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Toxin Plasmids of *Clostridium perfringens*

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SUMMARY

In both humans and animals, *Clostridium perfringens* is an important cause of histotoxic infections and diseases originating in the intestines, such as enteritis and enterotoxemia. The virulence of this Gram-positive, anaerobic bacterium is heavily dependent upon its prolific toxin-producing ability. Many of the ~16 toxins produced by *C. perfringens* are encoded by large plasmids that range in size from ~45 kb to ~140 kb. These plasmid-encoded toxins are often closely associated with mobile elements. A *C. perfringens* strain can carry up to three different toxin plasmids, with a single plasmid carrying up to three distinct toxin genes. Molecular Koch's postulate analyses have established the importance of several plasmid-encoded toxins when *C. perfringens* disease strains cause enteritis or enterotoxemias. Many toxin plasmids are closely related, suggesting a common evolutionary origin. In particular, most toxin plasmids and some antibiotic resistance plasmids of *C. perfringens* share an ~35-kb region containing a Tn*916*-related conjugation locus named *tcp* (transfer of clostridial plasmids). This *tcp* locus can mediate highly efficient conjugative transfer of these toxin or resistance plasmids. For example, conjugative transfer of a toxin plasmid from an infecting strain to *C. perfringens* normal intestinal flora strains may help to amplify and prolong an infection. Therefore, the presence of toxin genes on conjugative plasmids, particularly in association with insertion sequences that may mobilize these toxin genes, likely provides *C. perfringens* with considerable virulence plasticity and adaptability when it causes diseases originating in the gastrointestinal tract.

INTRODUCTION

The Gram-positive, anaerobic, spore-forming bacterium *Clostridium perfringens* is distributed ubiquitously throughout the environment, with a presence in soils, foods, sewage, feces, and the intestines of many healthy humans and animals (1–3). This bacterium also ranks among the most common and important pathogens of humans and livestock (1, 3, 4). *C. perfringens* causes histotoxic infections, including gas gangrene (clostridial myonecrosis), anaerobic cellulitis, and simple wound infections (3–5). It is also responsible for several human and animal diseases originating in the intestines; these illnesses typically manifest as enteritis or enterotoxemia, a condition where toxins produced in the intestines are absorbed into the circulation and then damage other internal organs such as the brain, lungs, or kidneys (3, 6).

The virulence of *C. perfringens* is attributable largely to its ability to produce at least 16 different toxins and extracellular enzymes (3, 7–11). However, no single strain produces this entire toxin panoply. A commonly used toxin typing classification system (1, 8, 9) assigns *C. perfringens* isolates to types A to E based upon their ability to produce four typing toxins, as indicated in Table 1. Besides expressing one or more of the typing toxins, some *C. perfringens* strains produce additional toxins, such as *C. perfringens* enterotoxin (CPE) or necrotic enteritis B-like toxin (NetB), which are also very important during certain diseases, as described below (1, 11, 12). Since the type A to E toxin typing scheme was developed before *cpe* or *netB* was identified, it does not address carriage of these (and other) toxin genes, indicating a need to update this historical classification system.

TABLE 1 *C. perfringens* toxinotypes

Type	Toxin produced			
	Alpha	Beta	Epsilon	Iota
A	+	−	−	−
B	+	+	+	−
C	+	+	−	−
D	+	−	+	−
E	+	−	−	+

It has now become clear that many important *C. perfringens* toxins are encoded by large plasmids (13–24). Other recent studies, described later in this review, have provided important insights into the diversity of *C. perfringens* toxin plasmids, the critical importance of these plasmids for pathogenesis, and the ability of toxin plasmids to transfer among *C. perfringens* strains. Given this progress, it is timely to summarize and interpret this information. In response, this review will first introduce the *C. perfringens* toxins, with an emphasis on those toxins that can be plasmid encoded, and then briefly discuss the contributions of the key plasmid-encoded toxins to *C. perfringens* diseases. Our focus will then shift to discussing the current understanding of *C. perfringens* toxin plasmid biology, addressing such issues as toxin plasmid diversity, replication, conjugative transfer, plasmid compatibility, and evolution.

CLOSTRIDIUM PERFRINGENS TOXINS

Properties of the key *C. perfringens* toxins are highlighted in Table 2, and these toxins will now be briefly described.

Chromosomally Encoded Toxins

Alpha-toxin (CPA or PLC). *C. perfringens* strains of all types can produce CPA, which is a zinc metallophospholipase C that has both phospholipase C (PLC) and sphingomyelinase activity (30, 31). Alpha-toxin cleaves charged phosphorylcholine head groups from the outer surface of host cell phospholipid bilayers, thereby disrupting the function of host cell membranes, leading to cell lysis and tissue necrosis.

Analysis of the CPA structure reveals that it has two biologically active domains (32): an N-terminal α-helical domain that

TABLE 2 Properties of the key *C. perfringens* toxins[c]

Toxin	Location[a]	Molecular mass (kDa)	LD_{50}[b] (mice)	Biological activity(ies)	Reaction to trypsin	Action(s)
CPA	C	43	3 μg	Necrotizing, hemolytic, contraction of smooth muscle	Susceptible	Phospholipase C; activates host cell signaling
CPB	P	35	<400 ng	Dermonecrosis, edema, enterotoxic	Susceptible	Pore former
ETX	P	34	100 ng	Dermonecrosis, edema, contraction of smooth muscle	Activation required	Pore former
ITX	P	Ia, 48; Ib, 72	40 μg	Necrotizing	Activation required	ADP-ribosylating action
PFO	C	54	15 μg	Necrotizing	Susceptible	Pore former
CPE	C/P	35	81 μg	Erythema, enterotoxic	Activation but not required	Pore former
CPB2	P	28	160 μg	Dermonecrosis, edema, enterotoxic	Susceptible	?
TpeL	P	191	600 μg	?	?	Glycosylates Ras
NetB	P	33	?	Hemolytic	?	Pore former

[a] C, chromosomal; P, plasmid (13–16, 18, 23, 25–27).
[b] Per kilogram of mouse after intravenous injection (10, 28, 29).
[c] Question marks indicate a lack of information on the relevant toxin property.

includes the single active site of the enzyme and a C-terminal β-sandwich domain that is essential for both cytolytic and toxic activity. Both domains are immunogenic, but only the C-terminal domain stimulates a protective immune response (33, 34). The C-terminal domain of CPA has structural similarity to C2 lipid-binding domains of eukaryotic proteins such as synaptotagmin and pancreatic lipase (30), suggesting an explanation as to why this membrane binding domain of CPA is required for its toxicity and is immunoprotective.

The lipid-soluble products of these reactions, diacylglycerol and ceramide, are important in host cell signaling pathways (31, 35). Therefore, direct disruption of the host cell membrane is not the only mechanism by which CPA causes cell lysis. It has also been shown that CPA activates the MEK/extracellular signal-regulated kinase (ERK) pathway and thereby induces oxidative stress in affected cells (36, 37) and interleukin-8 (IL-8) production by stimulating both the ERK1/2 and p38 mitogen-activated protein kinase (MAPK) pathways (38). Recent studies suggested that CPA may induce signal transduction changes after binding to a ganglioside GM1 receptor (38).

Perfringolysin O. Perfringolysin O (PFO) can be produced by all *C. perfringens* types; however, the *pfoA* gene is absent from many, if not all, type A food poisoning strains carrying a chromosomal enterotoxin gene (25, 39) and from Darmbrand-associated type C strains (40). PFO is a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins, which also includes listeriolysin O and streptolysin O (41–43). These CDCs are produced as soluble monomers, which oligomerize at the target cell surface to form a pore complex that then undergoes a conformational change and inserts into the membrane to form a large pore.

The mechanism by which PFO inserts into the host cell membrane is intriguing. The crystal structure of PFO reveals an elongated monomer that has three primarily β-sheet domains (D1, D2, and D4) and a domain (D3) with a core of four antiparallel β-sheets and four α-helices (44). Contact between D4 and the cell membrane leads to conformational changes in D3. The α-helices are converted into β-sheets that, together with the core D3 β-sheets, form two extended amphipathic transmembrane β-hairpins that, upon oligomerization, are capable of penetrating the cell membrane and forming a large pore that may be comprised of up to 50 monomeric subunits. In this process, the structure of each monomer is compressed by some 40 Å (45).

The formation of the PFO pore results in disruption of the cell's protective barrier, leading to an osmotic imbalance and ultimately to cell lysis. However, cell lysis may not be the major biological effect of PFO in an infected tissue. It is well established that both CPA and PFO are responsible for the characteristic lack of a leukocyte influx at the focus of a *C. perfringens*-mediated myonecrotic infection (46–48), and, like other CDCs, PFO is a Toll-like receptor 4 (TLR4) agonist that induces tumor necrosis factor alpha (TNF-α) and IL-6 expression and apoptosis in cultured macrophages by activating the p38 MAPK pathway (49).

Toxins That Can Be Either Chromosomally or Plasmid Encoded

***C. perfringens* enterotoxin.** *C. perfringens* enterotoxin (CPE) is produced by some type A, C, D, and E strains but not by any known type B isolates (14–16, 23, 50–52). The CPE primary amino acid sequence is (i) highly conserved, except for some type E strains that produce a slightly variant CPE (23), and (ii) unique, apart from some limited similarity (still of unknown significance) with the nonneurotoxic HA3 protein made by *Clostridium botulinum* (1, 53).

The CPE structure was recently solved by X-ray crystallography, which assigned this toxin to the aerolysin family of small pore-forming toxins (54, 55). Furthermore, those structural analyses, coupled with mutagenesis studies (56–61), indicated that CPE contains a C-terminal domain that binds to claudin receptors on host cells and an N-terminal domain, consisting of two halves, that is critical for pore formation by mediating oligomerization and membrane insertion.

CPE action begins by binding of the toxin to its receptors, which include certain members of the claudin tight junction protein family (62–68). Claudins are ~20- to 25-kDa proteins that consist of four transmembrane domains and two extracellular loops (ECLs) (69, 70). CPE binds, via a pocket on its C-terminal domain, to the second ECL of claudin receptors (71). Particularly important for this receptor binding interaction are (i) an Asn residue located near the middle of ECL2 on receptor claudins and (ii) Tyr residues present at amino acids 306, 310, and 312 in the CPE C terminus (1, 65, 67, 72).

After binding, CPE first localizes in a small, ~90-kDa complex (73). At 37°C, CPE in a small complex rapidly oligomerizes on the membrane surface to form a large (~450-kDa) prepore complex named CPE hexamer 1 (CH-1) (59, 66, 74). In addition to six copies of the toxin, CH-1 contains both receptor and nonreceptor claudins (the presence of nonreceptor claudins in CH-1 likely reflects a propensity for claudin-claudin interactions) (66). The CH-1 prepore complex, which forms in both cultured Caco-2 cells and the small intestine, then inserts into membranes by using a β-hairpin formed by CPE amino acids 81 to 106 (59, 61). This process results in formation of a cation-selective CPE pore that is initially permeable to molecules of <200 Da (1, 75–77).

CPE pore formation elevates cytoplasmic Ca^{2+} levels, thereby triggering calmodulin- and calpain-dependent host cell death via either caspase 3-mediated apoptosis (low CPE doses) or oncosis (high CPE doses) (78, 79). Ca^{2+} entry also induces morphological damage that exposes the basolateral cell surface, allowing CPE to interact with claudins and another tight junction protein named occludin (74, 80). This process leads to formation of a second large (~550-kDa) CPE complex, named CH-2, which contains six copies of CPE as well as occludin and both receptor and nonreceptor claudins (66). Whether CH-2 forms in the intestine is still unclear.

CPE induces necrosis, epithelial desquamation, and villus blunting in all sections of the small intestines, but it is particularly active in the ileum (8, 81). CPE-induced histological damage apparently causes intestinal fluid and electrolyte transport changes since (i) the onset of histological damage precedes the development of transport changes in CPE-treated rabbit small intestinal loops (82) and (ii) only those CPE doses causing histological damage are capable of producing fluid transport changes in rabbit small intestinal loops (81, 83). CPE effects on the colon appear to be more modest (84, 85), although this subject requires further study.

Plasmid-Encoded Toxins

Beta-toxin. Beta-toxin (CPB) has 20 to 28% amino acid sequence similarity with several pore-forming toxins of *Staphylococcus aureus* (86). This toxin is exceptionally sensitive to trypsin (87, 88). While the CPB structure-versus-function relationship has not yet been well studied, an older site-directed mutagenesis study suggested that CPB receptor binding activity may be localized in the C-terminal region of the toxin (89).

CPB forms ~12-Å channels that are selective for monovalent cations (90). The toxin shows specificity for only a few cultured cell lines, possibly due to the limited distribution of a still unidentified receptor. Evidence for CPB oligomer formation has been reported for beta-toxin-sensitive HL-60 cells (91).

In vivo, CPB causes necrotic enteritis, probably by targeting both enterocytes and endothelial cells (92). In addition, once produced in the intestines, CPB is absorbed (by unknown mechanisms) into the circulation to cause lethal enterotoxemia (3). The internal organs targeted by CPB during enterotoxemia are unknown.

Beta2-toxin. Despite its name, beta2-toxin (CPB2) has <15% sequence identity with CPB (28). Two major variants (with many subvariants) have been identified for this toxin (20, 93), which can be produced by all *C. perfringens* types. Interestingly, some *cpb2*-positive strains have a premature stop codon in their *cpb2* gene; however, *in vitro* aminoglycoside treatment induces ribosomal frameshifting to restore CPB2 expression by these strains (94). This observation may suggest that aminoglycoside treatment can sometimes stimulate CPB2 production *in vivo*, although there is still no conclusive evidence that CPB2 contributes to disease.

The cellular action and pathophysiological activity of CPB2 remain incompletely characterized. However, CPB2 is reportedly cytotoxic for CHO cells (28) although only at relatively high levels (20 μg/ml). This low potency of CPB2 may reflect its instability, perhaps due to protease susceptibility. This toxin can reportedly induce hemorrhagic necrosis in guinea pig intestine (28).

Epsilon-toxin. Epsilon-toxin (ETX) ranks as the most potent clostridial toxin after botulinum and tetanus toxins (95, 96). The toxin is secreted as a 296-amino-acid prototoxin, which is then proteolytically activated by digestive proteases such as chymotrypsin and trypsin or *in vitro* by *C. perfringens* lambda-toxin (97, 98). Recently, an unusual *C. perfringens* strain that can use a cytoplasmic protease to partially activate ETX was identified (99). Optimal activation of prototoxin is achieved with a combination of trypsin and chymotrypsin, which removes 13 amino acids from the N terminus and 29 amino acids from the C terminus (97, 98). Removal of the C-terminal amino acids is critical for producing active ETX, probably because those residues block toxin oligomerization (97, 100).

Like CPE, ETX belongs to the aerolysin family of pore-forming toxins (101). The mature ETX protein is comprised of three structural domains (101). These domains include (i) the N-terminal domain, which is thought to be important for receptor binding; (ii) the middle domain, containing a β-hairpin loop that likely mediates toxin insertion during pore formation; and (iii) the C-terminal domain, proposed to function during toxin oligomerization.

Relatively few mammalian cell lines are sensitive to this toxin (102), suggesting that the as-yet-unidentified ETX receptor is not widely distributed among host cells. ETX was recently shown to bind *in vitro* to hepatitis A virus cellular receptor 1 (HAVCR-1) (103, 104), which is produced in the kidneys, testis, and, to a lesser extent, colon (105). This observation is interesting since ETX binds strongly to the kidneys (106) and HAVCR-1 is expressed by an ETX-sensitive kidney cell line but not by several ETX-insensitive human cell lines (103). However, whether HAVCR-1 functions as an ETX receptor during disease is not known.

Once bound, ETX uses lipid rafts to oligomerize into heptamers (107). Recent findings suggest that the ETX oligomeric complex is ~700 kDa and contains, in addition to seven ~30-kDa ETX monomers, mammalian proteins such as caveolin-1 and -2 (108, 109). ETX oligomerization initially occurs on the membrane surface (100); the ETX prepore then rapidly inserts into the membrane to form an active pore with a diameter of 0.4 to 1 nm and a slight selectivity for anions (100, 110, 111). Pore formation in ETX-treated host cells results in rapid loss of intracellular K^+ and increased cytoplasmic levels of Cl^- and Na^+ (112). Unlike CPE, ETX causes only a slow increase in cytoplasmic Ca^{2+} levels in sensitive host cells (112). Instead, ETX-induced cytoplasmic K^+ loss triggers rapid cell death due to a necrosis process involving ATP depletion. It was recently suggested that at low doses, ETX can be internalized into host cells (113), but the pathophysiological importance of this observation is unclear.

Through an undefined mechanism, ETX increases intestinal permeability (114), which allows entry of the toxin into the circulation. The absorbed toxin then affects various organs such as brain, kidneys, and lungs (3, 106). Effects observed in naturally or

experimentally intoxicated animals include edema in multiple organs, which probably reflects the effects of ETX on endothelial cells (3). Intriguingly, most endothelial cell lines are not sensitive to ETX, perhaps because they have lost receptor expression during culture (95).

Iota-toxin. Iota-toxin (ITX) is a member of the clostridial binary toxin family and consists of separate IA and IB proteins that are produced as proproteins and then proteolytically activated when their N-terminal sequences are removed by host proteases (e.g., chymotrypsin) or *C. perfringens* lambda-toxin (115–118). Mature IA consists of an N-terminal domain that interacts with IB and a C-terminal domain with ADP-ribosyltransferase activity. Mature IB exhibits some similarity with *Bacillus anthracis* protective antigen (PA) but not in the receptor binding domain, which is consistent with IB and PA recognizing different receptors (115–118). IB has four domains, which mediate (i) IA interactions, (ii) internalization into host cells, (iii) oligomerization, and (iv) binding to host cell receptors (115–118).

ITX action begins with IB binding to its receptor(s). The lipolysis-stimulated lipoprotein receptor (LSR) has been identified as an ITX receptor (119) as well as a receptor for some other clostridial binary toxins, including *Clostridium difficile* transferase and *Clostridium spiroforme* toxin but not *C. botulinum* C2 toxin (119, 120). However, recent studies suggested that the multifunctional mammalian surface protein CD44 may also function as an ITX receptor or coreceptor (121).

In lipid rafts, bound IB toxin oligomerizes as a heptamer, which then binds IA (122, 123). Once formed, the holotoxin is endocytosed, and IA translocates into the cytoplasm from early endosomes (124, 125). Inside the cytoplasm, IA exerts its enzymatic activity, which involves ADP-ribosylating actin at Arg-177 to disassemble the host cell cytoskeleton (126). ITX can persist for at least 24 h inside host cells, which results in a delayed apoptosis (127).

NetB. The most recently identified toxin in the *C. perfringens* armory is NetB (11, 128), which is produced by many avian isolates of *C. perfringens* type A (129–132). Only one nonavian strain of *C. perfringens* has been shown to produce NetB, which is consistent with its key role in the pathogenesis of necrotic enteritis in chickens (11), as discussed below.

NetB is a 33-kDa secreted β-pore-forming toxin that is most closely related to CPB from *C. perfringens*, alpha-hemolysin from *Staphylococcus aureus*, and CytK from *Bacillus cereus* (11). Like most of these toxins, it is produced as a monomer and presumably oligomerizes on the host cell surface prior to membrane insertion, forming 1.6- to 1.8-nm pores in susceptible chicken leghorn male hepatoma (LMH) cells (11). The structures of both the soluble monomeric form of NetB (133) and a heptameric pore form of NetB (134) have recently been solved, and its structural similarity to *S. aureus* alpha-hemolysin was confirmed. Although the precise NetB receptor has not been identified, there is evidence for cell specificity, since not all chicken cell lines are susceptible to NetB (11). Recent studies have shown that NetB interacts with cholesterol to enhance pore formation (134) and that it formed pores with much higher single-channel conductance than alpha-hemolysin and varied in its ion selectivity, preferring cations over anions (133).

TpeL. The gene (*tpeL*) encoding TpeL is carried by some type A, B, and C strains (10, 16, 17) and reportedly can be expressed during sporulation under the control of Spo0A and the sporulation-specific sigma factor, SigE (135). TpeL (toxin *C. perfringens* large cytotoxin) is the largest known *C. perfringens* toxin, although some strains produce a truncated (~15-kDa-smaller), less active TpeL variant (10, 136). TpeL belongs to the clostridial glycosylating toxin (CGT) family, which includes toxins A and B of *C. difficile* as well as the lethal and hemorrhagic toxin of *Clostridium sordellii* and *Clostridium novyi* alpha-toxin. Like other CGTs, TpeL has an N-terminal domain mediating glycosyltransferase activity, a domain with autocatalytic activity, and a putative transmembrane domain that is thought to deliver the enzymatic domain into the cytoplasm (136). However, TpeL is distinguishable from other CGTs by its severely truncated C-terminal domain, which is notable since this region has been postulated to mediate CGT binding to cell surface receptors (10, 136, 137).

TpeL binds to unidentified receptors and is then endocytosed (136). After inositol hexakisphosphate-dependent cysteine protease cleavage and transport across the endocytic vesicle membrane, the enzymatic domain enters the cytoplasm from early endosomes. Due to its unique sugar binding motif, TpeL is the only CGT that can use both UDP-glucose and UDP-*N*-acetylglucosamine as donor substrates, although it prefers to utilize UDP-*N*-acetylglucosamine (136, 137). TpeL modifies the regulatory GTPase Ras at Thr35, which disrupts cell signaling, including Ras-Raf interactions and ERK activation (136). The role, if any, of TpeL in disease is still unclear, but it has been suggested that TpeL production might enhance virulence of avian necrotic enteritis strains (138).

Other toxins and secreted enzymes. In addition to the toxins described above, *C. perfringens* produces a slew of other toxins and secreted enzymes. These include another plasmid-encoded toxin named delta-toxin and several chromosomally encoded toxins (e.g., kappa-toxin, a collagenase, and mu-toxin, a hyaluronidase) and enzymes (e.g., clostripain, a cysteine protease) (8, 139, 140). Lambda-toxin, a 36-kDa thermolysin-like protease, is plasmid encoded and (as mentioned above) can activate ETX and the IA or IB component of ITX *in vitro* (97), although the importance of lambda-toxin in disease is unclear. Finally, *C. perfringens* produces several chromosomally encoded sialidases that are not essential when *C. perfringens* type A strain 13 causes gas gangrene in a mouse myonecrosis model (141); however, the NanI sialidase may still contribute to the early stages of a gas gangrene infection. This enzyme may also be important for type B or D disease originating in the gastrointestinal (GI) tract, since it increases ETX binding and mediates the *in vitro* adherence of CN3718, a type D strain, to enterocyte-like Caco-2 cells (142).

REGULATION OF PLASMID-ENCODED TOXIN PRODUCTION

The VirS/VirR Regulatory System

The classical two-component global regulatory system VirS/VirR, consisting of the VirS membrane sensor histidine kinase and the VirR transcriptional regulator, was discovered nearly 20 years ago, when it was shown to regulate the production of PFO, CPA, and some extracellular enzymes by type A strain 13 (143, 144). Later studies demonstrated that VirS/VirR directly regulates PFO production when VirR binds to VirR boxes located upstream of the *pfoA* gene (145–147). In contrast, this two-component system was found to indirectly control CPA production via a regulatory RNA molecule named VR-RNA (148, 149).

Of relevance for this review, the chromosomal VirS/VirR sys-

TABLE 3 Main diseases associated with *C. perfringens* in human and animals

Type	Major toxin(s)	Human disease(s)	Animal disease(s)
A	Alpha-toxin	Human myonecrosis (gas gangrene)	Gas gangrene in sheep, cattle, horses, and other spp.; yellow lamb disease in sheep
	Alpha-toxin, CPE	Human food poisoning; non-food-borne GI diseases	Enteritis in dogs, pigs, horses, foals, and goats
	Alpha-toxin, NetB	Not reported	Necrotizing enteritis in chickens
	Alpha-toxin, CPB2	Not reported	Possible enteritis in pigs; possible enterocolitis in horses
B	Alpha-toxin, beta-toxin, epsilon-toxin	Not reported	Necrotizing enteritis and enterotoxemia in sheep, cattle, and horses
C	Alpha-toxin, beta-toxin	Human enteritis necroticans	Necrotizing enteritis and enterotoxemia in pigs, lambs, calves, foals, and other spp. (usually neonatal)
D	Alpha-toxin, epsilon-toxin	Not reported	Enterotoxemia in sheep, goats, and cattle
E	Alpha-toxin, iota-toxin	Not reported	Enteritis in rabbits, lambs, and cattle

tem can also regulate the expression of several plasmid-carried toxin genes, as initially shown for *cpb2* transcription in strain 13, where VirS/VirR works via VR-RNA (150). More recently, VirS/VirR was found to control NetB and CPB production by type A or C strains, respectively (151–153). Interestingly, close contact with enterocyte-like Caco-2 cells increases production of CPB by type C strain CN3685, and this effect requires VirS/VirR (153). Furthermore, this two-component system is required for type C strain CN3685 to produce CPB *in vivo* and cause either lethal enterotoxemia or necrotic enteritis in animal models (152). However, VirS/VirR is not necessary for production of all plasmid-carried toxin genes, since a VirS/VirR null mutant of type D strain CN3718 still produces wild-type levels of ETX (154).

The Agr-Like Regulatory System

C. perfringens carries a chromosomal operon with partial homology to the *S. aureus* operon encoding components of the Agr quorum-sensing (QS) system. This *agr*-like operon was shown to regulate CPA and PFO production by *C. perfringens* type A strain 13, presumably by encoding components of a similar QS system (155, 156). It also controls the production of several plasmid-encoded *C. perfringens* toxins, including CPB2 and CPE expression in type A strain F5603 (157), CPB production in type C strain CN3685 (158) and type B strains CN1793 and CN1795 (159), and ETX production in type D strain CN3718 (154). However, this Agr-like regulatory system is not required for wild-type levels of production of all *C. perfringens* toxins, since inactivating this system in type B strains CN1793 and CN1795 had no effect on their ETX or CPB2 production (159).

The Agr-like regulatory system plays a role in the virulence of some *C. perfringens* strains. Specifically, by using *agrB* null mutants and their complemented derivatives, it was demonstrated that the Agr-like regulatory system is essential for CN3685 to cause either lethal enterotoxemia or hemorrhagic necrotic enteritis in animal models (158). The dependency of CN3685 virulence on the Agr-like regulatory system was shown, at least in part, to involve this system regulating intestinal CPB production (158).

Since the highly conserved *agr*-like operon present among most or all *C. perfringens* strains apparently does not encode the AgrA/AgrC two-component system of the *S. aureus* Agr QS operon (155, 156), it was proposed that *C. perfringens* uses the VirS/VirR system for responding to Agr-like regulatory system signaling (155). While this putative relationship may yet explain the regulation of some toxins by some *C. perfringens* strains, recent results indicated that Agr-like regulatory system signaling in this bacterium does not always require the VirS/VirR system (154). Specifically, while type D strain CN3718 was shown to depend upon the Agr-like regulatory system to produce wild-type levels of ETX, inactivating VirS/VirR had no effect on ETX production levels (154). This finding suggests that CN3718 regulates ETX production by using another of the ~20 *C. perfringens* two-component systems instead of, or in addition to, VirS/VirR.

C. PERFRINGENS DISEASES

The major diseases caused by *C. perfringens* are summarized in Table 3 and are briefly discussed below.

Diseases Involving Primarily Chromosomal Toxin Genes

Histotoxic infections of humans and animals. *C. perfringens* type A causes gas gangrene (clostridial myonecrosis) in humans (160–162). The disease is instigated by the infection of a wound by *C. perfringens* spores from the soil or GI tract; it is a typical disease of war, with gunshot wounds being one of the major causes of the traumatic damage that leads to infection. Surgical wounds, particularly those that affect the bowel, are also major causes of gas gangrene infections. Irrespective of its cause, injury leads to disruption of blood flow to the tissues and localized tissue ischemia, creating the conditions required for the germination of *C. perfringens* spores and the subsequent growth of vegetative cells and extracellular toxin production (5, 163). The result is extensive tissue necrosis that is characterized by an absence of a leukocyte influx into the infection site (160, 164). Genetic studies, which involved the construction and subsequent analysis of isogenic *plc* and *pfoA* mutants of a gas gangrene strain of *C. perfringens* type A, showed that CPA (PLC) is essential for virulence in the mouse myonecrosis model and that PFO, although not essential for disease, acts synergistically with CPA (46, 165). Unless promptly treated by

a combination of antibiotic therapy and surgical debridement, and potentially by amputation, the disease is almost invariably fatal.

Ruminants, horses, and swine are also highly susceptible to *C. perfringens* histotoxic infections, whereas carnivores are rarely affected (166). The main predisposing factors for gas gangrene in animals include castration, shearing, penetrating stake wounds, injury to the female reproductive tract during parturition, and injection sites (166–168). The typical gross appearance of these infections include severe edema, emphysema, discoloration of the overlying skin, coldness of the affected areas, and general signs of toxemia, while histologically, there is coagulation necrosis of tissues with marked leukostasis (166–168). Little information is available on the pathogenesis of naturally occurring gas gangrene in animals. However, CPA and PFO are presumably the main virulence factors, since gas gangrene in sheep, cattle, horses, and other animals presents with clinical, gross, and microscopic changes almost identical to those described for the mouse model of *C. perfringens* type A gas gangrene, where these two toxins are of paramount importance.

***C. perfringens* type A food poisoning.** *C. perfringens* type A food poisoning is a human syndrome that currently ranks as the second most common bacterial food-borne disease in the United States, where a million cases/year occur (1, 169). *C. perfringens* type A food poisoning usually develops when meat or poultry products become heavily contaminated with a CPE-positive type A strain. In ~75 to 80% of characterized cases, the causative type A strain carries a chromosomal, rather than a plasmid-borne, *cpe* gene (1, 170). The specific association of type A chromosomal *cpe* isolates with food poisoning likely involves the exceptional resistance properties of their spores (171–175). One major contributor to this resistance phenotype is the ability of type A chromosomal *cpe* strains to produce a unique small acid-soluble protein 4 (SASP-4) variant that binds spore DNA more tightly than the SASP-4 made by most other *C. perfringens* strains, thus offering greater protection against heat and other food-associated stresses (176, 177). Other factors such as reduced spore core size, which is indicative of a more dehydrated (and thus more stress-resistant) core, further contribute to the extreme resistance phenotype of spores made by most type A chromosomal *cpe* strains (174, 175).

Upon ingestion of heavily contaminated food, vegetative cells of a chromosomal *cpe* strain survive passage into the intestines, where they initially multiply but then soon sporulate (1); Spo0A and alternate sigma factors control both *in vivo* sporulation and CPE production (178–181). The toxin accumulates in the mother cell until it is released at the completion of sporulation, when the mother cell lyses. The released toxin then acts, as described above, to damage the intestines and trigger diarrhea and abdominal cramping (1). *C. perfringens* type A food poisoning symptoms typically have a ~12- to 16-h incubation period and then resolve within 24 h (1). However, fatalities can occur in the elderly or in patients with reduced intestinal activity from medication side effects (182, 183). It is thought that this lethality results when the medication reduces intestinal motility and interferes with CPE-induced diarrhea, thus prolonging contact between CPE and the intestinal mucosa. Based upon animal model studies (184), this longer presence of CPE in the intestines could facilitate absorption of the toxin into the circulation to cause a lethal enterotoxemia. The presence of CPE in the circulation leads to binding of the toxin to the kidneys and liver, causing a massive release of potassium, which can produce hyperkalemia-associated heart failure and death.

Diseases Involving Primarily Plasmid-Encoded Toxins

CPE-associated type A human non-food-borne gastrointestinal disease. Type A strains carrying a CPE plasmid cause ~5 to 10% of all cases of human non-food-borne GI diseases, including antibiotic-associated diarrhea or sporadic diarrhea (185). It was proposed that these cases involve true infections, but some could involve an overgrowth of normal *C. perfringens* flora, since type A strains harboring a *cpe*-carrying plasmid are present in the GI tract of some healthy people (186–188). These CPE-associated human non-food-borne GI diseases, which occur more frequently in the elderly, are typically more severe and longer-lasting than most cases of *C. perfringens* type A food poisoning (185). CPE is clearly important for the pathogenesis of these illnesses, as described below.

Type C enteritis necroticans of humans. *C. perfringens* type C isolates cause food-borne enteritis necroticans, which currently occurs sporadically throughout much of Southeast Asia and less commonly elsewhere (92, 189, 190).

After World War II, type C strains caused enteritis necroticans outbreaks (termed Darmbrand) in malnourished people in Northern Germany (191). A recent study showed that these Darmbrand strains carry and express both plasmid-borne *cpb* and *cpe* genes (40), although multilocus sequence typing (MLST) analyses conducted during that work also indicated that Darmbrand strains are otherwise genetically related to type A food poisoning strains carrying a chromosomal *cpe* gene. Of particular note, Darmbrand strains produce the same variant small acid-soluble protein as type A chromosomal *cpe* food poisoning strains, which likely contributes to the ability of these type C strains to form exceptionally resistant spores and thus facilitates their survival in the food environment.

In the 1960s to 1970s, type C-induced enteritis necroticans (known locally as pigbel) was very common in Papua New Guinea (PNG), causing >50% of the deaths occurring in children between 5 and 10 years of age (189, 190). The disease is clinically characterized by abdominal pain that develops 1 to 5 days after eating a high-protein meal. Pathologically, pigbel involves severe mucosal necrosis of the jejunum or ileum. The pathogenesis of pigbel in PNG is associated with a low-protein diet, which leads to limited production of pancreatic proteases. In addition, the major dietary item in the PNG highlands is the sweet potato, which contains a trypsin inhibitor. Therefore, when a child eats a meal containing sweet potato and meat contaminated with *C. perfringens* type C, coupled with a dietary background of protein subnutrition, little trypsin activity is present in the gut to degrade CPB. In Pigbel, type C isolates are usually introduced into the gastrointestinal tract by consumption of a contaminated meat (typically pork).

Avian necrotic enteritis. *C. perfringens* type A-mediated necrotic enteritis is of major importance to the poultry industry (192, 193). The onset of this disease usually requires predisposing conditions such as (i) switching the birds to a high-protein diet that favors the rapid growth of *C. perfringens* in the gastrointestinal tract or (ii) prior infection with *Eimeria* spp., which presum-

ably facilitates access to the enterocytes of either *C. perfringens* cells or their toxins.

The mechanism of pathogenesis of avian necrotic enteritis has been the subject of some controversy. For many years, CPA was thought to be the major toxin required for virulence, but it has now been shown that a *plc* null mutant is virulent in a chicken necrotic enteritis model (194). Nonetheless, CPA may still play a role in the disease process since CPA has at least some immunoprotective properties (195, 196). The essential toxin in avian necrotic enteritis is now established as NetB based upon studies using *netB* null mutants (11) and recent vaccination studies that provide evidence that NetB is immunoprotective (197, 198).

***C. perfringens* enteritis/enterotoxemia of other (nonhuman) mammals. (i) CPE-positive type A infections of animals.** Some case reports suggested that CPE also causes GI disease in domestic animals and possibly wild animals. For example, one study showed the presence of *cpe*-positive type A isolates and CPE in the small intestines of a goat kid suffering from necrotic enteritis (199). Additionally, fecal CPE and CPE-positive fecal isolates have been associated with canine diarrhea (200), and *cpe*-positive strains were suggested to cause recurrent diarrhea in dogs. In horses, fecal CPE was detected in ~20% of adults with diarrhea and ~30% of foals with diarrhea, while no fecal CPE was detected in healthy adult horses or foals (201).

(ii) CPE-negative *C. perfringens* type A. Type A strains are rarely implicated in enteric disease of animals (22, 202), but they do cause yellow lamb disease (203), which is a rare form of acute enterotoxemia in lambs characterized by severe hemolysis, jaundice, and hemoglobinuric nephrosis. Most of the clinical signs and lesions of yellow lamb disease are attributed to the effects of CPA, although there is little evidence to support this claim. CPB2-producing *C. perfringens* type A has also been linked to disease in several animal species, including horses, sheep, and goats (94, 204–206); however, this association is circumstantial and based mainly upon isolation of CPB2-positive *C. perfringens* from sick animals. Similarly, some studies have reported more isolation of CPB2-positive type A strains from sick than from healthy pigs (204, 206).

(iii) *C. perfringens* type B. Type B-mediated disease has been described in sheep, cattle, and horses; however, it is apparently restricted to parts of Europe, South Africa, and the Middle East (207). Disease by *C. perfringens* type B is characterized by sudden death or acute neurological signs with or without hemorrhagic diarrhea (3, 6, 208, 209).

Preliminary results suggest that both CPB and ETX are the most important toxins for the pathogenesis of type B infections in domestic animals (52). For example, without pretreatment with trypsin, CPB was found to be the main contributor to the lethal properties of type B supernatants using a mouse intravenous (i.v.) injection model, whereas seroneutralization studies with this model indicated that CPB and ETX are both important after trypsin pretreatment of type B supernatants (52). CPB is very sensitive to trypsin digestion, so animals with low levels of intestinal trypsin (such as neonates) are usually the most susceptible to infection by type B or C isolates (3, 6, 210). In contrast, ETX requires proteolytic activation via trypsin or other (intestinal or bacterial) proteases (97, 98). These opposing effects of trypsin on ETX and CPB activity suggest that when both toxins are present together in the intestine, such as during type B-associated infections, variations in intestinal conditions select for the predominant activity of ETX over CPB or vice versa. In animal model studies, at least some CPB produced by type B isolates remained active after trypsin treatment, but the overall lethality of most type B supernatants was lower after trypsin pretreatment (52).

(iv) *C. perfringens* type C. Type C disease has been described for multiple animal species, including, but not limited to, sheep, cattle, horses, and pigs (3). Most type C infections occur in neonatal animals due, as mentioned above, to the lower trypsin levels in these animals, which favor CPB activity. Type C infection is characterized by sudden death or colic and diarrhea, with occasional neurological clinical signs observed. Histologically, the hallmark of type C infection is necrosis of the intestinal wall, which starts in the mucosa but usually progresses to affect all layers of the intestine. Fibrin thrombi occluding superficial arteries and veins of the lamina propria and submucosa are characteristic of this condition (207), and it was postulated (although not yet definitely proven) that vascular damage by CPB is an early event in type C infections (211, 212).

(v) *C. perfringens* type D. Toxinotype D is by far the most common cause of clostridial enterotoxemia in sheep and goats and is occasionally the cause of clostridial enterotoxemia in other animal species (3). ETX is considered to mediate, in large part, the pathogenesis of *C. perfringens* type D disease; e.g., intravenous ETX injection in sheep and goats has been shown to reproduce most of the clinical signs and lesions of natural diseases in these species (213), and an intravenous ETX monoclonal antibody (MAb) was able to protect mice from intraduodenal challenge with type D strains (214). In enterotoxemia, ETX affects endothelial tight junctions in the brain (215), causing swelling and rupture of perivascular astrocyte processes (216). These effects are followed by increased capillary permeability (217), rapid extravasation of fluid (218), elevated intracerebral pressure, and parenchymal necrosis (215). In most animal species, type D disease is clinically characterized by neurological disease involving perivascular edema of the brain and, less frequently, by focal symmetrical encephalomalacia.

(vi) *C. perfringens* type E. Toxinotype E has been linked to hemorrhagic enteritis and sudden death in beef calves and lambs (219). These strains may also cause enterotoxemia in rabbits, although suspected type E-induced disease in rabbits must be differentiated from that caused by *C. spiroforme*, which also produces a toxin similar to iota-toxin (220).

DEMONSTRATING THE PATHOGENIC ROLE OF PLASMID-BORNE TOXINS BY MOLECULAR KOCH'S POSTULATES

The association of each *C. perfringens* type with specific diseases strongly suggests that plasmid-borne toxins are important for pathogenesis, since most typing toxins are plasmid encoded. However, the application of molecular Koch's postulate analyses has now firmly demonstrated the involvement of several plasmid-encoded toxins in *C. perfringens* diseases, as described below. Although chromosomally encoded toxins are not the primary focus of this review, it should be noted that molecular Koch's postulates were first applied in *C. perfringens* research to demonstrate the pathogenic importance of (i) CPA and PFO for gas gangrene in mouse myonecrosis models (46, 165) and (ii) CPE when type A chromosomal *cpe* food poisoning strains cause gastrointestinal pathology in rabbit small intestinal loops (12).

TYPE	*cpb*	*etx*	*iota*	*cpe*	*tpel*	*cpb2*	*netB*
Type A	—	—	—	70 seq 75 seq	ND	— 75	82 seq
Type B	65 90	65 seq	—	—	65 90	65 seq	—
Type C	65/90 110 75/85/110	—	—	75/85/90/110	65/90 65	75 65/75/90	—
Type D	—	48/75 65 75/85/110	—	75/85/110	—	65 45/75/85	—
Type E	—	—	97/135 65	97/135 65	—	70/85/90/97	—

F5603 family
F4969 family

FIG 1 Size diversity of *C. perfringens* plasmids encoding key toxins. Shared colors (other than black) indicate a similar (if not identical) plasmid; e.g., the 65-kb *etx*- and *cpb2*-carrying plasmid of type B strains is also apparently present in some type D strains. "seq" denotes a sequenced plasmid.

CPE-Associated Type A Non-Food-Borne Human GI Disease

The application of molecular Koch's postulates definitively demonstrated that CPE is essential for the ability of the type A plasmid CPE sporadic diarrhea isolate F4969 to cause gastrointestinal pathology in animal models (12). Specifically, while sporulating culture lysates of wild-type F4969 caused fluid accumulation and histological damage in rabbit ileal loops, no intestinal pathology was observed by using similar sporulating culture lysates of an F4969 mutant in which the *cpe* gene had been inactivated by allelic exchange. The inability of the mutant lysates to cause intestinal pathology was attributable specifically to the loss of CPE expression, since pathogenicity could be restored by complementing the F4969 *cpe* mutant with a plasmid carrying the wild-type *cpe* gene.

Type A Avian Necrotic Enteritis

Analysis of a *netB* mutant derived by allelic replacement revealed that, unlike its isogenic parent strain, it was no longer able to cause disease in a chicken necrotic enteritis model. The ability to cause avian necrotic enteritis was restored when the mutation was complemented with the wild-type *netB* gene, providing clear evidence that NetB is a key toxin in the disease process (11).

Type C Enteritis and Enterotoxemia

CPB is both sufficient and required for type C-induced enteric pathology, as shown recently by the use of purified CPB or isogenic toxin null mutants of type C isolate CN3685 (210, 221). Similar to natural type C infection, late-log-phase vegetative cultures of CN3685 cause necrotizing enteritis in rabbit small intestinal loops. When isogenic toxin null mutants were prepared by using TargeTron technology and then tested in the same model, a double *cpa pfoA* null mutant of CN3685 remained virulent. However, two independent *cpb* null mutants were completely attenuated for virulence in this animal model, and reversal of the *cpb* mutation restored CPB production and intestinal virulence. Additionally, preincubation of wild-type strain CN3685 with a CPB-neutralizing monoclonal antibody rendered the strain unable to cause intestinal pathology. Finally, highly purified CPB alone was able to reproduce the intestinal damage of wild-type CN3685, and this damage could be prevented by preincubating purified CPB with a CPB monoclonal antibody (210). Other studies using CN3685 and its isogenic derivatives later showed that CPB production is also very important for this type C strain to cause lethality in mouse and goat intraduodenal challenge models of type C enterotoxemia (222, 223).

TOXIN PLASMIDS OF *C. PERFRINGENS*

Plasmid Diversity

While early studies of *C. perfringens* plasmids focused primarily on antibiotic resistance and bacteriocin plasmids (224–232), the first linkage of *C. perfringens* toxin production with plasmids occurred over 30 years ago, when loss of CPB production was shown to correlate with the disappearance of a plasmid from a type C strain (233). Later studies then definitively localized several toxin genes to extrachromosomal DNA in a few *C. perfringens* strains (234). By using Southern blot analyses of pulsed-field gels, long-range and overlapping PCR techniques, and sequencing, it has now been firmly established (Fig. 1) that the genes encoding beta2-toxin (*cpb2*), epsilon-toxin (*etx*), iota-toxin (*iap/ibp*), beta-toxin (*cpb*), TpeL (*tpeL*), lambda-toxin (*lam*), NetB toxin (*netB*), and (sometimes) enterotoxin (*cpe*) are carried on large plasmids (13, 15–20, 40, 235).

Complete sequencing of *C. perfringens* toxin plasmids remains challenging due to the presence of these plasmids at low copy numbers in *C. perfringens* cells and because these strains often contain several plasmids that are closely related. Therefore, to sequence a plasmid of interest, it is often necessary to first move that plasmid into a plasmid-free recipient strain. Nonetheless, when this review was being prepared, the complete sequences had been determined for three *cpb2*-carrying plasmids, a plasmid carrying both the *etx* and *cpb2* genes, two *cpe*-carrying plasmids, two *netB*-carrying plasmids, and a plasmid carrying both the *cpe* and *iab/ibp* genes (Fig. 2) (13, 14, 19, 23, 235). The tetracycline resistance plasmid pCW3, which is often used as a paradigm plasmid for studying conjugative plasmid transfer in *C. perfringens*, has also been completely sequenced (Fig. 2) (236).

The *cpe*-carrying plasmids of type A strains. The first sequenced *C. perfringens* plasmids (Fig. 2) carrying functional toxin genes were the CPE-encoding plasmids from two type A strains

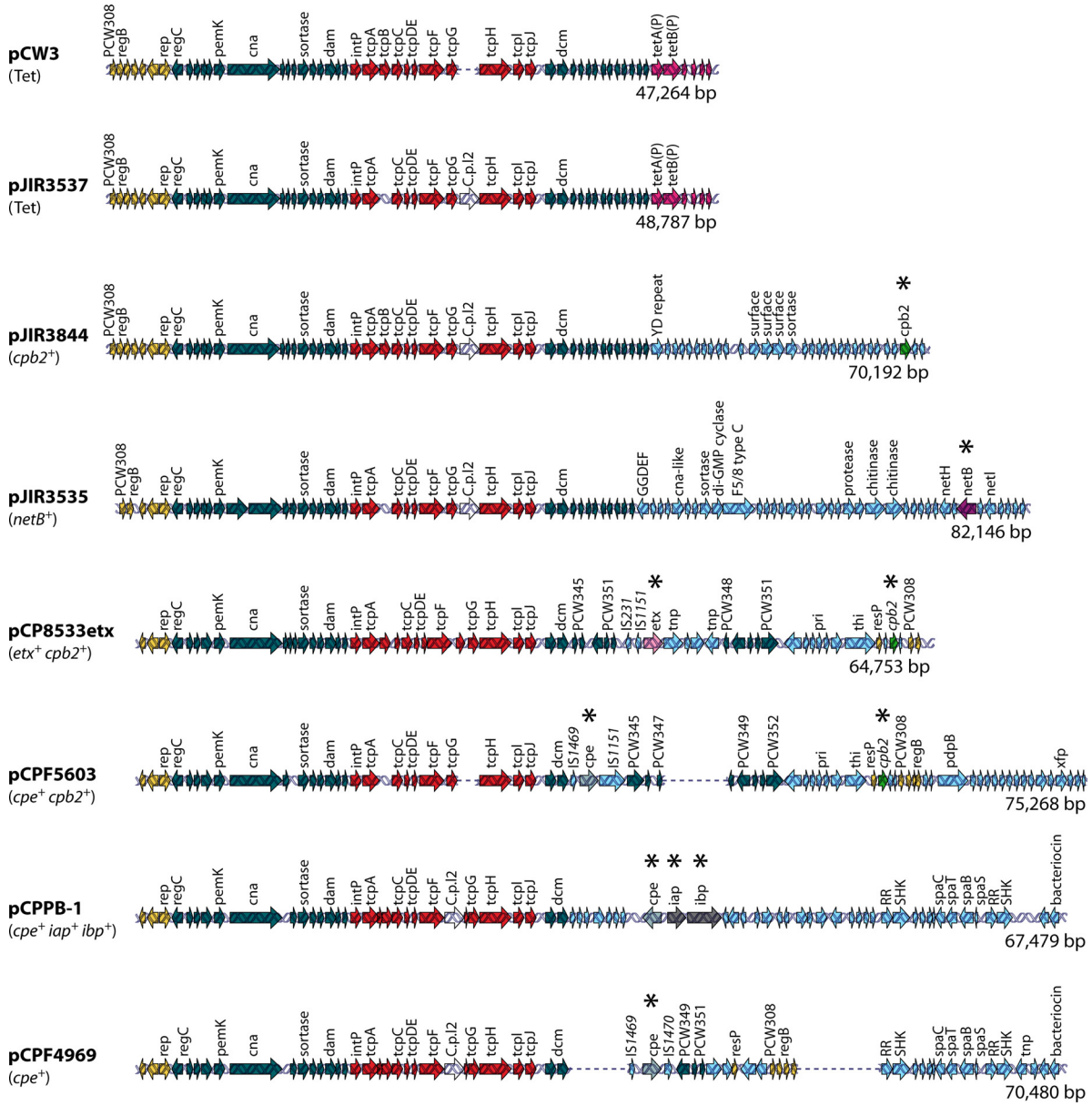

FIG 2 Comparative alignment of sequenced *C. perfringens* plasmids. Shown are sequence alignments for pCW3 (236); pJIR3537 (*tet*$^+$), pJIR3844 (*cpb2*$^+$), and pJIR3535 (*netB*$^+$) (19); pCP8533etx (*etx*$^+$ *cpb2*$^+$) (14); pCPF5603 (*cpe*$^+$ *cpb2*$^+$) (13); pCPPB-1 (*cpe*$^+$ *iota*$^+$) (23); and pCPF4969 (*cpe*$^+$) (13). Each arrow represents an ORF; ORF arrows shown are as follows: red arrows, the conserved *tcp* locus (note the adjacent *dcm* ORF); dark blue arrows, other conserved ORFs shared by these plasmids; light purple arrows, tetracycline resistance gene; green arrows, the *cpb2* toxin gene; purple arrows, the *netB* toxin gene; pink arrows, the *etx* gene; gray arrows, the *cpe* gene; dark gray arrows, the iota-toxin gene; yellow arrows, plasmid replication region; light blue arrows, regions unique to each plasmid. Asterisks denote a toxin gene. The GenBank accession numbers for the plasmid sequences are DQ366035 for pCW3, JN689220 for pJIR3537, JN689217 for pJIR3844, JN689219 for pJIR3536, AB444205 for pCP8533etx, AB236337 for pCPF5603, AB604032 for pCPPB-1, and AB236336 for pCPF4969. RR refers to response regulator, and SHK refers to sensor histidine kinase.

causing non-food-borne human gastrointestinal (GI) diseases (13). The 75.3-kb *cpe*-carrying plasmid (pCPF5603) of type A sporadic diarrhea isolate F5603 was shown to carry both *cpe* and *cpb2* toxin genes, whereas the ~70-kb plasmid pCPF4969 from type A sporadic diarrhea isolate F4969 lacks the *cpb2* gene.

Overlapping PCR surveys and pulsed-field Southern blot analyses established that most type A CPE-associated non-food-borne human GI disease isolates carry either a pCPF5603-like or a pCPF4969-like *cpe*-carrying plasmid (13, 237). These two *cpe*-carrying plasmid families share a ~35-kb conserved region encoding the *tcp* (transfer of clostridial plasmids) region, which can mediate *C. perfringens* toxin plasmid transfer, as discussed below. The pCPF4969 variable region contains genes encoding two putative bacteriocins and a two-component regulator similar to VirS/VirR, while the pCPF5603 variable region contains the functional *cpb2* gene and several metabolic genes. Some isolates carrying a pCPF4969-like plasmid also possess a second plasmid encoding CPB2 (13, 20).

The *netB*- and *cpb2*-carrying plasmids of *netB*-positive avian type A strains. A recent study (19) determined that NetB is encoded on a large conjugative plasmid in the type A avian necrotic enteritis strain EHE-NE18, which also carries two other large plasmids. High-throughput sequencing identified three closely related conjugative plasmids in this strain, including (i) the 82-kb plasmid pJIR3535, which encodes the *netB* gene and other potential virulence genes (Fig. 2); (ii) the 70-kb plasmid pJIR3844, which carries the *cpb2* gene (Fig. 2); and (iii) a 49-kb tetracycline resistance plasmid, pJIR3537, that is very closely related to pCW3 (Fig. 2). Each of these three plasmids contains a highly conserved 40-kb region encoding plasmid replication and transfer functions, including a *tcp* conjugation locus similar to that found in pCW3 and pCPF5603-like and pCPF4969-like *cpe*-carrying plasmids. Other workers (226, 235) determined the sequences of two plasmids from a different necrotic enteritis-causing strain of *C. perfringens*, CP1. These plasmids, pNetB-NE10 and pCpb2-CP1, had the same genetic organization and 99.1% and 97.9% identity to pJIR3535 and pJIR3844, respectively. These data provide evidence that the *netB*- and *cpb2*-carrying plasmids present in necrotic enteritis strains of *C. perfringens* are highly conserved. This conservation extends to the pathogenicity locus NELoc1 (located on *netB*-carrying plasmids) and the locus NELoc3 (located on *cpb2*-carrying plasmids), which were previously shown to be associated with necrotic enteritis strains (24). Analysis of other necrotic enteritis strains (235) showed that NELoc1 was more highly conserved than NELoc3, which is consistent with the fact that it carries the *netB* gene. These data also confirmed that the chromosomal NELoc2 region is associated with necrotic enteritis-causing strains, as originally suggested (24).

The toxin plasmids of type B strains. Type B strain CN8533 produces the two most lethal *C. perfringens* toxins, i.e., CPB and ETX. Sequencing (14) determined that this strain carries a ~64.7-kb *etx*-carrying plasmid, named pCP8533etx, with the *tcp* conjugative transfer region and open reading frames (ORFs) encoding additional potential virulence factors such as CPB2 or collagen adhesion protein (Fig. 2). Notably, the *cpb* gene is not carried by this plasmid. Interestingly, nearly 80% of the pCP8533etx ORFs are also present on pCPF5603 (Fig. 2). Furthermore, Southern blot analyses and overlapping PCR results indicated that most, if not all, type B isolates carry an *etx*-carrying plasmid that is very similar, if not identical, to pCP8533etx (14, 16).

The *cpb* gene has been localized, by Southern blotting analyses of pulsed-field gels, to ~90-kb plasmids in most type B isolates, although a few type B isolates carry a ~65-kb *cpb*-carrying plasmid that is distinct from their *etx*-carrying plasmid (16). The *cpb*-carrying plasmids of type B strains were also shown to possess the *tcp* locus, suggesting that they are conjugative (16). Overlapping PCR analysis revealed that the *tpeL* toxin gene is located ~3 kb downstream from the *cpb* gene in these plasmids (16). Finally, most type B isolates were shown to possess a third virulence plasmid carrying genes encoding urease and lambda-toxin (16).

The toxin plasmids of type C strains. While type B strains carry either 65-kb or 90-kb *cpb*-carrying plasmids (16), the *cpb*-carrying plasmids of type C isolates exhibit greater size diversity, ranging from ~65 kb to ~110 kb (17). Note that almost all large toxin plasmids in type C isolates carry the *tcp* genes, suggesting that they are conjugative (17). Southern blot analyses of pulsed-field gels run with restriction enzyme-digested DNA showed that these ~65-kb and ~90-kb *cpb*-carrying plasmids of some type C isolates resemble the equivalent-sized *cpb*-carrying plasmids of type B isolates; e.g., these two *cpb*-carrying plasmids also carry a *tpeL* gene ~3 kb upstream from their *cpb* gene (16, 17). However, in other *tpeL*-positive type C strains, the *tpeL* gene is located on a different plasmid from the *cpb*-carrying plasmid (17).

Some type C isolates possess ~75- or ~85-kb *cpb*-carrying plasmids that also carry the *cpe* gene (17). However, a few type C strains have their *cpe* gene on an ~110-kb plasmid that is distinct from their *cpb*-carrying plasmid (17, 40). Interestingly, among surveyed type C strains, no *cpe*-positive isolates were found to carry the *tpeL* gene (17). While some type C strains possess *cpb2* genes on plasmids ranging in size from ~65 to ~90 kb, those *cpb2*-carrying plasmids are distinct from the *cpb*-carrying plasmid present in these isolates (17).

Toxin plasmids of type D strains. Unlike type B *etx*-carrying plasmids, the *etx*-carrying plasmids of type D strains exhibit considerable size diversity (15). For type D isolates lacking the *cpe* or *cpb2* gene, the *etx* gene is generally present on an ~48-kb plasmid, although a few type D strains carry larger (~73- to 75-kb) *etx*-carrying plasmids (15). For type D isolates possessing the *cpe* and/or the *cpb2* gene, the *etx* gene is located on large plasmids ranging in size from ~75 to 110 kb (15). In these type D isolates, their *cpb2* gene is present on ~45- to 85-kb plasmids, most commonly 75-kb plasmids, while their *cpe* gene is carried on large plasmids ranging from 75 kb to, most commonly, ~110 kb (15). A few type D strains apparently carry the same 65-kb *etx*- and *cpb2*-carrying plasmid found in type B strains (14). For most type D isolates, their toxin plasmids also have the *tcp* locus genes essential for conjugative transfer (15), and conjugative transfer has been demonstrated for two type D *etx*-carrying plasmids (21).

Toxin plasmids of type E strains. Two major families of iota-toxin plasmids have been identified, the first of which includes large plasmids, varying in size from ~97 kb to ~135 kb, with a pCPF5603 backbone (18). These iota-toxin plasmids carry functional *iap/ibp* genes, but their adjacent *cpe* sequences are silent due to extensive mutations in the *cpe* gene (18, 22). This *iap/iab*-carrying plasmid family also encodes urease and lambda-toxin (18). The second iota-toxin plasmid family, which includes the recently sequenced plasmid pCPPB-1, carries expressed *iap/ibp* and *cpe* genes (23). This ~65-kb plasmid has a pCPF4969 backbone but does not encode lambda-toxin or urease (23). In all examined type E isolates, the *iap/ibp*-carrying plasmid has a *tcp* locus, strongly suggesting that these plasmids are conjugative (18, 23).

Relationship between *C. perfringens* toxin plasmids. Emerging evidence indicates that many, although not all, *C. perfringens* toxin plasmids are related to either pCPF5603 or pCPF4969 and carry the same *tcp* sequences also found in some conjugative antibiotic resistance plasmids, e.g., pCW3. For example, the *etx*-carrying plasmid present in most or all type B isolates, and a few type D isolates, resembles pCPF5603 (13, 14). Similarly, the *netB*-derived plasmids pJIR3536 and pNetB-NE10 share ~35 kb of conserved backbone (Fig. 2) with pCPF5603 and pCW3 (13, 19, 235, 236). As mentioned above, some type E iota-toxin-encoding plasmids share substantial similarity with pCPF5603 (18), while others more closely resemble pCPF4969 (23).

The similarity of many *C. perfringens* toxin plasmids may impact plasmid carriage and, by extension, toxin production and virulence. For example, no *C. perfringens* isolate has been found to carry both *iap/ibp* genes and the *cpb* or *etx* gene, suggesting fundamental plasmid incompatibility issues. However, some toxin

plasmid combinations can be stably maintained in a single *C. perfringens* cell; e.g., some chicken necrotic enteritis strains can carry three related plasmids, including two different toxin plasmids, while type B isolates carry their *cpb* and *etx* genes on separate plasmids (13, 16, 19). In this regard, it is notable that the *cpb*-carrying plasmids and *etx*-carrying plasmids in type B strains are much less diverse than the *cpb*-carrying plasmids in type C strains or the *etx*-carrying plasmids of type D strains (15–17), further suggesting that only certain plasmid combinations can be stably maintained in the same *C. perfringens* cell.

As mentioned above, most of the examined *C. perfringens* toxin plasmids carry the *tcp* locus, which mediates conjugative transfer of *C. perfringens* plasmids (see below). Therefore, when different *C. perfringens* strains make physical contact, conjugative exchange of their toxin plasmids may occur, which may sometimes be followed by the loss of one toxin plasmid in a recipient strain due to plasmid incompatibility. However, in certain situations (e.g., the type A *cpe*-positive strains that carry *cpe* and *cpb2* on separate plasmids, type B strains, and type A avian necrotic enteritis strains), the two toxin plasmids can be stably maintained together, thus enhancing virulence diversity.

Association of *C. perfringens* Toxin Genes with Insertion Sequences

As mentioned above, in 75 to 80% of type A food poisoning isolates, the *cpe* gene is chromosomal (1, 170, 187, 237) and located near an upstream IS*1469* sequence and flanking IS*1470* sequences (Fig. 3). This structure resembles that of a compound transposon (238); however, IS*1470*-mediated transposition of the *cpe* gene has not yet been demonstrated. This genetic organization differs from that of the plasmid-determined *cpe* loci (Fig. 3); i.e., in pCPF5603-like plasmids, the *cpe* gene is flanked by an upstream IS*1469* sequence and a downstream IS*1151* sequence, while the *cpe* gene in the pCPF4969 plasmid family is flanked by an upstream IS*1469* sequence and a downstream IS*1470*-like sequence (13).

Approximately 15% and 25% of type C and D isolates, respectively, carry plasmid-borne *cpe* genes that are identical to the type A *cpe* gene (26). However, the genetic organization of the *cpe* locus varies between these type C and D strains and the plasmid *cpe* locus found in type A strains (Fig. 3). Most *cpe*-positive type C isolates possess a *cpe* locus similar to that found in the chromosomal *cpe* locus of type A isolates, except that (i) the IS*1469* sequence is located upstream of an IS*1470* sequence and (ii) there is an IS*1151*-like sequence located downstream of the *cpe* gene in these type C strains (26). One unusual type C *cpe* locus that is missing the two copies of IS*1470* found in the *cpe* locus of most type C *cpe*-positive strains has been identified (26).

The type D *cpe* locus (Fig. 3) has a unique genetic organization (26). There are two copies of an ORF with 67% identity to a Tn*1456*-like transposase gene (COG4644) located upstream of the *cpe* gene. The region downstream of the *cpe* gene is organized similarly to the sequences downstream of the *cpe* gene in type A isolate F4969, except for the absence of an IS*1470*-like insertion sequence (IS) (26).

In all studied *cpe*-positive type E isolates, the iota-toxin genes are located in close proximity to the *cpe* promoter region, suggesting an insertional event (Fig. 3). In pCPF5603-like iota-toxin plasmids, this putative insertion appears to have silenced the *cpe* promoter, leading to a loss of *cpe* expression (18, 22). In these type E strains, the locus carrying *iab/ibp* genes and silent *cpe* sequences lies between two IS*1151*-like insertion sequences, but again, there is no direct experimental evidence that this putative compound transposon can transpose (18, 22). In contrast, for the pCPPB-1 family of iota-toxin plasmids, only one of three *cpe* promoters was apparently inactivated by insertion of the *iap/ibp* genes, so the *cpe* gene is still transcribed (23).

Two variations of the *etx* gene locus have been identified (Fig. 3). Most type B strains, and a few type D strains, have an *etx* locus similar to the pCPF5603 *cpe* locus, with IS*1151*-like and IS*231*-related transposase gene sequences located upstream of the *etx* gene. In contrast, the *etx* locus of most type D strains contains an IS*1151* sequence and a Tn*3*-like transposase gene upstream of the *etx* gene. All *etx* loci have the same mutator transposase sequence located downstream of their *etx* gene (14).

Similarly, all type B strains and some type C isolates have a similar *cpb* locus (16, 17), with the *cpb* gene downstream of IS*cpe7* and IS*1151* sequences but upstream of a Tn*3*-like transposase gene. The *tpeL* gene is also located downstream of this *cpb* gene (Fig. 3). In addition, another IS*cpe3* sequence gene is present upstream of the *tpeL* gene. Other type C strains have the same upstream IS*cpe7* and IS*1151*-like sequence but lack the downstream *tpeL* gene.

Evolution of Characterized *C. perfringens* Toxin Plasmids

Many *C. perfringens* toxin gene loci are located near the *dcm* gene (Fig. 3), which may represent a hot spot region for the insertion of toxin gene-carrying mobile genetic elements (13–18, 26). Some indirect evidence supports this hypothesis. For example, although IS-mediated movement of plasmid-borne *C. perfringens* toxin genes from one location to another has not been directly demonstrated, toxin gene-carrying circular DNA molecules that potentially represent transposition intermediates have been detected (15, 16, 18, 26, 40, 238). Specifically, those circular intermediates can carry the *cpe* genes of type A, C, and D isolates, the iota-toxin genes of type E isolates, the *cpb-tpeL* genes of type B isolates, or the *etx* genes of type D isolates. We postulate that IS-mediated movement of toxin genes may help to explain why some *C. perfringens* toxin genes are found on different plasmid backbones.

While overlapping PCR analyses have strongly suggested that some *C. perfringens* toxin plasmids have a different (but as-yet-uncharacterized) backbone from the pCPF5603- or pCPF4969-like toxin plasmids (15, 17), all of the sequenced toxin plasmids share considerable homology with these two *cpe*-carrying plasmid families and pCW3, the paradigm conjugative plasmid from *C. perfringens*. This observation provides considerable insight into the possible origin and evolution of the *C. perfringens* toxin plasmids (Fig. 4). Both the pCPF5603- and pCPF4969-like toxin plasmids contain two regions (*dam-rep*) and *tcp*, which are also present on the pCW3 tetracycline resistance plasmid (13, 14, 18, 23, 227, 235, 236). Since the *tcp* region has homology with Tn*916*, which is a conjugative transposon, it is conceivable that a Tn*916*-like transposon may have integrated into a plasmid, creating a conjugation-capable precursor plasmid (13, 236).

This putative conjugative precursor plasmid, which has not yet been identified, may then have acquired or lost genes by transposition or recombination events. In some cases, the acquired genes encoded antibiotic resistance. For example, the first *C. perfringens* plasmids shown to be capable of conjugative transfer, i.e., pCW3 (239) and pIP401 (226), both encode tetracycline resistance but lack toxin genes. Furthermore, pIP401 is a pCW3-like plasmid that acquired the chloramphenicol resistance transposon Tn*4451* (240).

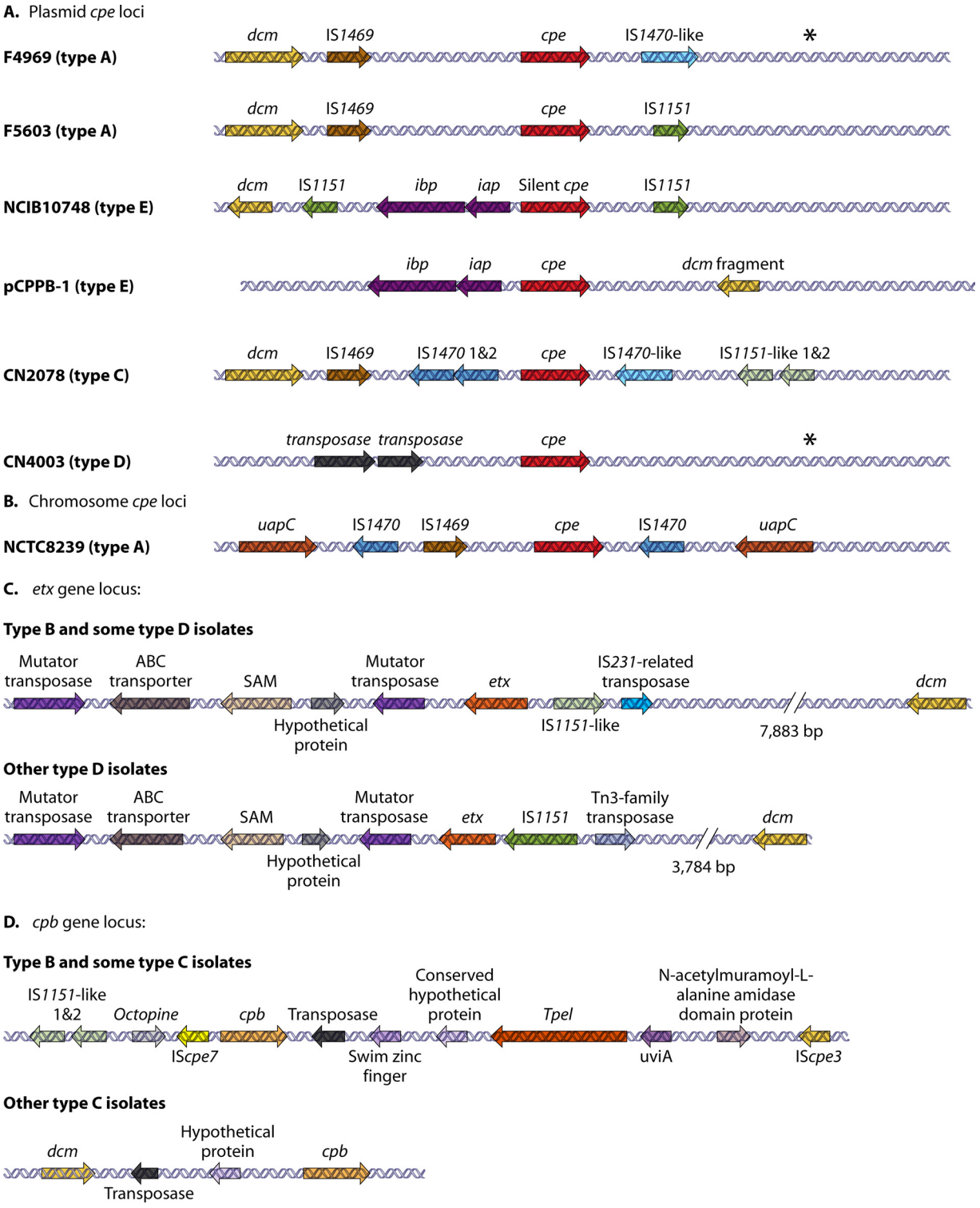

FIG 3 Organization of toxin (*cpe*, *etx*, and *cpb*) loci in type A, B, C, D, and E strains of *C. perfringens.* (A) Organization of plasmid-borne *cpe* loci in type A, E, C, and D strains. (B) Organization of the type A chromosome *cep* locus. (C) Organization of plasmid-borne *etx* loci in type B and D strains. (D) Organization of plasmid-borne *cpb* loci in type B and C isolates. Each arrow represents an ORF. Asterisks indicate a region with similarity to sequences present downstream of the *cpe* gene in F4969, except for the absence of an IS*1470*-like gene. (Panels A and B adapted from reference 26; panel C adapted from reference 14; panel D adapted from references 16 and 17.)

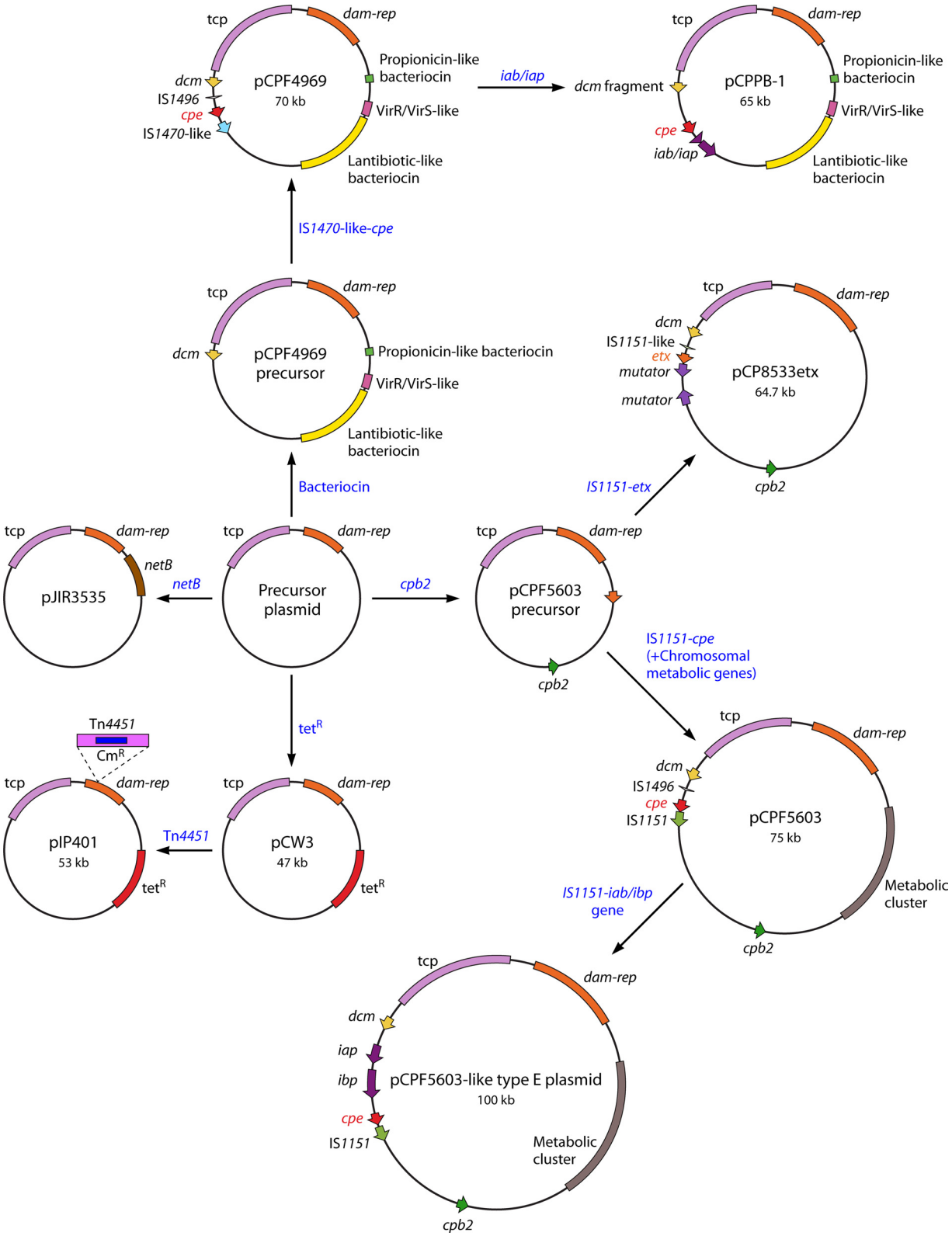

FIG 4 Model for evolution of characterized *C. perfringens* toxin plasmids. See the text for discussion of the possible evolution of pIP401 (230), pCW3 (236), pJIR3535 (*netB*$^+$) (19), pCP8533etx (*etx*$^+$ *cpb2*$^+$) (14), pCPF5603 (*cpe*$^+$ *cpb2*$^+$) (13), pCPPB-1 (*cpe*$^+$ *iota*$^+$) (23), and pCPF4969 (*cpe*$^+$) (13). Each box color depicts a different region of importance on the toxin plasmids, as indicated.

At other times, the putative conjugative precursor plasmid may have acquired mobile genetic elements carrying toxin genes. For example, if a mobile element carrying both IS*1470*-like sequences and the *cpe* gene integrated into the precursor plasmid, the result would have been a pCPF4969 toxin family plasmid. Alternatively, if this precursor plasmid acquired a mobile element carrying IS*1151*-*cpe* sequences or IS*1151*-*etx* sequences, it would have given rise to pCPF5603-like *cpe*-carrying plasmids or the pCP8533 *etx*-carrying plasmids, respectively. In one *C. perfringens* strain carrying pCPF5603, an IS*1151*–iota-toxin element apparently then inserted into the *cpe* promoter, silencing the *cpe* gene and creating the pCPF5603-like family of iota-toxin plasmids. In another *C. perfringens* strain carrying pCPF4969, we postulate that a similar mobile element carrying IS*1151*–iota-toxin genes inserted slightly upstream of the *cpe* gene, giving rise to the pCPBB-1 family of type E toxin plasmids carrying functional iota-toxin genes and *cpe* genes.

Conjugative Transfer of Toxin Plasmids

To date, five toxin plasmids have been shown experimentally to be conjugative, but virtually all of the large toxin plasmids of *C. perfringens* carry a *tcp* conjugation locus that is very closely related to the *tcp* conjugation region of pCW3 and therefore are highly likely to be conjugative. The first toxin plasmid shown to be conjugative was pMRS4969, a genetically marked derivative of the CPE plasmid pCPF4969 (241). Mixed-plate matings into suitable recipient strains of *C. perfringens* were used to demonstrate that pMRS4969 transferred by conjugation at a high frequency (2.0×10^{-2} to 4.6×10^{-4} transconjugants per donor cell). Cell-to-cell contact was required for transfer. The resultant transconjugants carried the same plasmid that was present in the donor strain and could also act as a conjugation donor, at a similarly high frequency, providing evidence that this plasmid carried a functional conjugation locus. Finally, Southern blots provided evidence that pMRS4969 carried regions that were also present on pCW3, which at the time had not been sequenced. It was postulated that these regions were involved in conjugative transfer, a postulate that was subsequently validated (236).

More recent studies (21) have demonstrated that the *etx*-carrying plasmids from two *C. perfringens* type D strains, CN1020 and CN3718, are also conjugative. Initial mating experiments using *etx*-carrying plasmid derivatives in which the *etx* gene was insertionally inactivated by the *catP* gene showed that both strains contained plasmids that also transferred at very high frequencies (2.9×10^{-1} to 3.8×10^{-2}). Once more, the transconjugants could act as donors in subsequent matings. These transfer frequencies were so high that further matings conducted with one of the wild-type type D strains yielded detectable transfer frequencies in the absence of any antibiotic selection (21). These experiments have shown that a toxinotype A strain can be converted to a genotypic toxinotype D strain by conjugation, a process which we postulate is likely to occur in the gastrointestinal tract, with potential disease significance (see Concluding Remarks). These results also illustrate the genetic plasticity of toxin types, since most toxin type genes are probably located on conjugative elements.

Finally, as described above, a chicken necrotic enteritis strain, EHE-NE18, has been shown to carry three closely related plasmids, encoding NetB toxin production, CPB2 toxin production, and tetracycline resistance, respectively (19). It was a relatively straightforward process to show that the tetracycline resistance plasmid, which was almost identical to pCW3, was conjugative. In addition, by separately genetically marking the *netB* and *cbp2* toxin genes, it was demonstrated that their host plasmids also were independently conjugative. Cotransfer experiments showed that when the transfer of the *netB*-carrying plasmid was selected, the rate of cotransfer of the tetracycline resistance plasmid was very high (90%), but when transfer of tetracycline resistance was selected, cotransfer of the *netB*-carrying plasmid was only 1% (19). Sequence analysis showed that all three plasmids carried a pCW3-like *tcp* conjugation locus. To our knowledge, this was the first time that a bacterial strain had been shown to carry three independently conjugative plasmids that all have virtually the same conjugation locus. A similar situation is also probably common among *C. perfringens* type B, C, and D strains, since they often carry two or more toxin plasmids with a *tcp* locus (15–17).

All conjugative *C. perfringens* plasmids identified to date have the *tcp* locus, which has been demonstrated to be essential for conjugative transfer of pCW3 (236, 242–245). Furthermore, either sequence analysis (13, 14, 23) or Southern hybridization analysis (14–18) indicated that many *C. perfringens* type B to E strains contain multiple large plasmids carrying toxin genes (*cpb*, *etx*, *iapA/iapB*, *cpb2*, and *tpeL*) and a *tcp* locus, which we assume is a reasonable predictor of their conjugative potential. Similarly, it has been shown that necrotic enteritis strains of *C. perfringens* type A also carry multiple plasmids that all have the *tcp* locus (19, 235).

Functional Analysis of the *tcp* Conjugation Locus

Analysis of the conjugation mechanism in *C. perfringens* has focused on the tetracycline resistance plasmid pCW3 (224, 227, 228), which is 47,263 bp and encodes 51 potential open reading frames (236). As mentioned above, a conjugation locus of 11 genes, *intP* to *tcpJ*, has been designated the transfer of clostridial plasmid (*tcp*) locus. This locus is present in all known conjugative *C. perfringens* plasmids and is related to the conjugation locus from the Tn*916* conjugative transposon family. Detailed mutagenesis and complementation studies (244) have shown that many of these *tcp* locus genes are required for the optimal conjugative transfer of pCW3 (Fig. 5).

Plasmid conjugation systems generally consist of two components: (i) a relaxosome-DNA complex that includes a plasmid-encoded relaxase enzyme, which binds to the plasmid and nicks one strand at the origin of transfer (*oriT*) site, and (ii) a membrane-bound transfer apparatus through which a relaxase–single-stranded-DNA (ssDNA) complex passes from the donor strain into the recipient (246). There is no apparent relaxase gene carried on pCW3 (236), but the first gene in the putative *tcp* operon is *intP*, which likely encodes a tyrosine recombinase that may act as the functional equivalent to a relaxase in the pCW3 transfer process.

The *tcpA* gene product is essential for conjugation: *tcpA* mutants cannot transfer, and conjugation proficiency is restored by complementation with the wild-type *tcpA* gene (243). The next gene, *tcpB*, appears to be a truncated variant of *tcpA* that is probably derived from a gene duplication event. It is not present in many of the conjugative *C. perfringens* plasmids and is not required for conjugative transfer. TcpA apparently functions as the coupling protein that docks the putative relaxosome complex to the conjugation apparatus. It has two N-terminal transmembrane domains and a cytoplasmic domain that includes an FtsK-like ATPase domain found in proteins of the DNA translocase superfamily (247). These proteins include FtsK and SpoIIIE, which are

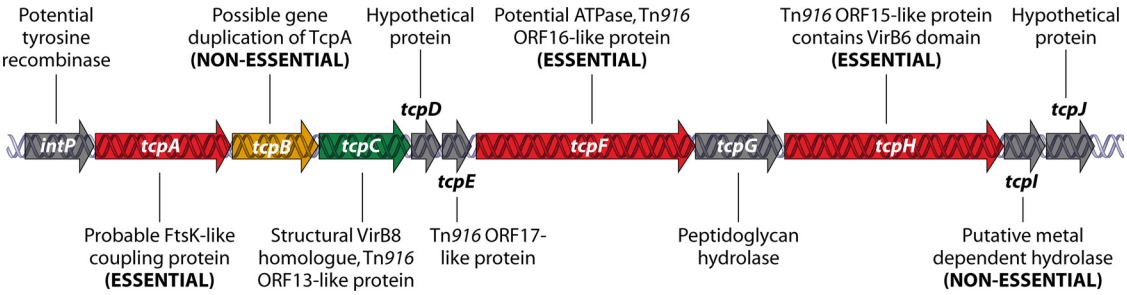

FIG 5 The pCW3 *tcp* locus. The genetic organization and protein products of the *tcp* locus are shown. (Adapted from reference 244 with permission of the publisher [copyright 2012 Blackwell Publishing Ltd.].)

involved in double-stranded-DNA (dsDNA) translocation, and coupling proteins from plasmid conjugation systems. Mutagenesis studies showed that both the ATPase motifs present in TcpA and an FtsK-like RAAG motif are essential for TcpA function (243).

Since TcpA was proposed to act as a coupling protein, it was anticipated that it should undergo protein-protein interactions with other components of the *C. perfringens* conjugation apparatus. TcpA forms homodimers and interacts with TcpC, TcpG, and TcpH, all of which are encoded within the *tcp* locus (248). Mutation of the ATPase domain of TcpA reduced TcpA homodimer formation, and deletion of the putative TcpA N-terminal transmembrane domains also affected plasmid transfer. Analysis of the latter derivative showed that it had reduced TcpA self-interaction as well as less interaction with TcpC and TcpH.

Mutagenesis of the *tcpC* gene revealed that it was required for optimal conjugative transfer; a *tcpC* mutant has a transfer frequency that is 5 orders of magnitude lower than that of the wild type. Again, complementation restored activity to wild-type levels (244). The TcpC protein has 24% amino acid sequence identity to ORF13 from Tn*916* (236), but neither of these proteins has any domains suggesting protein function. However, TcpC does have an N-terminal transmembrane domain, and it was shown that this domain is required for TcpC function, for its independent localization to the *C. perfringens* cell envelope, and for its protein-protein interactions (244). The soluble C-terminal region of TcpC has been purified, and its crystal structure was determined to a resolution of 1.8 Å (244). TcpC crystallized as a trimer, with each monomer consisting of two domains: the central and C-terminal domains. These domains are structurally related to each other and to the structure of the periplasmic region of VirB8, which is an important component of the type IV T-DNA conjugation system from *Agrobacterium tumefaciens*. VirB8 is thought to function as a scaffolding protein that promotes transfer complex assembly and stabilization (249, 250). Note that although TcpC and VirB8 have structural similarity, they have no amino acid sequence identity. Protein-protein interaction studies showed that TcpC interacts with itself, TcpA, TcpG, and TcpH. Within the structure of the TcpC trimer, the central domains are located internally and form most of the contacts within the trimer, which is consistent with this domain being important for TcpC homooligomerization. In contrast, the C-terminal domain occupies most of the external surfaces, and protein-protein interaction analysis showed that this domain is most important for interactions with TcpA, TcpG, and TcpH (244). These results suggest that TcpC may play a role similar to that of VirB8 in the assembly and stability of the pCW3 conjugation apparatus.

TcpD and TcpE are small putative transmembrane proteins of 115 and 122 amino acids, respectively (236). TcpD has no sequence similarity to proteins in the database, but TcpE has 27% identity to the ORF17 conjugation protein from Tn*916*, the precise function of which is unknown. Similarly, the role of the *tcpD* and *tcpE* genes, as well as the *tcpJ* gene, which encodes a hypothetical protein, during the conjugative transfer of pCW3 is unclear.

The TcpF protein contains a conserved ATPase domain, is related to ORF16 from Tn*916*, and is likely to provide at least some energy required to drive plasmid DNA transfer. It is essential for conjugation, since mutagenesis of the *tcpF* gene eliminates pCW3 transfer, which subsequently can be restored by complementation (236). Immunofluorescence studies have shown that TcpF and TcpH are localized to the poles of donor cells, suggesting that pCW3 is transferred through a conjugation apparatus located at the cell poles (242).

TcpH is a large 832-amino-acid protein that has similarity to ORF15 from Tn*916* and has been shown to be essential for pCW3 transfer (236). It has an N-terminal region with eight putative transmembrane domains, including a VirB6-like region, and a putative cytoplasmic C-terminal domain. TcpH is located in the cell envelope of *C. perfringens* (242). Mutagenesis studies have shown that the N-terminal domain (amino acids 1 to 581), a conserved $_{242}$VQQPW$_{246}$ motif, and transmembrane domains 5 to 8 (from amino acids 311 to 450), which include the VirB6-like domain, are essential for TcpH function (242). A combination of bacterial two-hybrid experiments and protein-protein interaction studies showed that TcpH interacts with itself, TcpA, and TcpC (242, 248) and that the N-terminal domain, but not the VQQPW motif, is required for these interactions (242). It is proposed that TcpH is the major structural protein of the pCW3 conjugation apparatus and that it forms the transmembrane channel through which the plasmid DNA complex passes from the donor to the recipient cell.

Peptidoglycan hydrolases are commonly associated with bacterial conjugation systems, presumably facilitating the formation of the conjugation apparatus in the cell wall. Unusually, the *tcp* locus appears to encode two functionally distinct peptidoglycan hydrolases, TcpG and TcpI. Mutagenesis studies have shown that TcpG, but not TcpI, is required for optimal transfer of pCW3 (245). TcpG has peptidoglycan hydrolase-like activity. It has two putative catalytic domains, an N-terminal muramidase-like FlgJ glucosaminidase domain and a C-terminal NlpC/P60 endopeptidase

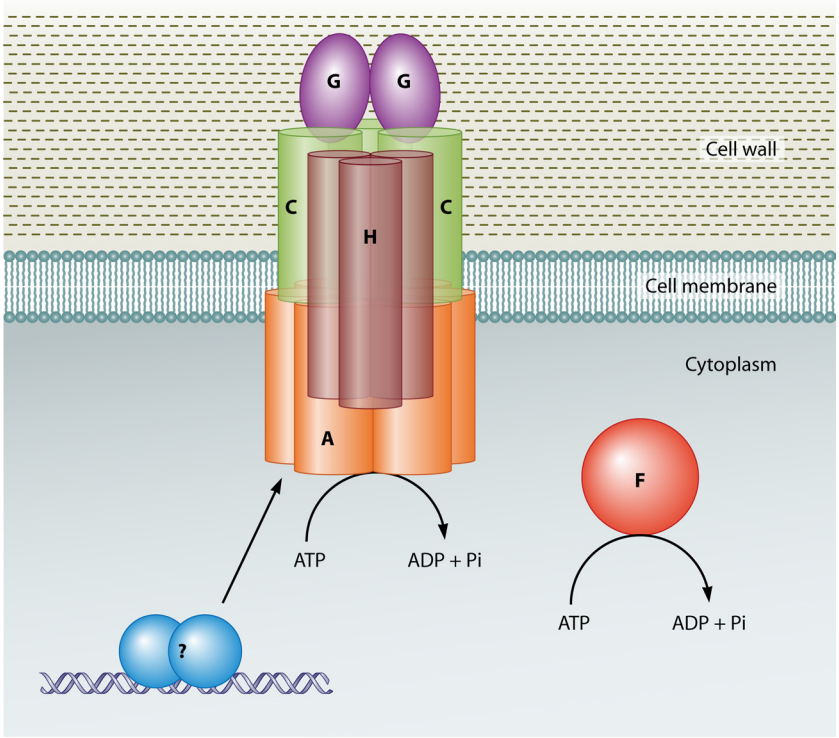

FIG 6 Putative model of the pCW3 conjugation apparatus. The relative locations and known protein-protein interactions of the TcpA (A) (orange), TcpH (H) (brown), TcpC (C) (green), TcpG (purple), and TcpF (F) (red) proteins are based on data from previous studies (236, 242, 244, 245, 248). (Based on a model prepared by Jessica Wisniewski, Monash University.)

domain, both of which have been shown to be functional (245). TcpG interacts with TcpA (248) and TcpC (244) but not with TcpH (242). Based on these data, a model for the pCW3 conjugation apparatus has been proposed (244, 248), as shown in Fig. 6. In this model, the VirB6-like TcpC protein acts as a scaffolding protein that helps form a complex at the cell envelope. This complex includes TcpC, the coupling protein TcpA, the peptidoglycan hydrolase TcpG, and TcpH, which is proposed to form the cell wall pore through which the plasmid DNA is transported into the recipient cell. Further genetic and structural studies are required to determine the roles of the putative IntP, TcpD, and TcpE proteins and to determine how these proteins, and TcpF, interact with the conjugation apparatus.

Replication of Toxin Plasmids

An important factor in the ability of plasmids to replicate autonomously is the self-encoded plasmid replication initiator protein, often referred to as the Rep protein. This protein recognizes plasmid-specific DNA sequences and determines the point from which replication starts. Rep proteins generally share signature domains that enable them to be assigned to one of several replication initiation families (251).

Unexpectedly, analysis of the nucleotide sequences of the large conjugative clostridial plasmids, including pCW3, failed to identify a potential replication protein based on amino acid sequence identity or domain searches. Subcloning of pCW3 and analysis of the replication ability of the resultant derivatives identified a 3,918-bp fragment that encoded the ability to replicate independently in *C. perfringens* (236). Subsequent transposon mutagenesis studies of a recombinant shuttle plasmid containing this region then led to the identification of the *rep* gene carried by pCW3. Transposon insertions that mapped to the *rep* gene resulted in an inability of the shuttle plasmid to replicate in *C. perfringens.* The region upstream of the *rep* gene contained four 17-bp direct repeats that were postulated to act as the iteron-like sequences that presumably would be required for the Rep-mediated initiation of plasmid replication (236). The putative Rep protein had no similarity to proteins or motifs of known function in the databases but had a pI of 10, in keeping with its proposed function as a DNA binding protein. An almost identical Rep protein, with 95 to 100% amino acid sequence identity, is encoded by all of the sequenced conjugative toxin and resistance plasmids described in this review, providing evidence that all of these plasmids replicate by the same mechanism. The fact that these Rep proteins are unique to *C. perfringens* may explain why this family of plasmids has not been detected in any other species (236). The tetracycline and chloramphenicol resistance plasmid pIP401, which is closely related to pCW3, can transfer by conjugation from *C. perfringens* to *C. difficile*, but the resultant transconjugants appear to be unstable (252), presumably because the Rep protein is not functional in *C. difficile*. Nonetheless, such conjugation events may explain why very closely related mobilizable chloramphenicol resistance transposons, Tn*4451* and Tn*4453*, are found in *C. perfringens* and *C. difficile*, respectively (253), and how the pCW3-encoded Tet(P) tetracycline resistance determinant has moved to *Clostridium septicum* and *Clostridium sordellii* (254). Similar events could explain the presence of related toxin genes in several different clostridial species (see Concluding Remarks).

Finally, it is very common for *C. perfringens* strains to carry

several very closely related, but independently conjugative, plasmids that carry different toxin or resistance genes (15–19). There is no real precedent for this observation in other bacterial species, which raises the question as to what is the basis for the compatibility of these plasmids. It was suggested (19) that differences in a *parRMC* locus located upstream of the common *rep* gene may be responsible. In agreement with this hypothesis, other workers (235) analyzed the known *parRMC* loci of all of the sequenced plasmids that carry the *tcp* locus and divided them into four distinct groups. These groups are consistent with the known compatibility of the conjugative plasmids, but it is essential that this hypothesis be verified experimentally.

CONCLUDING REMARKS

It has now been established that *C. perfringens* maintains a large pool of closely related plasmids that are potentially moving from one cell to another via conjugation. These plasmids also have regions that seem to act as hotspots for the integration of mobile elements that are associated with plasmid-carried toxin genes. The net consequence is that some *C. perfringens* cells now carry multiple (at least up to three) different toxin plasmids. However, plasmid incompatibility issues apparently place some limitations on the total repertoire of toxin plasmids that can be maintained by a single *C. perfringens* bacterium. Perhaps the best evidence for toxin plasmid incompatibility issues is the absence of certain toxin plasmid combinations; e.g., *C. perfringens* strains carrying both iota-toxin- and *cpb*-harboring plasmids are never identified.

It is also now clear that a single toxin gene can reside on many different plasmids among the *C. perfringens* population. Since these plasmids often share large (~35 kb) regions of identical sequences, there must be strong selective pressure to maintain this large pool of different toxin plasmids, or, considering their extensive shared regions of sequence identity, homologous recombination would otherwise rapidly lead to the evolution of plasmids that carry the conjugation locus and numerous toxin genes. However, there does appear to be some evolutionary movement toward that eventual outcome, as individual *C. perfringens* plasmids that can carry up to three different toxin genes have been identified.

C. perfringens likely maintains a large number of toxin genes on different conjugative plasmids because this strategy offers enormous virulence plasticity and adaptability. One example illustrating this principle would be the presence of *cpb* and *etx* genes on two different plasmids. Type C strains carrying only a CPB plasmid cause disease in hosts with lower intestinal trypsin levels due to age, diet, or disease, which allows CPB to persist and act for a longer duration in the intestines. In contrast, type D strains carrying an ETX plasmid cause illness in animals with normal protease levels, which proteolytically activates ETX. Type B strains, which have acquired both the CPB and ETX plasmids, have the versatility to cause disease at either low or normal intestinal protease levels.

C. perfringens is not the only pathogenic clostridial species that utilizes toxin plasmids for virulence. The neurotoxins of *Clostridium botulinum* and *Clostridium tetani* can also be plasmid encoded, and some botulinum toxin-encoding plasmids were recently shown to be conjugative, possibly involving a truncated *tcp*-like locus (255, 256). However, *C. perfringens* is remarkable for carrying so many different plasmid-encoded toxins. Why?

Studies are now revealing that the ability to produce plasmid-encoded toxins extends the disease spectrum of *C. perfringens*; i.e., these toxins are often important when this bacterium causes enteritis or enterotoxemia. Simple type A isolates (i.e., those strains producing chromosomally encoded PFO and CPA but no plasmid-encoded toxins) are virulent, since they cause histotoxic infections (4, 257). However, these simple type A strains rarely cause enteritis or enterotoxemia. The limited intestinal pathogenicity of type A strains producing only PFO and CPA is consistent with the common presence of these strains as innocuous normal intestinal flora and studies showing that inactivation of *pfoA* or *plc* genes in type C strains (210), or *plc* genes in NetB-producing type A strains (194), has little effect on the ability of those toxin null mutants to cause infections originating in the intestines.

Instead, when causing enteritis or enterotoxemia, *C. perfringens* usually relies upon plasmid-encoded toxins; the chromosomal *cpe* type A strains causing most cases of *C. perfringens* type A food poisoning represent the exception to this generalization, but even these strains use a toxin gene that is apparently associated with a mobile element (238) that may have mobilized from a plasmid (40). This strong association between *C. perfringens* plasmid-encoded toxins and enteritis or enterotoxemia likely involves, at least in part, the conjugative nature of the many toxin plasmids carried by this bacterium; that is, when a *C. perfringens* cell carrying a conjugative toxin plasmid is introduced into the intestines, it may then transfer its toxin plasmid to the normal resident *C. perfringens* strains. This *in vivo* plasmid transfer would likely impact the virulence properties of the recipient strain, as we have demonstrated *in vitro* for transconjugants receiving an *etx*-carrying plasmid (21), where the type A recipient strains were converted to ETX-producing type D strains. It is important that conjugative transfer of toxin plasmids between invading and resident *C. perfringens* strains be demonstrated experimentally *in vivo*, but this is not a simple task.

Since *C. perfringens* strains in normal flora are presumably under selective pressure for colonization and persistence in the intestines, this putative conjugative transfer of toxin plasmids to colonization-proficient *C. perfringens* strains in normal flora should help to establish and amplify infections originating in the intestines. For example, this effect could explain why the symptoms of CPE-associated non-food-borne GI diseases, which are caused by type A plasmid *cpe* strains, are more severe and of longer duration than the symptoms of *C. perfringens* type A food poisoning, which typically involves type A strains carrying a chromosomal *cpe* gene (241).

The putative *in vivo* augmentation of pathogenicity virulence by conjugative toxin plasmid transfer is likely to be important for establishing *C. perfringens* diseases originating in the intestines but should typically represent only one early step in pathogenesis. Factors altering the intestinal host defenses, the normal flora microbiome, or the intestinal environment also contribute to most cases of *C. perfringens* enteritis or enterotoxemia. For example, age, diet, or disease can reduce trypsin activity in the intestines, prolonging the presence of active CPB in the intestines (3). Alternatively, changing the normal intestinal flora by diet, antibiotic use, or coinfections with other pathogens is often necessary for a toxin plasmid-carrying *C. perfringens* strain to multiply sufficiently to reach pathogenic levels in the intestines or to gain access to the intestinal mucosa (3).

The presence of many *C. perfringens* toxin genes on conjugative plasmids may also have far-ranging virulence consequences. Some plasmid-borne toxin genes may have conjugatively transferred to

other clostridial spp. Perhaps, in combination with the mobilization of these toxin genes by associated insertion sequences, this interspecies plasmid transfer may have enhanced the pathogenicity of the recipients. For example, this process could explain the presence of ITX-like binary toxins in several other pathogenic clostridial spp., e.g., *C. difficile* and *Clostridium spiroforme*. Similarly, the presence of *tpeL* genes on conjugative plasmids in *C. perfringens* is notable given the widespread distribution among pathogenic clostridial spp. of genes encoding large glycosylating toxins. Conceivably, future toxin plasmid transfers could create additional strains with unique or enhanced virulence attributes.

Finally, while virulence plasmids clearly play a major role in many *C. perfringens* infections, it is becoming apparent that strain clonality is also an important contributing factor for pathogenicity. The concept of distinct *C. perfringens* lineages was first reported after MLST of type A chromosomal *cpe* strains versus other *C. perfringens* strains (39), and this conclusion has been supported by additional MLST studies (258) and DNA microarray analyses (259). Later, Darmbrand strains were shown to share a close genetic relationship with type A chromosomal *cpe* strains, even though these type C strains carry plasmid-borne *cpe* and *cpb* genes (40). The shared genomic background between the chromosomal *cpe* type A and type C Darmbrand strains allows production of exceptionally resistant spores, which in turn likely increases the transmissibility of these strains during food-borne illness. Similarly, it is now being established that type A avian necrotic enteritis strains are another distinct *C. perfringens* lineage. Besides carrying several virulence-associated plasmids, including the *netB*-carrying plasmid, type A necrotic enteritis strains also typically possess the unique NeLoc2 chromosomal pathogenicity locus (235, 260). It is not yet clear whether NeLoc2 directly enhances the virulence fitness of these strains or instead helps to retain these virulence plasmids.

While much has been learned recently regarding the biology and virulence contributions of *C. perfringens* toxin plasmids, many important questions remain unanswered regarding these plasmids and their toxins. For example, the receptors for most of the plasmid-encoded toxins have not yet been identified. In addition, the structure-versus-function relationship for many of the plasmid-encoded toxins is incompletely understood. With respect to the toxin plasmids themselves, further insights will be gained by sequencing and studying those toxin plasmids that are not closely related to pCPF5603 or pCPF4969. Similarly, the issue of toxin plasmid incompatibility remains to be elucidated experimentally, and the possible interspecies transfer of conjugative *C. perfringens* toxin plasmids should be investigated. The contribution of *C. perfringens* clonality to toxin-mediated diseases is an emerging topic that requires mechanistic study. Finally, further studies are needed to evaluate whether the many non-toxin-encoding genes carried on toxin plasmids also contribute to virulence and whether plasmids encode other, still unrecognized, toxins. These and many other intriguing issues are the subject of planned future studies in our laboratories.

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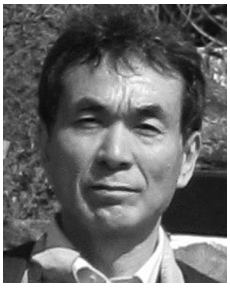

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