulation of their production, are all carried on two virulence plasmids, pXO1 (181.6 kb) and pXO2 (96.2 kb) (141, 142) (Fig. 3). Both plasmids are required for virulence; strains without pXO1 are avirulent because they do not produce the anthrax toxins, whereas strains lacking pXO2, such as the Sterne vaccine strain, are greatly attenuated because they do not have the capsule.

pXO1 has 203 ORFs including the structural genes for all three components of anthrax toxin (142). These toxin genes are located on a 44.8-kb pathogenicity island that is flanked by inverted copies of IS1627 and includes an 8.7-kb Tn3-like transposon, TnXO1, that carries the *gerX* operon, which is involved in spore germination (143). Toxin and capsule production, and therefore virulence, is regulated by AtxA, which is encoded by the pathogenicity island located on pXO1, and AcpA, which is encoded by pXO2. There is regulatory crosstalk between genes that are encoded on the plasmids and the chromosome; for example, AtxA,

which has been designated as a global regulator, also regulates chromosomal genes, including S-layer genes (144, 145). The pXO1 pathogenicity island also encodes an S-layer adhesin, BslA, which is essential for the adherence of vegetative *B. anthracis* cells to human cells (146).

pXO2 has 110 ORFs, including the *cap* capsule operon and the *acpAB* regulatory genes (141, 147, 148). Capsule production is tightly regulated and is only produced in the presence of bicarbonate ions.

Virulence Plasmids of *B. cereus* and *B. thuringiensis*

The carriage of different virulence plasmids traditionally was considered a major contributor to the phenotypic properties that were critical for the speciation of *B. anthracis*, *B. cereus*, and *B. thuringiensis*. For example, pXO1 and pXO2 were regarded as major defining characteristics of *B. anthracis*. However, recent findings suggest a need to reconsider traditional species assignments that were based largely upon plasmid-mediated pathogenic phenotypes. *B. thuringiensis* strains have been identified that produce the pXO2-encoded polyglutamate capsule that was historically associated with *B. anthracis* (149). Additionally, several *B. cereus* strains have been isolated from patients in Louisiana and Texas who were suffering from severe pneumonias that clinically presented like inhalational anthrax (150). Analysis of G9241, one of those *B. cereus* strains, reveals that it carries a pXO1 plasmid that is 99.6% identical to the *B. anthracis* pXO1 plasmid and expresses all three toxin components (151). Interestingly, G9241 is encapsulated but lacks pXO2; instead, it carries another large plasmid, pBC210, that is predicted to encode a polysaccharide (not polypeptide) capsule. Animal studies indicate that both pBC210 and pXO1 are required for the full virulence of G9241 (151). Finally, *B. cereus* strains carrying both pXO1 and pXO2 have been isolated in Africa from great apes suffering from anthrax (152, 153). Collectively, these studies indicate that possession of pXO1 and pXO2 no longer defines *B. anthracis*.

Although pXO1 and pXO2 do not appear to be conjugative, they carry genes encoding components of a type IV secretion system similar to those of a classical conjugation apparatus (154) and are capable of being mobilized by conjugative plasmids (155, 156). While pXO2 does not encode its own transfer, a plasmid from *B. thuringiensis*, pAW63, which is closely related to pXO2, is conjugative (148). Comparative analysis

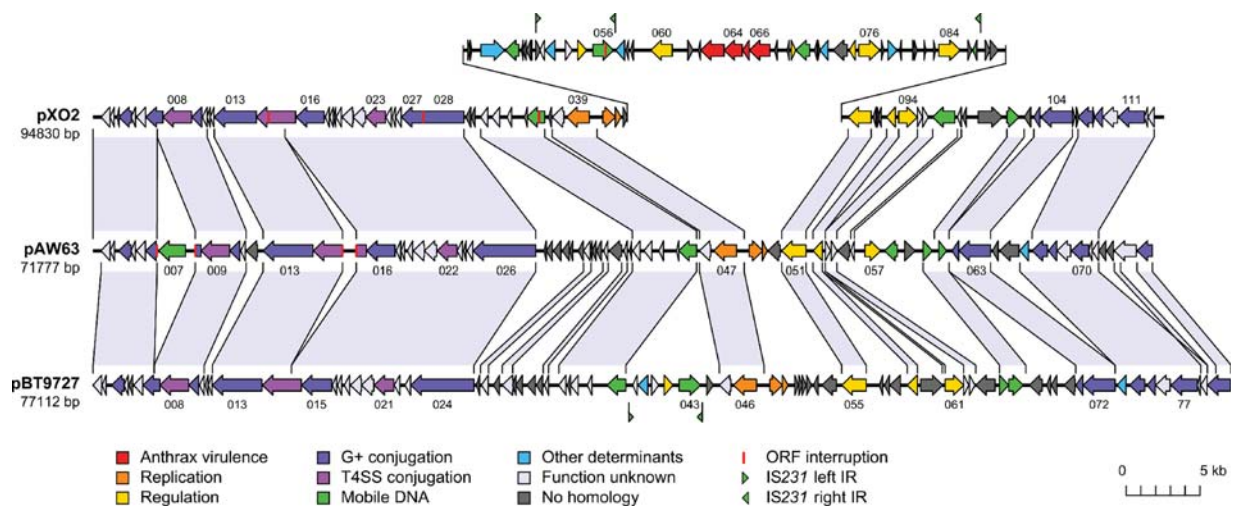
has shown that 50 of the 76 ORFs found on pXO2 have significant sequence similarity to genes on pAW63; the genetic organization of these genes is also very similar and is closely related to that of pBT9727 from *B. thuringiensis* (Fig. 7). By contrast, pXO2 carries a 37-kb pathogenicity island that is absent from the *B. thuringiensis* plasmids (148). pAW63 carries a 42-kb conjugation region, and most of these genes are also present on pXO2, but the precise reason why pXO2 is not conjugative is not known. These data provide clear evidence that although pXO1 and pXO2 are not conjugative, there are conjugative plasmids that are closely related to these plasmids and that conjugation and plasmid mobilization have played a major role in the wide distribution of these plasmids throughout the *B. cereus* group.

B. cereus is commonly responsible for two different types of human food poisoning, an emetic form and a diarrheal form (157). The diarrheal form is caused by several enterotoxins that are encoded by genes with a chromosomal location in *B. cereus*, although genes encoding one of these enterotoxins (Nhe) have been identified on a large plasmid in *Bacillus weihenstephanensis* (157). The emetic form of *B. cereus* food poisoning is caused by cereulide, a cyclic dodecapeptide with a molecular mass of 1.2 kDa (157). The *ces* genes responsible for cereulide synthesis are present on pCER270 (originally named pBCE4810). This large (~270 kb), low-copy-number plasmid is related to pXO1 but lacks

the pathogenicity island encoding EF and LF (pCER270 is apparently restricted to only a few clonal clusters of *B. cereus*). *B. cereus* also causes opportunistic human infections, but the involvement of plasmids in those diseases remains to be determined (157).

B. thuringiensis is an important insect and nematode pathogen but only very rarely a mammalian pathogen. The *B. thuringiensis* Cry (crystal) or Cyt (cytolytic) toxins are produced during stationary phase and then localize in paracrystalline bodies (158–160). Sequestration of these toxins in crystalline inclusions offers protection from bacterial cytoplasmic proteases; these inclusions are eventually solubilized by the alkaline pH of the insect midgut, and the solubilized toxins are then proteolytically activated by insect intestinal proteases (159). Different members of the Cry and Cyt toxin family exhibit toxicity for specific insects or nematodes, and some of these toxins act synergistically. Receptors for these toxins vary and can include insect plasma membrane proteins such as cadherin-like proteins, aminopeptidase N, and alkaline phosphatase (161). The Cry and Cyt toxins are considered beta pore-forming toxins that create channels in epithelial cells to damage the insect midgut (162). A single *B. thuringiensis* strain can carry up to seven different genes encoding crystal-associated toxins (163). Expression of these toxin genes occurs during stationary phase, and these toxins can represent up to 20 to 30% of the protein in sporulating cells (159), due to a combination of

FIGURE 7 Comparative genetic organization of pXO2, pAW63, and pBT9727. Shared regions are indicated by shaded segments. The pathogenicity island on pXO2 is raised above the map. Reproduced from reference 148 with permission of the authors. doi:10.1128/microbiolspec.PLAS-0024-2014.f7



transcriptional, posttranscriptional, and posttranslational regulatory effects (159, 160, 164).

B. thuringiensis strains carry from 1 to 17 plasmids, with sizes ranging from 3 to ~120 kb (164, 165). Most *cry* and *cyt* genes are encoded on large (>70 kb) plasmids that are often transmissible, due to either their own conjugative ability or to mobilization by helper plasmids (164, 166–168). A single plasmid can encode up to six different Cry and Cyt toxins (169). Recent studies showed that pBMB0228, which is an unusual 18-kb plasmid encoding two nematocidal Cry toxins, is a co-integrate of two plasmids, each with a *cry* gene (168). pBMB0228 is mobilizable and can resolve into its two separate plasmids after conjugative transfer; those plasmids can later fuse back together. In addition to often being located on conjugative plasmids, the dispersion of *cry* genes is probably assisted by their association with various IS elements, including IS231, IS232, IS240, IS*Bt1*, and IS*Bt2*, and transposons, including Tn4430 and Tn5401 (159, 160, 170).

CONCLUSIONS

We now know that toxin plasmids play an important role in the virulence of the spore-forming bacteria, but there is still a lot to learn about the biology of these plasmids and their molecular epidemiology. These plasmids represent fertile ground for future research. We need to elucidate the precise mechanism by which the conjugation process occurs in *C. perfringens* and how *C. perfringens* cells can maintain so many closely related but distinct plasmids. The role of several *C. perfringens* toxins in disease still remains to be determined, as do any synergistic effects on disease. There is little functional data available about the mechanism of replication and conjugative transfer of the toxin plasmids of the neurotoxic clostridia or the bacilli. Finally, there is a need for a systematic plasmid sequencing approach to determine the molecular epidemiology and evolution of the virulence plasmids of the *B. cereus* group.

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NOTE ADDED IN PROOF

Recently other workers have identified a novel binary toxin, BEC, that is produced by *C. perfringens* isolates from two outbreaks of human food-borne gastroenteritis in Japan. The BECa and BECb components are related to the equivalent ITX proteins and the becAB genes are encoded on 54.5-kb plasmids that are related to pCP13 (173).

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