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Enterotoxic Clostridia: *Clostridium perfringens* Enteric Diseases

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CHAPTER SUMMARY

In humans and livestock, *Clostridium perfringens* is an important cause of intestinal infections that manifest as enteritis, enterocolitis or enterotoxemia. This virulence is largely related to the toxin producing ability of *C. perfringens*. This chapter primarily focuses on the *C. perfringens* type F strains that cause a very common human food poisoning and many cases of nonfoodborne human gastrointestinal (GI) diseases. The enteric virulence of type F strains is dependent upon their ability to produce *C. perfringens* enterotoxin (CPE). CPE has a unique amino acid sequence, but belongs structurally to the aerolysin pore-forming toxin family. The action of CPE begins with binding of the toxin to claudin receptors, followed by oligomerization of the bound toxin into a prepore on the host membrane surface. Each CPE molecule in the prepore then extends a β-hairpin to form, collectively, a β-barrel membrane pore that kills cells by increasing calcium influx. The *cpe* gene is typically encoded on the chromosome of type F food poisoning strains but is encoded by conjugative plasmids in nonfoodborne human GI disease type F strains. During disease, CPE is produced when *C. perfringens* sporulates in the intestines. Beyond type F strains, *C. perfringens* type C strains producing beta toxin and type A strains producing a toxin named CPILE or BEC have been associated with human intestinal infections. *C. perfringens* is also an important cause of enteritis, enterocolitis and enterotoxemia in livestock and poultry due to intestinal growth and toxin production.

INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming rod with a ubiquitous environmental distribution, including a presence in the soil and sewage [1–3]. Given its anaerobic nature, it is unsurprising that *C. perfringens* is also a component of the normal gastrointestinal (GI) tract microbiota of humans and other animals [1, 2, 4]. Additionally, this bacterium is an important cause of intestinal and histotoxic infections in human and other animals [2, 5, 6].

The pathogenicity of *C. perfringens* largely reflects the toxin-producing proficiency of this bacterium, which can make at least 20 different toxins, including many with GI tract activity

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[6–10]. However, toxin production patterns vary greatly amongst different strains. This variability permits classification of *C. perfringens* isolates based upon their production of certain toxins referred to as typing toxins. Historically, five *C. perfringens* types were recognized, but this system was recently expanded (11) to include seven types (A-G), as shown in Table 1.

Due to their medical importance, this chapter will primarily focus on type F strains of *C. perfringens*. It will also briefly mention other *C. perfringens* strains causing human or livestock GI diseases.

C. perfringens TYPE F FOOD POISONING

Epidemiology—Type F strains are the causative agent of *C. perfringens* type F food poisoning (formerly referred to as *C. perfringens* type A food poisoning prior to the recent typing system revision), which is the 2nd most common bacterial food-borne illness [12, 13]. A million cases of this disease occur annually in the USA, involving ~$400 million in economic losses [12–14]. The high prevalence of *C. perfringens* type F food poisoning is attributable to several factors [1]. First, type F strains are common in foods [15, 16], providing ample opportunity for infection if that food is incompletely cooked or held prior to ingestion. Second, this bacterium has a very short doubling time of ~10 minutes, which allows it to grow quickly in contaminated food to reach the bacterial load (>10^6 vegetative cells/gram of food) needed to initiate GI disease. Last, most *C. perfringens* type F food poisoning strains have an outstanding ability to survive in incompletely cooked or improperly held foods due to: i) the heat tolerance of their vegetative cells, which grow up to at least 50°C [17] and ii) the even greater heat, cold and food preservative resistance of their spores, as discussed later.

C. perfringens type F food poisoning outbreaks are typically large, averaging ~100 cases [1]. This large outbreak size is attributable to at least two factors. First, *C. perfringens* type F food poisoning is strongly associated with institutional settings because such facilities need to prepare and store foods in advance of serving, which provides sufficient time for *C. perfringens* to grow and reach pathogenic loads in improperly cooked or held foods. Second, public health officials often overlook small food poisoning outbreaks not involving fatalities; therefore, small outbreaks of this food poisoning typically go unidentified.

The most important contributing factor to *C. perfringens* type F food poisoning outbreaks is holding foods under improper temperatures [1, 13]. Because of the relative heat tolerance of *C. perfringens* vegetative cells, food should either be stored under refrigeration or held at temperatures >65°C to prevent growth of this bacterium. Incomplete cooking is the 2nd leading contributor to development of this food poisoning since it allows *C. perfringens* spores to survive and then germinate in undercooked foods. Beef roasts and turkey are among the most common vehicles for this food poisoning because those large food items are difficult to cook thoroughly [1, 13].

The laboratory plays an important role in identifying *C. perfringens* type F food poisoning outbreaks [1]. The most reliable approach to identify this food poisoning involves serologic demonstration of *C. perfringens* enterotoxin (CPE) in feces from food poisoning victims.

Several commercial assays for fecal CPE detection are available. Alternatively, it can also be informative to demonstrate, by PCR, the presence of *cpe*-positive *C. perfringens* in foods or feces.

Pathogenesis—*C. perfringens* type F food poisoning begins with the ingestion of food contaminated with vegetative cells of a type F strain [1]. Most of those bacteria die upon exposure to gastric acid but, if the ingested food was heavily contaminated (i.e., $>10^6$ to 10^7 vegetative cells/gram of food), some survive to pass into the intestines. After initial growth, those *C. perfringens* cells then undergo *in vivo* sporulation. The trigger for this response is unclear but may involve exposure of ingested *C. perfringens* cells to low pH in the stomach or to bile salts or phosphate in the intestines [18–20].

Considerable evidence implicates CPE as the toxin responsible for the GI symptoms of *C. perfringens* type F food poisoning (reviewed in [1]). First, this toxin is detectable in the feces of nearly all *C. perfringens* food poisoning victims, but is absent from the feces of healthy people. Second, the CPE concentrations present in feces of food poisoning victims are similar to the CPE concentrations that cause GI effects in animal models. Third, type F strains are much more virulent in animal intestinal disease models than are most CPE-negative type A strains, which (like type F strains) produce alpha toxin but (unlike type F strains) do not produce CPE. Importantly, the GI effects of type F strains can be neutralized with a CPE antibody. Fourth, human volunteers fed purified CPE developed the GI symptoms of *C. perfringens* type A food poisoning. Last, and perhaps most persuasive, are studies fulfilling molecular Koch's postulates, which showed that an isogenic *cpe* null mutant of a type F food poisoning strain is fully attenuated in a rabbit small intestinal loop model of GI disease and complementation of that mutant to regain CPE production also fully restored virulence [21]

Only sporulating *C. perfringens* cells produce CPE [1, 22, 23]. Once made, this toxin is not secreted but is instead freed into the intestinal lumen only when the mother cell lyses to release its mature endospore upon the completion of sporulation [24]. CPE then binds to the intestinal epithelium and exerts its cytotoxic action, described later, causing villus shortening and intestinal epithelial desquamation. Animal model studies suggest this intestinal damage is responsible for the onset of the GI symptoms associated with *C. perfringens* type F food poisoning [25–27], although contributions by other processes (such as CPE effects on paracellular permeability) cannot be excluded.

All regions of the small intestine respond to CPE, but the ileum is particularly sensitive [28, 29]. Interestingly, while CPE binds to, and initially damages, villus tips in the small intestine, the toxin eventually damages the entire villus [25]. Recent cell culture studies, described later, identified a bystander killing effect that may help to explain this *in vivo* observation [30].

Based upon an initial study where CPE did not affect the rabbit colon [31], it was originally believed that *C. perfringens* type F food poisoning only involves the small intestine. However, later studies demonstrated that purified CPE does affect the rabbit colon [32] and it can also damage human colonic tissue *ex vivo* [33]. Furthermore, colonic damage has

been observed in some human *C. perfringens* type F food poisoning victims [34]. Recent studies suggest that CPE acts focally in the human colon, mainly damaging cells with exposed receptors [35].

Clinically, *C. perfringens* type F food poisoning usually involves diarrhea and abdominal cramps that develop within ~8–16 h of ingesting contaminated food [1]. This incubation period largely reflects the ~8–12 h needed for *C. perfringens* to complete sporulation and then release CPE into the intestinal lumen. In most cases, the GI symptoms of this food poisoning persist for 12–24 h before self-resolving. However, it has long been recognized that this disease can be fatal in the elderly or debilitated; it is estimated that ~26 people die from *C. perfringens* type F food poisoning every year in the USA [12].

In addition, two unusually severe *C. perfringens* type F food poisoning outbreaks occurred during the past 15 years, resulting in deaths of several relatively healthy and younger people [34, 36]. Both outbreaks involved psychiatric institutions, which is relevant because the severity of these outbreaks appears attributable to the victims having predisposing medical conditions prior to contracting *C. perfringens* type F food poisoning. Specifically, the severely affected individuals in both outbreaks had been receiving psychoactive medications that can induce severe constipation or fecal impaction side-effects. Those side-effects likely prevented the GI tract flushing effects of the diarrhea that typically occurs during *C. perfringens* type F food poisoning. The absence of diarrhea in these cases is thought to have prolonged intestinal contact with CPE, thus enhancing disease severity. Studies with a mouse model [37] supported this hypothesis by showing that prolonged intestinal contact with purified CPE increases uptake of this toxin into the circulation. This allowed CPE to damage internal organs, such as the liver and kidneys, resulting in a hyperpotassemia that induces cardiac arrest. Those mouse model studies suggest that pre-existing severe constipation or fecal impaction can facilitate development of a lethal enterotoxemia following *C. perfringens* type F food poisoning [37].

The CPE Protein—CPE is a single 319 amino acid polypeptide (M_r 35,317, pI 4.3) [38]. Its primary amino acid sequence lacks significant sequence homology with other proteins, except for some limited sequence homology (of unknown significance) with a non-neurotoxic protein of *C. botulinum* [39]. The secondary structure of CPE is ~80% β-pleated sheet and ~20% random coil [28].

The structure of CPE was solved in a series of studies that began with crystallization and x-ray diffraction analysis of the C-terminal domain of CPE, which mediates binding of the toxin to receptors, as discussed below. That initial study [40] showed this CPE domain consists of a nine-stranded β-sandwich, with most strands in an antiparallel arrangement. Tyrosine and leucine residues implicated ([41] and as described later) in receptor binding line a pocket located on the surface of this C-terminal CPE domain, where they can readily interact with receptors [40].

The complete structure of native CPE was then solved independently by two groups in 2011 [42, 43]. Those structural analyses (Fig. 1, left panel) revealed that, in addition to the C-terminal domain mediating receptor binding activity, this pore-forming enterotoxin also has

an N-terminal domain required for CPE oligomerization and pore formation. The 34 N-terminal amino acids of CPE appear to be disordered, explaining why their presence reduces CPE-induced cytotoxicity (see below). Although lacking primary sequence homology with other proteins, CPE structurally belongs to the aerolysin pore-forming toxin family, which also includes *C. perfringens* epsilon toxin. A region in the N-terminal domain named TM1 (for transmembrane 1), which is important for pore formation (as discussed later), is largely present in an α helix when the native CPE monomer is in solution. This is distinctive since comparable sequences in other members of the aerolysin pore-forming toxin family are present in a β strand [42, 43].

Structure vs function relationships have been extensively mapped for the CPE protein. Early deletion mutagenesis research [44], coupled with chemical cleavage analyses [45], revealed that the C-terminal domain of the toxin is sufficient for receptor binding. That research also showed the N-terminal domain must also be present to obtain cytotoxicity and cause GI tract pathology. Later studies using recombinant CPE fragments and synthetic peptides showed that even the 30 C-terminal amino acids of CPE alone possess some receptor binding activity [46]. Subsequent site-directed mutagenesis results identified three tyrosine residues (Y306/Y310/Y312) and a leucine triplet (L223/L254/L315) in the C-terminal half of CPE as being important for receptor binding [41].

While full-length CPE (amino acids 1–319) is cytotoxic, removing up to the first 45 amino acids from CPE by deletion mutagenesis increases cytotoxic activity by 2–3 fold [47]. This activation effect, which does not correspond to removal of a leader sequence since CPE is not a secreted protein, may have pathologic relevance, i.e., purified trypsin and chymotrypsin also induce a 2–3 fold increase in CPE-induced cytotoxic activity by removing the first 25 or 37, respectively, N-terminal amino acids from the native toxin [48, 49]. However, deleting amino acids beyond residue 45 renders CPE noncytotoxic [50]. Subsequent site-directed mutagenesis studies identified several amino acids located between residues D45 and G53 of the native toxin as mediating CPE oligomerization [51]. In particular, residue D48A is required for both CPE oligomerization and cytotoxicity [51].

Besides oligomerization, the N-terminal domain of CPE mediates a second function during CPE-induced cytotoxicity. This CPE domain contains TM1, a region consisting of alternating hydrophobic and hydrophilic residues spanning from amino acids 80 to 106 of native CPE [52, 53]. As mentioned earlier, the TM1 region is mainly localized within an alpha helix in the N-terminal domain of native CPE. However, once CPE oligomerizes, the TM1 α helix is thought to unwind to form a β-hairpin loop that participates in pore formation (as described in the next section).

CPE-induced Cytotoxicity

CPE binding—The cytotoxic action of CPE begins (Fig. 2) with binding of this toxin to its receptors [54]. Conclusive insights into CPE receptors were provided by expression cloning studies that identified two CPE-binding host proteins, later determined to be claudins-3 and −4 [55, 56]. Claudins are a family of ~21 to 27 kDa single polypeptides that play a critical role in maintaining the normal barrier and fence properties of the tight junction [54]. Humans are thought to produce 27 different claudins [54]. Structural analyses [57] revealed

that claudins consist of a four transmembrane domain bundle and two extracellular loops, referred to as ECL-1 and ECL-2. Claudins also possess a short (~7 amino acid) N-terminal sequence and a C-terminal tail (~20–60 amino acid), both of which are located in the host cell cytoplasm. The sequence of the C-terminal tail is variable, allowing discrimination between different claudins using antibodies raised against synthetic peptides with a claudin tail sequence.

Experiments established that, in addition to claudins-3 and −4, CPE can use claudins −6, −7, −8-, −9 and −14 as receptors [54, 58, 59]. The CPE binding affinity amongst those claudin receptors varies greatly, with some (e.g., claudin-3 and −4) binding CPE strongly, but others (e.g., claudin-8 and −14) binding CPE with only moderate affinity [59]. Furthermore, not all claudins are CPE receptors, as claudins-1, −2, −5, −10, −13 and 20–24 cannot bind significant amounts of CPE at physiologically-relevant toxin concentrations [54, 58, 59, 61, 62].

Substantial research has investigated the basis for the different CPE binding abilities between claudins. Early studies used chimeric claudins [54, 58] to demonstrate the importance of ECL-2 for CPE binding. Later work [54, 59, 62] identified specific amino acids in ECL-2 that contribute to CPE binding. In particular, an N residue is conserved in the ECL-2 of receptor binding claudins, while the corresponding residue is never an N (usually it is a D or S) in claudins unable to bind the toxin [62]. Site-directed mutagenesis studies [62] directly confirmed the importance of this conserved N residue for CPE binding. ECL-2 residues adjacent to the essential N residue vary among the receptor claudins and those variations modulate the CPE binding affinity of different receptor claudins [62–64]. Structural biology studies demonstrated that the N residue and adjacent P and L/S residues dock tightly in the tyrosine pocket of the C-terminal CPE domain [57, 65].

Recently, the structure of the CPE C-terminal domain bound to a claudin receptor was solved [57, 66] (Fig. 1, right panel), which provided a major surprise by showing that CPE interacts with ECL-1, as well as with ECL-2. ECL-1 sequences are conserved in all claudins, explaining why previous chimeric claudin studies did not pick up CPE interactions with ECL-1. The strong sequence conservation amongst the ECL-1 of different claudins is also consistent with a model where ECL-2 is the primary determinant of CPE binding ability amongst the claudins, although CPE does also interact with the conserved ECL-1 in receptor claudins. Supporting the importance of CPE interactions with ECL-1 for receptor binding were site-directed mutagenesis studies showing that mutation of ECL-1 residues A39-I41 affects CPE binding to claudin receptors [57].

Post binding steps in CPE action—Binding of CPE to a claudin receptor results in the formation of a small complex of ~90 kDa [67]. Co-immunoprecipitation and electroelution results [68] suggest this small complex can contain a non-receptor claudin (e.g., claudin-1), as well as CPE bound to the claudin receptor. Heteromer gel shift studies [68] indicated that 6 small CPE complexes then oligomerize to form the CH-1 large complex, with a molecular mass of ~425–500 kDa. It is believed that CH-1 is initially present as a prepore on the membrane surface of host cells but each CPE in the prepore then unwinds its TM1 α helix to form a β-hairpin loop [52, 53]. Similar loops likely extend from each of the 6 CPE

molecules present in the prepore oligomer, forming a β-barrel that inserts into the membrane to create an active pore. In support of this model, site-directed mutagenesis identified several TM1 variants that have normal binding and oligomerization properties but are impaired for membrane insertion or for formation of a functional pore [52].

CPE pore formation leads to a rapid efflux of some cytoplasmic ions (e.g., potassium), but an influx of other extracellular ions, including calcium [28, 69–72]. CPE-induced calcium influx is particularly important since it activates, in a toxin dose-dependent manner, calpain and calmodulin to cause cell death [70, 73]. At low CPE doses (~1 μg/ml), modest pore formation occurs to cause a limited calcium influx and a mild activation of calpain that triggers caspase-3 dependent apoptosis [70, 73]. However, high CPE doses (~10 μg/ml) form large numbers of pores, allowing a massive calcium influx that results in strong calpain activation to cause a necrotic death involving oncosis [70, 73].

Cells affected by CPE develop morphologic damage [71, 74]. This allows even more CPE binding to the abundant claudin receptors present on the basolateral cell surface [75]. In Caco-2 cells, CPE-induced cell damage also permits some receptor-bound CPE to interact with another tight junction protein named occludin to form an ~600 kDa complex named CH-2 [68, 75, 76]. The importance of this complex for CPE activity, particularly *in vivo*, is not clear since only the CH-1, (i.e., not the CH-2) complex is detectable in CPE-treated rabbit small intestine [37].

CPE Induces a Bystander Killing Effect

The human body is a complex mixture of host cells, including cells that produce claudin CPE receptors and respond to this toxin, as well as other cells that do not make CPE receptors and are not directly sensitive to this toxin. Therefore, a recent study [30] asked whether CPE effects on sensitive host cells might impact nearby CPE-insensitive host cells. When a co-culture of CPE-sensitive and CPE-insensitive cells was treated *in vitro* with the enterotoxin, many of the CPE-insensitive cells died. This bystander killing effect was shown to involve a factor, possibly a 10–30 kDa serine protease, that is released from CPE-sensitive cells when they undergo CPE-induced, caspase 3-mediated apoptosis. The cytotoxic host factor released from those dying cells then induces a caspase 3-mediated apoptosis in the naturally CPE-insensitive cells. Collectively, these results suggest that a similar bystander killing effect may amplify CPE action *in vivo* and thus contribute to disease.

CPE Genetics

Less than 5% of global *C. perfringens* isolates carry the gene (*cpe*) encoding the enterotoxin [77]. Those *cpe*-positive strains belong to types C, D and E, as well at type F [77–81]. In type F strains, the *cpe* gene can be present on either the chromosome or large plasmids [82–84]; no single type F strain has been found that carries both a chromosomal and plasmid-borne *cpe* gene. In other types, the *cpe* gene is always present on large (>70 kb) plasmids [78, 79, 82, 85].

Whether located on plasmids or the chromosome, the *cpe* open reading frame sequence is highly conserved amongst type C, D and F strains [84, 86]. However, type E strains can possess either silent *cpe* sequences (due to loss of the *cpe* promoter and the presence of

mutations, including a nonsense mutation, in the *cpe* ORF) or a functional *cpe* gene that encodes a variant CPE with ~10 amino acid sequence differences from the classical *cpe* ORF found in type C, D and F strains [85, 87]. Whether those amino acid sequence differences affect CPE cytotoxic activity is unknown.

Most (~70%) type F food poisoning strains carry a chromosomal *cpe* gene [1, 88–91]. This association is not due to the chromosomal *cpe* strains producing either more CPE or a CPE variant with greater cytotoxicity [84]. Instead, it is attributable to at least two factors, i) the exceptional resistance of the spores made by most chromosomal *cpe* strains [17], which likely facilitates their survival in incompletely cooked foods and ii) the vast majority of *cpe*-positive strains present in retail raw meats/fish having a chromosomal vs. plasmid-borne *cpe* gene, providing the chromosomal *cpe* strains with much greater opportunity to cause food poisoning [1, 16]. The reason for the greater prevalence of chromosomal *cpe* strains in meats/fish is unknown.

Genome sequencing of strain SM101, a transformable derivative of the type F food poisoning strain NCTC8798, showed that its *cpe* gene is located in a highly variable region of the chromosome [92]. Other chromosomal *cpe* strains appear to carry a similarly organized *cpe* locus [86, 93, 94]. The presence of the *cpe* gene in a highly variable chromosomal region is likely due to the integration of Tn*5565*, a putative 6.4 kb transposon where the *cpe* gene and an upstream IS*1469* sequence are both flanked by IS*1470* sequences [95]. The putative Tn*5565* insertion event apparently occurred in the chromosomal locus between *nadC*, encoding a putative quinolate phosphoryltransferase gene, and *uapC*, encoding a putative purine permease (Fig. 3). The ability of adjacent insertion sequences to mobilize the chromosomal *cpe* gene is suggested by PCR studies, which detected circular DNA species carrying the *cpe* gene in DNA extracted from chromosomal *cpe* strains; those circular DNAs may be transposition intermediates carrying the *cpe* gene [95].

MLST studies [80, 97] showed that, beyond their *cpe* gene location, chromosomal *cpe* type F strains differ phylogenetically from type F plasmid *cpe* strains and most other *C. perfringens* strains, with the exception of the type C strains causing a form of enteritis necroticans known as Darmbrand in post-World War II Germany (further discussion later). Type F chromosomal *cpe* strains and type C Darmbrand strains are both also unusual [80] among *C. perfringens* strains by, i) lacking the *pfoA* gene that encodes perfringolysin O and ii) carrying a *sasp4* gene that encodes a variant small acid soluble protein-4 to impart exceptional spore heat resistance (further description later). However, an important distinction between the type F chromosomal *cpe* strains and the type C Darmbrand strains is that, when Darmbrand strains carry a *cpe* gene, it is located on a large plasmid rather than on the chromosome [80].

The *cpe* plasmids of type F strains belong predominantly to the pCPF5603 and pCPF4969 plasmid families [82, 98]. Both of those plasmid families likely evolved from a common precursor plasmid. The pCPF5603 family later acquired a cluster of metabolic genes, as well as the gene encoding beta2 toxin, while the pCPF4969 family picked up genes encoding a VirS/VirR-like two component regulatory system and a putative bacteriocin. The *cpe* gene in both of these *cpe* plasmid families is associated with insertion sequences (Fig. 3), including

the presence of a 5′ IS*1469* sequence near their *cpe* genes. However, the *cpe* loci in these two *cpe* plasmids lack the upstream IS*1470* sequence upstream found in the chromosomal *cpe* locus. In addition, while an IS*1470* sequence is located downstream of the *cpe* gene in pCPF4969 plasmids, that insertion sequence is absent from the *cpe* locus of the pCPF5603 plasmids. Instead, pCPF5603 plasmids carry an IS*1151* sequence downstream of their *cpe* gene. The presence of these insertion sequences near these *cpe* genes may help to mobilize this toxin gene into circular forms that could be transposition intermediates, as shown for type C and D strains carrying *cpe* plasmids [86].

Insertion sequences are also located adjacent to the plasmid *cpe* gene (Fig.2) in type C, D and E isolates [85, 86], as well as the silent *cpe* sequences present on plasmids in some type E strains [86]. However, the *cpe* locus is organized differently in those strains vs type F strains (Fig. 2). In addition, the plasmids carrying *cpe* genes or silent *cpe* sequences in non-type F strains are often larger, ranging up to ~135 Kb, than the pCPF5603 or pCPF4949 plasmids [82, 99]. Some *cpe* plasmids in type C-E strains share partial similarity with pCPF5603 or pCPF4969 and they can also carry other toxin genes [78, 79, 99], e.g., the *cpb* gene encoding beta toxin, the *etx* gene encoding epsilon toxin or the *iap* and *ibp* genes encoding iota toxin [78, 79, 82, 99].

Nearly all *cpe* plasmids, including the pCPF5603 and pCPF4969 plasmids, carry a region named *tcp*, for transfer of clostridial plasmids [82]. This carriage is important because the *tcp* locus has been shown to mediate conjugative transfer of another *C. perfringens* plasmid named pCP13 [82, 100]. Those pCP13 results predicted that the pCPF5603 and pCPF4969 *cpe* plasmids should also be conjugative. This postulate was confirmed for pCPF4969 when mixed mating studies demonstrated conjugative plasmid transfer of this *cpe* plasmid [83, 101].

In summary, both the chromosomal and plasmid *cpe* genes are associated with insertion sequences, which may facilitate mobilization of the *cpe* gene within a *C. perfringens* cell. Additionally, the *cpe* gene is often present on conjugative plasmids, which allows transfer of the *cpe* gene from one *C. perfringens* strain to another. If this conjugative transfer happens during infection, it could contribute to pathogenesis, as explained later in the nonfoodborne human GI disease section of this chapter.

Regulation of CPE Production

When CPE is produced during sporulation it can account for >15% of the total protein inside the sporulating *C. perfringens* cell [1]. This abundant CPE production is not attributable to a gene dosage effect since most food poisoning strains carry only a single copy of the *cpe* gene. Instead, Northern blot and reporter studies indicated that *cpe* expression is primarily regulated at the transcriptional level, with the *cpe* gene transcribed as a monocistronic message that becomes detectable soon after the onset of sporulation [1, 28, 102–104].

The presence of three promoters upstream of the *cpe* start codon likely explains the strong production of CPE during sporulation [1, 103]. The close association between CPE production and sporulation is attributable to those *cpe* promoters sharing significant homology with SigK-or SigE-dependent promoters in *Bacillus subtilis* [103]. Those

promoter sequence homologies are important because SigK and SigF are sporulation-associated sigma factors in many *Bacillus* and *Clostridium spp* [105]. A direct role for SigK and SigE in regulating both CPE production and *C. perfringens* sporulation was demonstrated experimentally using *sigK* and *sigE* null mutants [106].

A later study [107] established a role for SigF, another sporulation-associated sigma factor, in regulating both CPE production and *C. perfringens* sporulation. In contrast to early PCR studies [106] suggesting that SigK is the first sporulation-associated sigma factor made during *C. perfringens* sporulation, the later study [107] using Western blots detected no SigK or SigE production by a *sigF* null mutant. Those Western blot results indicate that, during early sporulation either, i) SigF controls production of both SigK and SigE or ii) only very low (below Western blot levels of detection) SigK levels are produced before SigF is made. In addition, those Western blot studies [107] demonstrated that SigF controls production of a fourth sporulation-associated sigma factor named SigG. It was also shown that SigG is necessary for *C. perfringens* sporulation, but not for CPE production [107]. An emerging model from these and other studies is shown in Fig. 4. Briefly, *C. perfringens* sporulation initiates with phosphorylation of Spo0A by an unknown kinase. Phosphorylation of Spo0A [108], a master regulator of stationary phase gene expression, leads to production of SigF [107]. Once produced, SigF signals the sporulating cell to make SigE, SigG and SigK. SigE and SigK then direct expression of the *cpe* gene in the sporulating *C. perfringens* cell. All four sporulation-associated sigma factors contribute to the gene expression needed for sporulation [106, 107].

Several other regulators, i.e., CcpA, CodY and the Agr-like quorum sensing system, have been shown to regulate sporulation and CPE production, but the precise nature of their involvement is unclear at present [109–111].

Other Virulence Factors: Small Acid Soluble Protein-4

The vegetative cells and, especially, spores of type F strains carrying a chromosomal *cpe* gene are usually much more resistant to heating (cooking), cold (storage in refrigerators/freezers) and food preservatives than cells and spores of most other *C. perfringens* strains [17, 112, 113]. The exceptions are type C Darmbrand strains, which also form highly-resistant spores and cause human food-borne disease [80]. For example, the spores of both type F chromosomal *cpe* strains and type C Darmbrand strains have a D_{100} value (D_{100} is the time needed at 100°C to reduce viability by one log) of ~1 h vs. a D_{100} value of only ~1–2 min for spores made by other *C. perfringens* strains [17, 80].

Small acid soluble proteins (SASPs) provide important protection to spore DNA. *C. perfringens* produces four SASPs [114, 115]. Early studies [114] showed that differences in SASPs 1–3 do not explain the exceptional heat resistance properties of spores made by type A chromosomal *cpe* strains or type C Darmbrand strains. Instead, later studies demonstrated that those chromosomal *cpe* type F strains and type C Darmbrand strains forming highly heat-resistant spores produce a variant SASP-4 with an Asp at residue 36, unlike the SASP-4 of other *C. perfringens* strains which have a Gly at this residue [80, 115]. Studies with isogenic mutants and complementing strains confirmed the importance of this variant SASP-4 for the extreme spore resistance properties of type F chromosomal *cpe* stains and,

presumably, type C Darmbrand strains [114]. The greater protection offered by the SASP-4 Asp_{36} variant appears to involve tighter DNA binding, particularly to AT-rich DNA sequences, by the Asp_{36} vs. Gly_{36} SASP-4 variant [116]. While their SASP-4 variant plays a major role in the strong spore resistance properties of type A chromosomal *cpe* strains, other factors may also contribute [117, 118].

Immunology

There is no evidence that immune responses contribute substantially to the rapid resolution of most *C. perfringens* type F food poisoning cases. Instead, symptoms of this illness are thought to quickly abate due to CPE-induced diarrhea flushing unbound toxin and *C. perfringens* from the GI tract. Most people have CPE antibodies in their serum but there is no evidence that prior exposure to *C. perfringens* type F food poisoning provides long-term protection against future acquisition of this illness [119, 120].

Treatment and Prevention

Treatment of *C. perfringens* type F food poisoning is symptomatic. However, recent studies showed that mepacrine could be a potential therapeutic since it can inhibit CPE action *in vitro* [121].

As mentioned earlier, the best approach to prevent this food poisoning is to thoroughly cook foods and then hold them at temperatures <4°C or >65°C. No vaccine is licensed for prevention of *C. perfringens* type F food poisoning studies. However, the C-terminal domain of CPE, which is noncytotoxic and does not cause GI tract effects, contains a neutralizing epitope [1, 48]. Immunization of mice with a conjugate consisting of a peptide containing this CPE epitope coupled to a thyroglobulin carrier induced high titers of CPE-neutralizing antibodies in mice, suggesting this epitope could be a vaccine candidate [122].

CPE-ASSOCIATED NON-FOODBORNE HUMAN GI DISEASES

C. perfringens type F isolates are also responsible for 5–15% of all non-foodborne human GI disease cases [2, 123, 124], which include antibiotic-associated diarrhea (AAD) and sporadic diarrhea. In CPE-associated AAD, patients receiving antibiotics become colonized, often from the nosocomial environment, by type F strains of *C. perfringens* [124–126]. CPE-associated nonfoodborne GI disease cases often last longer (up to several weeks) and are typically more severe than most cases of *C. perfringens* type F food poisoning, particularly in the elderly [124–126].

The more chronic duration of CPE-associated nonfoodborne GI diseases may be attributable to their superior intestinal colonizing ability compared to type F food poisoning strains. *In vitro* studies [127] showed that nonfoodborne human GI disease strains adhere much better than type F chromosomal *cpe* strains to human enterocyte-like Caco-2 cells. This enhanced adherence correlates with the ability of the CPE-associated nonfoodborne GI disease strains, but not chromosomal *cpe* type F food poisoning strains, to produce NanI sialidase [127]. Studies [127] with *nanI* null mutants of a nonfoodborne human GI disease strain confirmed a role for this sialidase in Caco-2 cell adherence, where it likely trims the host cell surface to promote bacterial adherence via a combination of surface charge reduction and exposure of

adhesin receptors. In addition, sialic acid generated by NanI from host sources such as enterocyte/colonocyte surfaces or GI tract mucus may facilitate the intestinal growth of *C. perfringens* [128]. Interestingly, NanI can also increase the activity of CPE and several other intestinally-active *C. perfringens* toxins by enhancing their binding to host cells [129, 130].

As mentioned earlier, virtually all *C. perfringens* type F isolates causing CPE-associated nonfoodborne human GI diseases carry a plasmid *cpe* gene, in contrast to the chromosomal *cpe* gene present in most *C. perfringens* type F food poisoning isolates [82, 83, 89, 93, 94]. The presence of the *cpe* gene on plasmids, which are conjugative [101], may contribute to the development of CPE-associated nonfoodborne diseases. Unlike *C. perfringens* type F food poisoning, which develops following the ingestion of massive numbers of bacteria, CPE-associated nonfoodborne GI diseases likely involve ingestion of low numbers of *C. perfringens*[2]. Therefore, inside the GI tract, the presence of the *cpe* gene on plasmids may result in conjugative transfer of this toxin gene to *C. perfringens* in the normal intestinal microbiota, thereby helping to establish and amplify the infection during CPE-associated nonfoodborne GI disease.

OTHER HUMAN GI DISEASES CAUSED BY *C. perfringens*

Enteritis necroticans

C. perfringens type C strains cause a disease named enteritis necroticans (EN), which was first identified in malnourished people in post-World War II Germany, where it was known as Darmbrand [80]. EN, known locally as pigbel, was the leading cause of childhood death in Papua New Guinea during the 1960's-70's [131, 132]. For special occasions, villagers in the Papua New Guinea highlands would barbecue a pig in a pit dug in the ground, thus allowing contamination of the meat with type C strains present in the environment (soil or pigs). When that food is ingested, beta toxin (CPB) was produced in the intestines and then caused both a necrohemorrhagic enteritis and enterotoxemia. While the presence of high trypsin levels in the intestines of well-fed people with a normal diet rapidly inactivates CPB, the diet in the highlands population was poor in protein (which reduces trypsin production) but rich in sweet potatoes (which have a trypsin inhibitor), rendering the local people (especially children) highly susceptible to CPB. Type C-induced EN outbreaks occur sporadically in malnourished populations in other developing countries [131]. Individual cases are observed occasionally in developed countries, particularly in people with underlying pancreatic diseases such as diabetes [133].

Beta toxin (CPB) is necessary for type C strains to cause EN [134]. However, some strains (including many Darmbrand strains) produce only low amounts of CPB, often along with low amounts of CPE [135]. For those strains, it has been shown [135] that CPB and CPE can act synergistically in the intestines to cause pathology. CPB, also a pore-forming toxin [6], has been poorly studied. For example, the receptor for this toxin remains unknown.

The *cpb* gene is always plasmid-borne in type C strains; occasionally this gene is located on the same plasmid carrying a *cpe* gene [79, 82]. Plasmids carrying the *cpb* gene are predicted to be conjugative since they typically carry the *tcp* locus; however, this has not yet been

evaluated [82]. Adjacent to the *cpb* gene are insertion sequences that may mobilize the *cpb* gene [79, 82].

The only treatment for EN is prompt intestinal surgery to remove affected portions of the small intestine [131]. Immunization with a CPB toxoid during the 1980's-90's greatly reduced the prevalence of EN in Papua New Guinea [131, 132].

Another *C. perfringens* foodborne human disease?

Recently, there were reports [7, 8] of several Japanese food poisoning outbreaks that apparently involved *cpe*-negative *C. perfringens* type A strains. Analysis of those strains determined they produce a binary toxin whose A and B components share 44% and 37% identity, respectively, with *C. perfringens* iota toxin. This new binary toxin (named CPILE or BEC by different groups) structurally resembles iota toxin and, like iota toxin, can ADP-ribosylate actin [136, 137]. It causes fluid accumulation in rabbit ileal loops and null mutants that do not produce the binary toxin lose their intestinal virulence, however, complementation of those mutants has not yet performed [7]. Additional research is needed to address the overall importance of strains producing CPILE/BEC for human foodborne disease, e.g., how common are outbreaks caused by these bacteria both inside and outside of Japan?

C. perfringens ANIMAL DISEASES OF INTESTINAL ORIGIN

C. perfringens type B, C and D strains, and perhaps certain type A and E strains, can cause enteritis, colitis, enterocolitis and/or enterotoxemia in livestock, while some type A strains are responsible for necrotic enteritis of poultry (Table 2). Like *C. perfingens* human enteric diseases, these animal diseases are not intoxications but true infections resulting from *C. perfringens* producing toxins in the intestines. Molecular Koch's postulate analyses have confirmed a role for epsilon toxin in type D infections, beta toxin in type C infections and NetB toxin in avian necrotic enteritis [9, 134, 138]. Each of those pore-forming toxins is plasmid-encoded [82]. Detailed discussion of these animal diseases is beyond the scope of this chapter, so readers are directed to several recent reviews on this subject [6, 139–141].

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Because of page limitations, this chapter has cited published reviews for many older studies.

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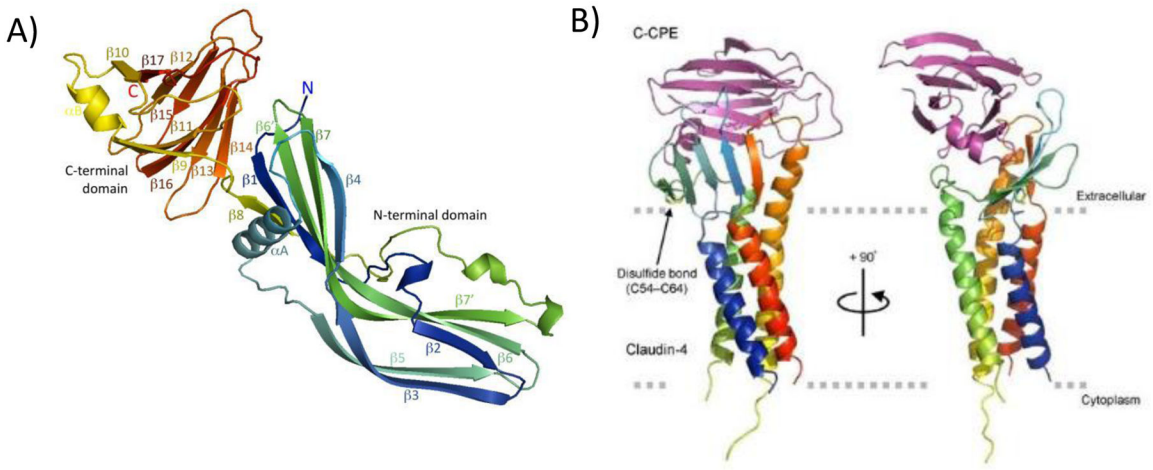

Figure 1. Structure of CPE (Panel A) and the C-terminal CPE structure bound to a claudin-4 receptor (Panel B).

Panel A: structure of the CPE monomer, colored from blue at the N-terminus to red at the C-terminus. Note the presence of two distinct domains, including the C-terminal domain (red/yellow) mediating receptor binding and the blue-green N-terminal domain mediating oligomerization, membrane insertion, and pore formation. The alpha helix labeled alpha A is located at the TM1 region that becomes a β-hairpin when CPE is assembled in the prepore. β-hairpins from the seven CPE molecules in the prepore are then thought to form a β-barrel that inserts into membranes to form the active pore. Panel B: Structure of the C-terminal CPE region (C-CPE) bound to the human claudin-4 receptor. The claudin receptor is rainbow-colored while C-CPE is purple. The membrane orientation of the claudin receptor, including the transmembrane helices, is also shown. Panels A and B are reproduced with permission from [42, 66] respectively.

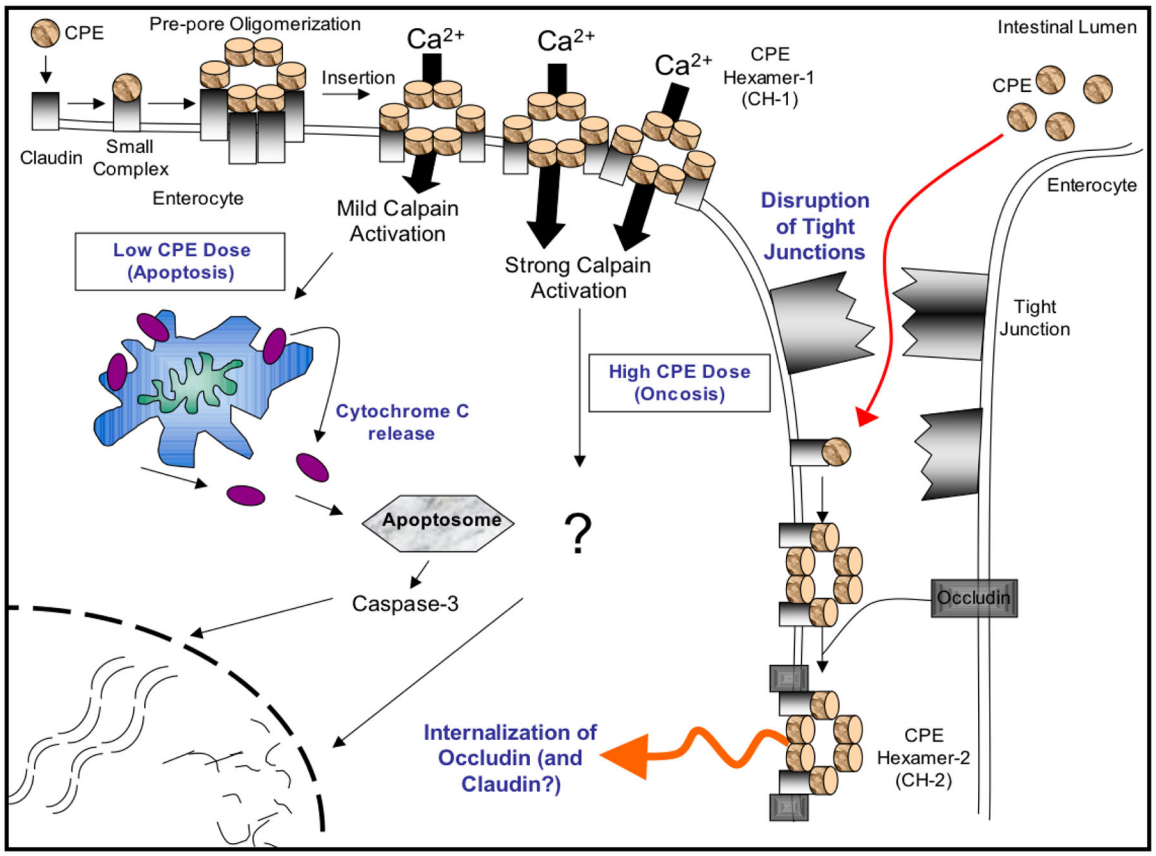

Figure 2. Model for the CPE mechanism of action.

Top left, CPE binds to receptors forming a small complex that contains both receptor claudins and nonreceptor claudins, as well as CPE. At 37°C, several small complex interact to form a prepore on the membrane surface. Portions of CPE in the prepore insert into the membrane to form a pore that allows Ca^{+2} entry into the cytoplasm. With high CPE doses, there is a massive Ca^{+2} entry that causes strong calpain activation to trigger oncosis (a form of necrotic cell death). At low CPE doses, there is a more limited Ca^{+2} entry that causes a mild calpain activation; this results in mitochondrial membrane depolarization, cytochrome C release and caspase-3 activation to cause death by apoptosis. Dying CPE-treated cells undergo morphologic damage that exposes their basolateral surface to CPE, resulting in formation of a second large complex containing occludin (as well as CPE and claudins), which induces internalization of these molecules. This effect may contribute to paracellular permeability changes, at least in cultured cells. Reproduced with permission from [54].

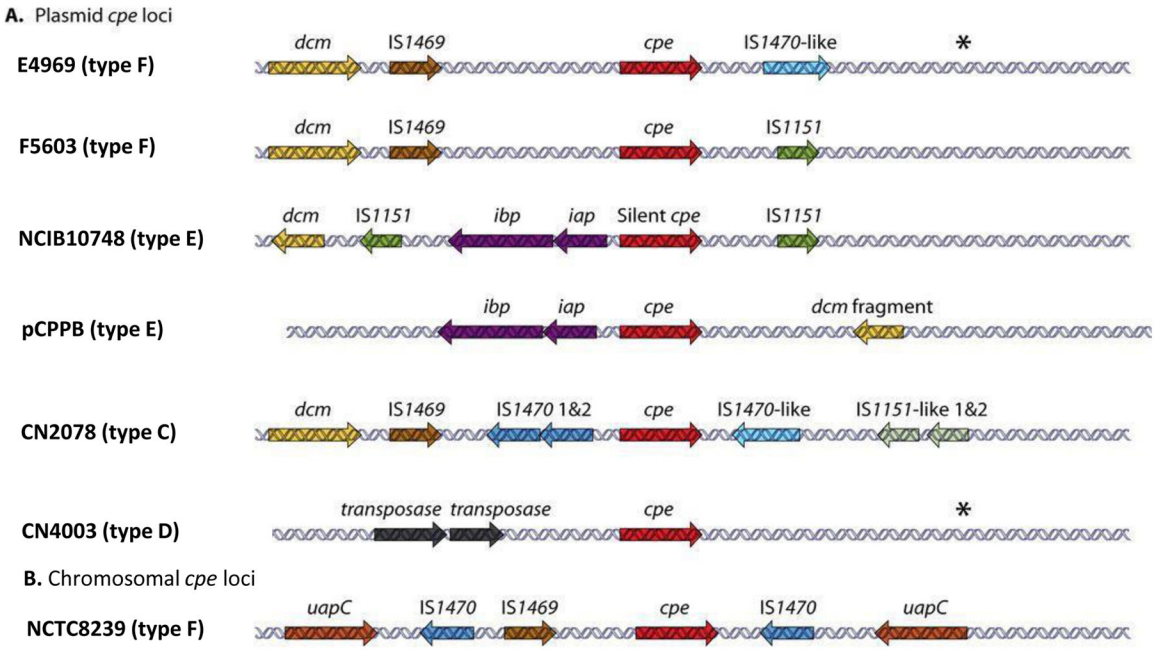

Figure 3. Organization of *cpe* loci in type C, D, E and F strains of *C. perfringens*.

(A) Organization of plasmid-borne *cpe* loci in type F, E, C, and D strains. (B) Organization of the type F chromosome *cpe* locus. Asterisks indicate a region with similarity to sequences present downstream of the *cpe* gene in F4969, except for the absence of an IS1470-like gene. Modified with permission from [82].

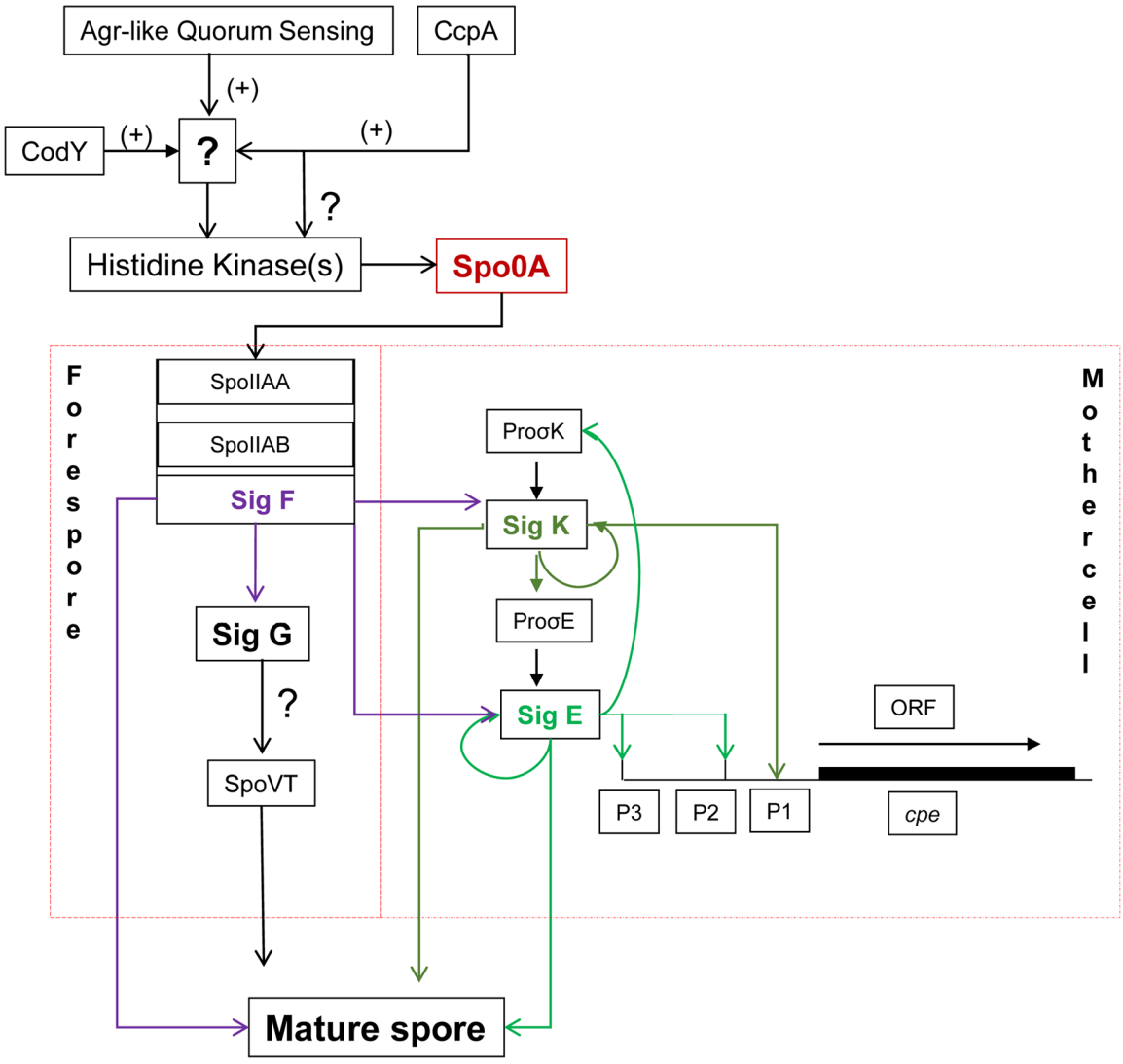

Figure 4: Model of *C. perfringens* sporulation and enterotoxin production.

Via the Agr-like quorum sensing system, CcpA and CodY and unidentified intermediates, a histidine kinase(s) affects spooA expression and/or possibly Spo0A phosphorylation to initiate sporulation. This triggers a sigma factor cascade, where SigF controls production of three other sporulation-associated sigma factors (SigE, SigG and SigK). SigE and SigK then regulate CPE production during sporulation by enhancing *cpe* expression. All four sigma factors are needed for sporulation. Reproduced with permission from [142].

Table 1.

Revised *C. perfringens* toxin-based typing scheme.

Toxinotype	Toxin Production					
	α-toxin *(plc or cpa)*	**β-toxin *(cPb)***	**ε-toxin *(etx)***	**ι-toxin *(iap and ipb)***	**CPE *(cpe)***	**NetB *(netB)***
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/−	-
D	+	−	+	−	+/−	−
E	+	-	-	+	+/−	-
F	+	-	-	-	+	-
G	+	−	−	−	−	+

+ means this toxin is produced by all strains of this type; − means this toxin is produced by no strains of this type ; +/− means this toxin is produced by some strains of this type

Table 2:

Clostridium perfringens enteric diseases of livestock and poultry

Type	Main enteric disease	Toxin
A	Necrotic enteritis in chickens, turkeys and possibly other avian species	NetB (necrotic enteritis B-like toxin)
	Possible necrotizing and hemorrhagic enteritis in dogs and foals	NetF ? (necrotic enteritis F-like toxin)
B	Lamb dysentery; possible necrotizing enteritis in horses	CPB (beta-toxin), ETX (epsilon toxin)
C	Necrotizing enteritis in neonatal calves, foals, lambs, piglets and possibly other species	CPB (CPE may be synergistic in some cases)
D	Enterotoxemia in sheep, goats and cattle	ETX
E	Possible hemorrhagic enteritis in cattle and rabbits	ITX (iota toxin)
F	Possible enteric disease in horses, dogs and other species	CPE (enterotoxin ?)
G	Avian Necrotic Enteritis	NetB