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***Clostridium perfringens* Sporulation and Sporulation-Associated Toxin Production**

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Abstract

The ability of *Clostridium perfringens* to form spores plays a key role during the transmission of this Gram-positive bacterium to cause disease. Of particular note, the spores produced by food poisoning strains are often exceptionally resistant to food environment stresses such as heat, cold and preservatives, which likely facilitates their survival in temperature-abused foods. The exceptional resistance properties of spores made by most type A food poisoning strains and some type C foodborne disease strains involves their production of a variant small acid soluble protein-4 that binds more tightly to spore DNA compared to the small acid soluble protein-4 made by most other *C. perfringens* strains. Sporulation and germination by *C. perfringens* and *Bacillus* spp. share both similarities and differences. Finally, sporulation is essential for production of *C. perfringens* enterotoxin, which is responsible for the symptoms of *C. perfringens* type A food poisoning, the second most common bacterial foodborne disease in the USA. During this foodborne disease, *C. perfringens* is ingested with food and then, using sporulation-specific alternate sigma factors, this bacterium sporulates and produces the enterotoxin in the intestines.

The ability of the Gram-positive, anaerobic rod *Clostridium perfringens* to form resistant spores contributes to its survival in many environmental niches, including soil, waste water, feces and foods (1). In addition, sporulation and germination play a significant role when this important pathogen causes disease (2-3). As introduced in the next section of this

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chapter, spores often facilitate the transmission of *C. perfringens* to hosts and then germinate *in vivo* to cause disease.

Toxin production is well-appreciated as a critical factor for the pathogenicity of *C. perfringens* (1). At least 17 different *C. perfringens* toxins have been described in the literature; however, individual isolates produce only portions of this impressive toxin arsenal. Consequently, *C. perfringens* strains are commonly classified into one of five types (A-E) based upon their ability to produce four “typing” toxins, i.e., alpha, beta, epsilon and iota toxins. While all isolates produce alpha toxin, type B strains also express beta and epsilon toxin, type C isolates also make beta toxin, type D strains also produce epsilon toxin and type E isolates also express iota toxin. Besides producing one or more of the typing toxins, sporulating cells of some *C. perfringens* strains produce additional toxins such as *C. perfringens* enterotoxin (CPE), or a recently identified toxin named TpeL (1-4). The connection between CPE production and sporulation has disease relevance, as introduced below.

The importance of spores for *C. perfringens* disease

In humans and several important livestock species, *C. perfringens* causes a spectrum of diseases that remain important medical and veterinary concerns. The most notable of those *C. perfringens* diseases are, i) histotoxic infections such as clostridial myonecrosis, also known as traumatic gas gangrene (5), and ii) diseases such as enteritis or enterotoxemias that originate in the intestinal tract (1-2). As will now be described, spores can play an important role in the transmission of all these illnesses.

C. perfringens is the most common cause of traumatic human gas gangrene, which remains challenging to treat even using modern medical approaches (5). *C. perfringens* type A causes clostridial myonecrosis when spores or vegetative cells gain entry into muscle tissue via a wound. Spores can germinate if low oxidation-reduction (Redox) conditions are present in the muscle tissue; the resultant vegetative cells then grow rapidly to further reduce tissue Redox conditions, promoting additional bacterial growth. The growing *C. perfringens* vegetative cells produce alpha toxin and perfringolysin O, which cause local and regional necrosis in muscle, allowing rapid and progressive spread of the infection. In addition, these toxins can enter the systemic circulation to induce organ damage, circulatory problems and death (5).

Many cases of human or animal enteritis and enterotoxemia (i.e., absorption of toxins from the intestines into the circulation, from where they damage nonintestinal organs) are also caused by *C. perfringens* (2). Spores often play a critical role in transmission of the *C. perfringens* illnesses originating in the intestines, particularly during two human food-borne illnesses. The first of those diseases, i.e., *C. perfringens* type A food poisoning, is caused by CPE-producing type A strains and ranks as the second most prevalent bacterial food-borne illness in the USA at 1 million cases/year (6). While the enterotoxin (*cpe*) gene can be either chromosomal or plasmid-borne, ~75% of all *C. perfringens* food poisoning cases are caused by type A strains carrying a chromosomal *cpe* gene (1). Food poisoning typically occurs when type A chromosomal *cpe*-positive strains are ingested with foods and then sporulate in

the small intestine, where they produce CPE (further discussion later) (1). The second *C. perfringens* food-borne illness of humans is enteritis necroticans, which is caused by beta toxin-producing type C strains (7). Historically, enteritis necroticans was first observed in post-World-War II Germany, where it was known as Darmbrand (7). However, enteritis necroticans is most often associated with childhood infections in Papua New Guinea (where the disease is known locally as PigBel because it often follows ingestion of contaminated pork), although it occasionally occurs in developed countries (7). In both *C. perfringens* type A and type C food-borne diseases, improper cooking or holding of foods plays a critical role in transmission. This temperature abuse facilitates the survival of resistant *C. perfringens* spores present in foods; those spores later germinate and cause illness when the food is ingested (as discussed later in the chapter).

CPE-producing type A strains carrying a plasmid *cpe* gene are responsible for 2-15% of all cases of non-food-borne human gastrointestinal (GI) diseases, such as antibiotic-associated diarrhea (8). These illnesses are primarily acquired by ingesting spores that are present in the environment. Although less studied, spores could also contribute to *C. perfringens* enteritis and enterotoxemias in livestock, which can be caused by all types (A-E) of this bacterium.

After introducing *C. perfringens* spore ultrastructure and describing the basic processes of sporulation and germination in this bacterium, the remainder of this chapter will focus on recent insights into, i) the resistance properties that allow spores to contribute to *C. perfringens* disease transmission and ii) the molecular basis for the expression of CPE and TpeL by sporulating cells.

The ultrastructure of *C. perfringens* spores

C. perfringens spores (Fig. 1) contain several different structural layers, all of which contribute to spore resistance properties (9, 10). Unlike some spore-forming species (11), *C. perfringens* does not possess an exosporium. Instead, the outermost layer of the *C. perfringens* spore is the spore coat. The composition and function of the spore coat in *C. perfringens* has not yet been carefully studied; however, in other Gram-positive spore formers, the spore coat is thought to be comprised of >50 spore-specific proteins and provides protection to the spore from reactive chemicals and lytic enzymes (12-14). In *C. perfringens*, a small fraction of the spore population (~5%) is defective in spore coats (15). Those spores are permeable to lysozyme (15) so they can germinate inside the host under specific conditions.

The layers that underlie the coat are common for all known Gram-positive spore formers, including *C. perfringens*. Beneath the coat is the outer membrane, which does not provide protection to dormant spores but is essential for spore formation and is presumably lost by shearing forces after short periods. The spore peptidoglycan cortex underlines the outer membrane, with a structure similar to that of peptidoglycan in a growing cell wall (16). The cortex plays an essential role in spore core dehydration and therefore directly contributes to spore resistance to environmental stress and chemicals. In studied spore-formers, and presumably also in *C. perfringens*, the spore peptidoglycan has three novel structural modifications that contribute to its resistance to cell wall hydrolases typically found in

growing cells: (i) only one-quarter of cortex N-acetylmuramic acid (NAM) residues are substituted with short peptides, giving the cortex a lower degree of crosslinking than the germ cell wall; (ii) about one-quarter of the NAM residues carry a single L-alanine modification not present in the glycan strands of the germ cell wall; and (iii) nearly every second muramic acid residue in the cortex PG is converted to muramic- δ -lactam (MAL) (17, 18), which seems to be the recognition substrate element for the CLEs which uniquely hydrolyze the PG cortex, but not the germ cell wall during spore germination (19, 20). The germ cell wall underlines the spore PG cortex, has no role in spore resistance and is converted into the growing cell wall during spore outgrowth. The spore inner membrane underlies the germ cell wall and is the last layer of protection of the spore core. The spore inner membrane is significantly compressed resulting in highly immobile lipids (12), resulting in a low permeability to small molecules including water and DNA damaging chemicals (12, 21).

The core is the inner-most layer of the *C. perfringens* spore and contains the spore DNA, RNA and most enzymes. Three major factors, including the low water content of the core (20-50% of wet weight), its high levels of Ca-dipicolinic acid (Ca-DPA) (25% of core dry weight) and the saturation of DNA with small acid soluble proteins (SASPs, discussed further below) together contribute to the resistance properties of these spores (22, 23).

Sporulation of *Clostridium perfringens*

To survive unfavorable conditions, *C. perfringens* initiates the process of sporulation by undergoing an asymmetrical division of its cytoplasm membrane. This process gives rise to two compartments, i.e., a small compartment (termed the forespore), and a large compartment (termed the mother cell), each with a complete genome. As sporulation progresses through a series of morphological and biochemical changes, the forespore becomes the mature *C. perfringens* spore that is eventually released to the environment upon lysis of the mother cell (24).

The sporulation process in spore-forming bacteria, including *C. perfringens*, is initiated by the integration of a wide range of environmental and physiological signals induced from changes in cell density, the Krebs cycle and nutrient starvation (25). In *C. perfringens*, initiation of sporulation requires the presence of inorganic phosphate (Pi) in the environment (26). In contrast, in sporulation medium containing Pi, *C. perfringens* was blocked at a very early stage of sporulation (i.e., the absence of polar septation and DNA partitioning) in cells reaching the stationary phase of growth (26). Importantly, Pi can neutralize the inhibitory effect of glucose at the onset of sporulation and induces *spo0A* expression, indicating that Pi acts as a key signal triggering spore-formation in *C. perfringens* (26). As introduced earlier, *C. perfringens* sporulation directly contributes to pathogenesis since sporulation leads to the synthesis of CPE and consequently to intestinal damage of epithelial cells (27). Coupling this finding with the fact that Pi is normally present in the GI tract of humans and animals, it appears that *C. perfringens* has efficiently adapted to sporulate in the GI tract. By extension, it can be speculated that Pi directly contributes to pathogenesis and survival of the progeny of *C. perfringens* type A food poisoning strains, i.e., it induces the production of CPE to cause diarrhea that disseminates metabolically dormant spores into the environment. These

spores are able to withstand unfavorable conditions and remain viable for long periods of time.

Global regulation between the transition state and sporulation has not been studied in as great detail in *C. perfringens* as for the model sporulation system, *B. subtilis*. However, several studies have shown significant differences in the molecular regulation of sporulation between *C. perfringens* and *B. subtilis* (28, 29). As for *B. subtilis*, glucose has been found to act as a catabolic repressor of sporulation in *C. perfringens* (30). The transcriptional regulator carbon catabolite protein (CcpA) of the LacI/GalR family of repressor in *C. perfringens* (31) regulates many catabolite repressor effects from glucose. Interestingly, and in contrast to *B. subtilis*, CcpA is required for the efficient sporulation of *C. perfringens* (31). Initiation of sporulation in *B. subtilis* is mediated by the phosphorylation state of the master regulator of sporulation, i.e., the transcriptional factor Spo0A, which is present in all sequenced *Clostridium* species, including *C. perfringens* (32–35). Although the genome of CPE-negative *C. perfringens* strain 13 has a premature stop codon in *spo0A* (36), other *C. perfringens* isolates, including SM101 (a CPE-positive transformable derivative of a type A food poisoning strain), possess an intact *spo0A* gene (37). Evidence of a master regulatory role for Spo0A in *C. perfringens* sporulation was provided by a study showing that a *spo0A* knock out mutant of SM101 was unable to form spores. This *spo0A* phenotype was restored upon complementing the mutant with a recombinant plasmid carrying a wild-type *spo0A* copy (37). Interestingly, complementing the SM101 *spo0A* mutant with wild-type *spo0A* from other clostridial species revealed that Spo0A homologues can also induce the initiation of sporulation in enterotoxigenic *C. perfringens* (38).

The environmental signals that drive the initiation of sporulation are sensed by sporulation-specific orphan histidine kinases, which have not yet been identified in *C. perfringens*. Those histidine kinases integrate the sporulation signals and trigger a complex phosphorelay that increases the concentration of Spo0A in a phosphorylated state (Spo0A~P) (39, 40). Once threshold levels of Spo0A~P are reached, many genes (including those required for polar septum formation) become up- or down-regulated, leading to a series of biochemical and morphological events (41). For example, expression of small acid soluble protein-4 (SASP4, discussed later) and CPE by *C. perfringens* is dependent upon Spo0A (37, 42).

In *C. perfringens*, the morphological events during sporulation are divided into seven stages (I–VII), resembling those of sporulating *B. subtilis* cells. In *B. subtilis*, four major sporulation-specific sigma (σ) factors (σ^F , σ^E , σ^G and σ^K) regulate this sporulation process. The homologs of these genes are present in *C. perfringens*, where two recent studies (28, 29) demonstrated the expression and function of these sigma factors (Fig. 2). Those studies also presented evidence for both similarities and differences between the sporulation of *C. perfringens* and that of *B. subtilis*. Sporulation similarities between these bacteria include (i) Spo0A and SigF mediate control of expression of the other sporulation-associated sigma factors; (ii) SigG is expressed in the late sporulation stage; and (iii) sporulation requires production of all four sporulation-associated alternative sigma factors. Difference in sporulation include (i) *C. perfringens* lacks a *B. subtilis*-like phosphorelay and (ii) the expression of a key mother cell transcription factor (SpoIID) depends on σ^E -associated RNA polymerase in *B. subtilis*, but not in *C. perfringens*.

In detail (Fig. 3), *C. perfringens* transcribes *sigF* as part of a *spoIIA* tricistronic operon containing the *sigF*, *spoIIA* and *spoIIAB* genes in the early sporulation stage (28). The bacterium then uses SigF to regulate the production of other sporulation-associated sigma factors (28). SigE and SigK, but not SigF or SigG, are initially made as inactive proproteins and then proteolytically-activated to their mature form (28–29). Formation of *C. perfringens* mature spores requires expression of all four sigma factors.

Most, if not all, *C. perfringens* strains possess (43–45) an Agr-like quorum-sensing (QS) system involving proteins homologous to, (i) AgrD, which is the putative precursor signaling peptide of this Agr system, and (ii) AgrB, a membrane protein that is thought to modify AgrD to the active form. A recent study (44) used an *agrB* mutant to demonstrate that the Agr-like QS system is also required for the initiation of sporulation in *C. perfringens*. Specifically, inactivating the *agrB* gene in *C. perfringens* strain F5603 decreased sporulation by ~1,500 fold. Furthermore, inactivation of the *agrB* gene in F5603 results in reduced or lost expression of SigF and SigG, respectively, which are needed for sporulation. In addition, this *agrB* mutant produced less Spo0A, which is also necessary for *C. perfringens* sporulation; in fact, this reduced Spo0A production may explain why the *agrB* mutant produced reduced amounts of alternative sigma factors and sporulates poorly. Collectively, these results indicate that, in *C. perfringens*, the Agr-like QS system regulates sporulation at an early stage, i.e., by controlling Spo0A and SigF synthesis.

Recent studies identified two sporulation repressors, named *virX* mRNA and CodY protein (46, 47). The global regulator CodY repressed sporulation in a *C. perfringens* type D strain, and resulted in spores with lower germination ability (46). The *virX* gene encodes a regulatory RNA that significantly inhibits sporulation and CPE production in the type A SM101. Because transcription levels of *sigE*, *sigF* and *sigK* were higher in an isogenic *virX*-null mutant compared to wild-type SM101, it appears that *virX* RNA negatively regulates spore formation through the sporulation-specific sigma factors (47).

A wide variety of genes are expressed during sporulation in *C. perfringens*. Whole-genome expression profiling of the sporulation process in type A food poisoning strain SM101 was recently performed using DNA microarrays (48). This analysis revealed that a large number of genes showing sporulation-associated upregulated expression are homologs of known *Bacillus* genes involved in sporulation or germination. Expression levels of 106 SM101 genes exceeded 5 log₂-fold increases during sporulation. Similarly, 294 SM101 genes showed up-regulation between 3 and 5 log₂-fold increases and 451 genes were up-regulated at a lower level (2 to 3 log₂-fold increases) in SM101 sporulation cultures.

Germination of *C. perfringens* spores

C. perfringens spores can remain in dormancy for extended periods of time and survive extreme environmental conditions (see next section). However, in the presence of favorable conditions they can return to life, with outgrowth in less than 20 min (19). Spore germination is also an important factor for *C. perfringens* food-borne disease transmission. From a practical food safety perspective, the process of germination is of considerable interest because, (i) germination of *C. perfringens* spores in food stuffs can lead to food

poisoning; and (ii) upon germination, these spores lose their resistance and become susceptible to mild decontamination treatments. Therefore, understanding the molecular mechanism of *C. perfringens* spore germination might allow modulation of the germination process in foods by either inhibitors or artificial germinants that could allow the control of spore contamination loads with milder treatment conditions.

Germination is also important for transmission of other *C. perfringens* diseases. As already mentioned, spore germination in wounds can lead to clostridial myonecrosis. Furthermore, germination of *C. perfringens* spores in the intestines is presumably important during CPE-associated non-food-borne human GI diseases, since these illnesses are thought to be transmitted by ingestion of environmental spores. Those spores would need to germinate in the human intestinal tract before they could colonize and then cause disease.

Germination does not require metabolism and is initiated by small molecules called germinants, which can include amino acids, sugars, purines, nucleosides and salts (19, 49, 50). Germinant specificity can vary significantly between bacterial species and strains, and this selectivity is likely to be influenced by adaptation to specific environmental niches. Indeed, significant differences in specificity of germinants are observed between spores of *C. perfringens* food poisoning strains versus non-food-borne GI disease isolates. Spores of *C. perfringens* food poisoning isolates are able to germinate in the presence of KCl, NaPi (pH 6.0), L-asparagine or exogenous 1:1 chelate of Ca^{2+} and Ca-DPA, while spores of non-food-borne GI disease isolates initiate germination in the presence L-alanine, L-valine and with the mixture of KCl and L-asparagine (51, 52). Also, spores of a non-food-borne GI isolate germinated to a greater extent than spores of a food poisoning isolate in the presence of cultured intestinal epithelial cells (53). These results support the hypothesis that spores of food poisoning isolates have adapted to food niches (i.e., processed meat products) where nutrients like KCl and NaPi are highly abundant, while spores of non-food-borne GI disease isolate are better adapted to germinate in the host's intestinal epithelium environment.

In addition to recognizing the aforementioned germinants, bacterial spores in the host encounter several host-derived components that are capable of inducing germination. Among these are lysozyme, which is released by Peyer patches in the small intestine (54), present in the serum (55), and comprises part of the antibacterial arsenal of phagocytic cells. Lysozyme can trigger germination of spores of *C. perfringens* strain SM101 by directly degrading the spore peptidoglycan cortex (15). This germination pathway might have implications for the pathogenesis of *C. perfringens* (15), especially for superdormant spores that are extremely slow to germinate (56).

The germinant receptors (GRs) that recognize these nutrient germinants are relatively low abundance proteins that localize to the spore inner membrane and belong to the GerA family of GRs (19, 21, 49). In *B. subtilis* spores, three tricistronic operons (*gerA*, *gerB* and *gerK*) encode the three major GRs, with different receptors responding to different germinants (57). In contrast, *C. perfringens* has no tricistronic *gerA*-like operon and only a monocistronic *gerAA* that is far from the *gerK* locus. This *gerK* locus contains a bicistronic *gerKA-KC* operon and a monocistronic *gerKB* upstream of, and in the opposite orientation from, *gerKA-KC* (58, 59). Interestingly, the tricistronic *gerA* operons found in *B. subtilis*

accounts for ~ 50% of GRs, while the *gerK* locus found in *C. perfringens* accounts for nearly 5% of the GRs present in sequenced genomes of endospore-forming members of *Bacillales* and *Clostridiales* (49). In *C. perfringens* strain SM101, gene knock-out studies have identified the main receptors for L-asparagine, KCl, AK, and NaPi as the products of the bicistronic operon *gerKA-KC*, while the products of *gerAA* and *gerKB* play auxiliary roles in germination (51, 52, 60). Further gene knock-out and protein localization studies demonstrated that GerKC is the essential GR for germination of *C. perfringens* spores and also that GerKC is located in spore inner membrane (58).

GerKA-KC and GerKB receptors are also required for viability and outgrowth of *C. perfringens* spores (51, 60). Binding of nutrient germinants to GRs located in the spore's inner membrane triggers the release of monovalent ions (Na^+ and K^+) and the spore core's depot of dipicolinic acid as a 1:1 chelate with Ca^{2+} (Ca-DPA) is replaced by water (49). Although the precise mechanism of monovalent ion release remains unknown, GrmA-like antiporter homologues (named GerO and GerQ, which are not to be confused with the coat protein GerQ in *B. subtilis* and the germination receptor GerQ in *B. anthracis*) in *C. perfringens* strain SM101 were shown to be involved in transport of K^+ and/or Na^+ , and GerO was also required for normal germination (61). However, because both GerO and GerQ are expressed in the mother cell compartment during sporulation, it is likely that their effect on spore germination is primarily during spore formation (61).

A major event after germinant nutrient binding to GRs is the release of the large deposit of Ca-DPA in the spore core (19). The precise mechanism of Ca-DPA release remains to be fully understood, although proteins encoded by the *spoVA* operon are thought to be involved in Ca-DPA movement (49). Instead of a hexacistronic *spoVA* operon as in *B. subtilis*, *C. perfringens* carries a tricistronic (*spoVAC*, *spoVAD* and *spoVAE*) *spoVA* operon (49). Interestingly, *C. perfringens* SM101 *spoVA* null mutant spores lacking DPA are stable and germinate well, suggesting that Ca-DPA is not required for either *C. perfringens* spore stability or signal transduction from GRs to downstream effectors as is the case in *B. subtilis* (49). This release of ions allows a slight hydration of the spore core that does not restore enzymatic activity but does lead to a decrease in spore wet heat resistance (19). Taken together, the aforementioned events constitute an initial stage of germination known as Stage I (19).

Once Ca-DPA is released from the spore core (signaling the start of Stage II of germination), a series of biochemical events take place, with the hallmark being the hydrolysis of the peptidoglycan cortex of the spore (19). The cortex, which acts as a strait jacket restricting spore core hydration and therefore expansion, is hydrolyzed by cortex lytic enzymes (CLEs) that will specifically degrade the cortex (19). Two CLEs are present in the *C. perfringens* spore; one of these, SleC, is synthesized as an inactive zymogen and is the sole essential CLE for cortex hydrolysis of *C. perfringens* food poisoning isolates. SleC is a bifunctional enzyme with lytic transglycosylase and *N*-acetylmuramoyl-L-alanine amidase activity on cross-linked peptide moieties in the cortex (62). In contrast to the case of *B. subtilis* CLE CwlJ, which is activated by Ca-DPA released from the spore core (63), *C. perfringens* SleC is controlled by the Csp proteins (64) that belong to the subtilisin family of serine proteases.

Csp proteins are localized in the cortex and activate cortex hydrolysis by converting pro-SleC to active SleC (64).

In contrast to SleC, the second CLE, SleM, is synthesized in a mature form with N-acetylmuramidase activity (65) and has little role in cortex hydrolysis of spores made by chromosomal *cpe* food poisoning isolates (66). Interestingly, complementation of a *sleC* mutant of a food poisoning isolate with wild-type *sleC* from a non-food-borne human GI disease isolate only partially restored the germination phenotype, suggesting that the precise role of both CLEs (i.e., SleC and SleM) in non-food-borne GI disease isolates might be different from that in chromosomal *cpe* food poisoning isolates (67). Significant differences between spores of both food poisoning and non-food-borne GI disease isolates also exist in regard to Csp proteins. While spores of chromosomal *cpe* food poisoning isolates possess only one Csp protein, CspB (64), spores of non-food-borne isolates have three Csp proteins (i.e., CspA, CspB, and CspC) encoded by a tricistronic operon (68). Studies with *C. perfringens* chromosomal *cpe* strain SM101 demonstrated that CspB alone is localized to the spore coat and alone is sufficient for converting pro-SleC to active SleC and activate cortex hydrolysis (58, 64). Degradation of the spore peptidoglycan cortex allows full core hydration, remodeling of the germ cell wall, resumption of metabolism, degradation of SASPs and complete loss of spore resistance properties (19).

Resistance properties of *C. perfringens* spores

One reason why *C. perfringens* is such a successful food-borne pathogen is because it can form resistant spores that allow survival in improperly held or incompletely cooked foods. Specifically, spores provide *C. perfringens* with resistance against such common food environment stresses as low or high temperatures, osmotic pressure, chemical preservatives and pH. For example, while vegetative cells of this bacterium cannot survive even brief exposure to 55°C, spores of some *C. perfringens* strains can survive boiling for an hour or longer (7, 69).

Importantly, *C. perfringens* spores exhibit significant strain-to-strain differences in their food environment stress resistance properties (7, 69–71). As mentioned earlier in this chapter, type A strains with a chromosomal *cpe* gene are strongly associated with food poisoning; it is those isolates that also typically form the most resistant spores, regardless of their geographic origin, date of isolation, or isolation source (72). For example, in terms of decimal reduction values (D_{100} value or the time that a culture must be kept at 100°C to obtain a one log reduction in viable spore numbers), the spores of type A chromosomal *cpe* food poisoning isolates are, on average, ~60-fold-higher than the D_{100} values for spores of type A isolates carrying a plasmid *cpe* gene or *cpe*-negative type A strains. Notably, spores produced by some chromosomal *cpe* food poisoning isolates have D_{100} values exceeding 2 h (69).

Of epidemiologic significance, a survey detected both spores and vegetative cells of *cpe*-positive type A isolates in non-outbreak raw meats and seafood sold retail in the USA (73). Importantly, those *cpe*-positive type A retail food isolates all carried a chromosomal *cpe* gene. Furthermore, the spores made by each of these raw food isolates exhibited

exceptionally strong heat resistance, indicating that the spore heat-resistant phenotype is an intrinsic trait of most type A chromosomal *cpe* isolates, rather than a survivor trait selected by cooking. This spore resistance phenotype should be an important virulence determinant since it likely favors survival of type A chromosomal *cpe* isolates in improperly warmed or incompletely cooked foods.

Storage of foods at low temperatures (in refrigerators or freezers) is another important food safety approach. The spores of most chromosomal *cpe* strains also show exceptional cold resistance compared with the spores of type A plasmid *cpe* strains or *cpe*-negative strains. For example, after a 6 month storage at 4°C or -20°C, the average log reduction in viability for spores of plasmid *cpe* or *cpe*-negative strains was about three to four-fold greater, respectively, compared against the average log reduction in viability of spores made by chromosomal *cpe* strains (71). These results suggest that the chromosomal *cpe* strains are strongly associated with food poisoning not only because of their exceptional spore heat resistance properties, but also because their spores are unusually tolerant of storage at low temperature (71).

Other factors besides temperature are also used to control the presence of pathogens in foods. For example, commercial curing of meats often involves use of sodium nitrite, which can inhibit outgrowth of clostridial spores or, at high concentrations, kill bacterial spores. One study (70) showed that the spores of type A chromosomal *cpe* isolates exhibit significantly better tolerance of, and survival against, nitrite-induced stress compared against the spores of other type A isolates. This nitrite resistance should further facilitate the ability of the chromosomal *cpe*, type A strains to cause foodborne illness.

Mechanisms of *C. perfringens* spore resistance

C. perfringens spore resistance depends upon a synergistic interplay of multiple factors, which include sporulation temperature, mineralization of the core with DPA and its cations, binding of α/β -type small soluble proteins (SASPs) to spore DNA, and core water content. Spore core water content is directly affected by sporulation temperature; a higher sporulating temperature produces *C. perfringens* spores with higher heat resistance. Spores of an *spmA/B* null mutant have more core water content, which directly reduces heat resistance by 50% (74). A *spoVA* null mutant makes spores with two-fold more core water than wild-type spores and those mutant spores also exhibit lower resistance to moist heat, UV radiation and chemical treatment (75). A possible explanation for this decreased resistance is that increased hydration decreases SASP binding to spore DNA (75). The degree of cross-linking of the spore PG cortex also plays a major role in *C. perfringens* spore resistance since *dacF/B* null mutant strains, which have significantly increased cross-linking of muropeptides, make spores with decreased heat resistance (74). However, the degree of cross-linking of muropeptides does not affect the core water content of *C. perfringens* spores (74).

SASPs bind to and saturate spore DNA, providing protection from various environmental stresses. The *C. perfringens* genome encodes four major α/β -type SASPs (i.e., SASP1, SASP2, SASP3 and SASP4). When SASP1, 2 and 3 levels in sporulating cells were reduced

by >90% using antisense-RNA-mediated down regulation approaches, a 5-fold reduction in spore heat-resistance was observed (76). (73, 74) Those spores also showed greater sensitivity to chemicals (i.e., nitrous acid, hydrogen peroxide, formaldehyde and HCL) and UV radiation. However, SASP1, 2 and 3 levels could not explain the resistance differences observed between spores of type A chromosomal *cpe* isolates vs. spores of other *C. perfringens* since all strains produce similar amounts of SASP1, 2 and 3 and no consistent sequence variation in these proteins occurs amongst *C. perfringens* strains (76, 77). (73, 74)

However, an Asp is found at residue 36 of the SASP4 made by most, if not all, of the type A chromosomal *cpe* isolates forming highly resistant spores (78). In contrast, Gly is consistently present at this SASP4 residue in those *C. perfringens* strains producing more sensitive spores. An important contribution of the Asp 36 SASP4 variant to the exceptional heat, sodium nitrite and cold resistance properties of spores made by most chromosomal *cpe* food poisoning strains has been directly demonstrated using *ssp4* null mutants (78). Furthermore, electrophoretic mobility shift assays (EMSA) and DNA binding studies showed that SASP4 variants with an Asp at residue 36 binds DNA more efficiently and tightly than do SASP4 variants with a Gly at residue 36 (Fig. 4A). Results from saturation mutagenesis experiments (42) indicated that both amino acid size and charge at SASP4 residue 36 are important for tight DNA binding and for spore resistance properties. It was also shown that *C. perfringens* SASP4 binds preferentially to AT-rich DNA sequences, while SASP2 binds better to GC-rich DNA sequences (Fig. 4B). Since the *C. perfringens* genome is more than 70% AT rich, these binding preferences may help to explain why SASP4 plays such an important role in providing cold, heat and nitrite resistance by protecting spore DNA. However, maximal spore resistance requires production of all four *C. perfringens* SASPs.

While SASP4 variations are clearly a major determinant of relative stress resistance for *C. perfringens* spores, the *ssp4* null mutant of a type A chromosomal *cpe* strain still showed somewhat more resistance than did wild-type spores of other type A isolates, indicating that other factors also contribute to the exceptional spore resistance associated with chromosomal *cpe* strains. Several studies (9, 10) have attempted to correlate structural features of *C. perfringens* spores with the striking heat resistance of chromosomal *cpe* positive isolates. Factors analyzed included the core, cortex, coat and total spore size (9, 10), but the most significant correlation noted between *C. perfringens* spore structure and heat resistance was for the ratio of core volume and core plus peptidoglycan layer, with a lower ratio giving higher heat resistance (10). However, in general, the ultra-structural features of *C. perfringens* spores are similar to those of other spore former species, so it is likely that the main differences involved in spore resistance differences between *C. perfringens* spores action at the molecular, rather than structural, level.

Multilocus Sequence Typing (MLST) analyses (79) of 8 housekeeping genes determined that chromosomal *cpe* isolates represent a distinct genetic cluster within the global *C. perfringens* population, i.e., these studies (72) identified many genetic differences in typical type A chromosomal *cpe* isolates besides their carriage of a chromosomal *cpe* gene, a variant *ssp4* gene, and ability to produce highly resistant spores. These type A chromosomal *cpe* isolates have apparently now evolved to excel at causing food-borne disease.

Since those initial MLST studies, molecular analyses have also been performed on type C strains (7), the only non-type-A isolates that cause human enteritis necroticans. In post-World War II Germany, this disease was named Darmbrand, and recent molecular analyses (7) of these Darmbrand isolates showed they carry both beta (*cph*) and *cpe* genes on large plasmids. Even though these Darmbrand isolates carry a plasmid-borne *cpe*, they produce highly heat-resistant spores. Interestingly, these type C Darmbrand strains produce the same variant Ssp4 made by chromosomal *cpe* strains. MLST analysis indicated that these type C Darmbrand strains and type A chromosomal *cpe* strains also share a similar genetic background. This helps to explain why both Darmbrand strains and type A chromosomal *cpe* strains are so well-suited to cause human foodborne illness, *i.e.*, besides producing enterically-active toxins, they both produce spores that are highly resistant to food environment stresses so that those surviving spores can later germinate into vegetative cells that are ingested in foods. In terms of evolution, it is likely that these strains emerged by entry of different mobile genetic elements (conjugative plasmids and/or transposons) into *C. perfringens* strains with a similar background, including production of the Asp36 SASP variant.

Sporulation-associated toxins

***Clostridium perfringens* enterotoxin (CPE)**

As already introduced, CPE-producing type A strains of *C. perfringens* cause the second most common bacterial food-borne illness in the USA, along with many cases of non-food-borne human GI diseases such as antibiotic-associated diarrhea. During this food poisoning (1), spores often germinate in temperature-abused foods, followed by rapid multiplication of the resultant vegetative cells in those contaminated foods (note that *C. perfringens* has a doubling time of only ~10 minutes). After the food is ingested, many vegetative cells are killed by exposure to the low pH of the stomach. However, when a food was sufficiently contaminated, some ingested bacteria survive and pass into the small intestines. Initially these bacteria multiply, but they soon commit to *in vivo* sporulation, possibly when they encounter Pi in the intestinal tract. It is during this sporulation in the small intestines that CPE is produced during *C. perfringens* type A food poisoning.

Considerable evidence implicates CPE as the toxin responsible for the GI symptoms that characterize both *C. perfringens* type A food poisoning and CPE-associated non-food-borne human GI diseases (1). For example, ingestion of purified CPE plus bicarbonate was shown to be sufficient to induce diarrhea and cramping in human volunteers (1). Additionally, studies fulfilling molecular Koch's postulates demonstrated that CPE production is essential for the GI pathogenicity of CPE-positive type A human food poisoning and non-food-borne human GI disease isolates in animal models (27).

CPE can induce substantial small intestinal histologic damage, which includes villus blunting along with epithelial necrosis and desquamation (1). This damage is thought to cause intestinal fluid and ion loss, effects that manifest clinically as diarrhea (1). Evidence with experimental animals suggests that CPE can sometimes be absorbed into the systemic circulation, where it can bind to and damage the liver and other organs. These effects can

lead to a lethal increase in serum potassium levels, which could explain some deaths associated with *C. perfringens* type A food poisoning (80).

The histologic damage that occurs in the CPE-treated small intestine is a consequence of the cellular action of this toxin (Fig. 5). This action starts with CPE binding to receptors that include certain members of the claudin family of tight junction proteins (1). There are ~ 27 members of the claudin family, but only some claudins can serve as CPE receptors (1). An Asn residue located in the middle of the second extracellular loop of receptor claudins is necessary for a claudin to bind CPE (81) and amino acid residues near this Asn residue modulate the affinity of CPE binding properties (82). Once bound to a claudin receptor, the toxin becomes localized in a small complex of ~90 kDa that can contain, at minimum, a CPE, claudin receptor and a claudin non-receptor (1).

At 37°C, several small complexes rapidly oligomerize to form a larger CPE complex named CH-1 (for CPE hexamer-1). CH-1 contains, at minimum, six CPE molecules as well as both receptor claudins and claudin nonreceptors (83). Formation of CH-1, which is ~450 kDa in size, occurs on the host cell surface; however, once formed, this CH-1 prepore soon inserts into the plasma membrane to form a pore.

CPE pore formation increases the permeability of mammalian cells for the second messenger calcium, amongst other ions. Calcium influx plays a critical role in CPE-induced cell death (84). At low CPE doses, where small amounts of the CH-1 pore form, there is a modest calcium influx and host cells die by a classical caspase-3 mediated apoptosis. At high CPE doses, where more CH-1 pores form, a massive calcium influx triggers mammalian cell death via oncosis.

Substantial morphologic damage, such as cell rounding, develops in CPE-treated host cells. This effect damages the tight junction and exposes the basolateral surface of the cell, which allows formation of a second large CPE complex named CH-2. CH-2 is ~600 kDa in size; like CH-1 it contains six CPE molecules and both receptor and nonreceptor claudins. However, CH-2 uniquely contains another tight junction protein named occludin (83). The consequences of CH-2 formation are less clear than for CH-1 formation but may include formation of additional pores, further damage to the tight junction by sequestering even more tight junction proteins in CPE complexes, and inducing the internalization tight junction proteins into the host cell cytoplasm.

The CPE protein is a single polypeptide consisting of 319 amino acids, with a molecular weight of ~35 kDa. The structure of the toxin was recently solved and shown to consist of two major domains with a resemblance to the aerolysin-like pore forming toxin family (85, 86). When this structural information is collated with results from structure/function mutagenesis studies (87), it revealed that claudin binding activity is mediated by the C-terminal domain of CPE. This binding involves several tyrosine residues located near the extreme C-terminus of the toxin that interact with the ECL-2 loop of receptor claudin. In contrast, the N-terminal domain of the toxin, which consists of two halves, mediates both CPE oligomerization and pore formation. The extreme N-terminal sequences of the toxin are susceptible to cleavage by intestinal proteases such as trypsin or chymotrypsin and thus may

be removed in the intestines during disease. Proteolytic removal of these sequences increases cytotoxicity by about 2 to 3-fold, as it exposes the CPE residues (notably residue D48) to promote toxin oligomerization (2).

During sporulation, some *C. perfringens* strains produce very large amounts of CPE, which can comprise up to 20% of the total protein present in a sporulating cell (2). The *cpe* gene is transcribed as a message of ~1.2 kb, beginning ~3 h after inoculation into Duncan-Strong sporulation medium (2). The CPE protein becomes detectable by Western blotting at 4-5 h post-inoculation into Duncan-Strong medium. CPE is not secreted from sporulating cells, but instead accumulates in the cytoplasm until the mother cell lyses to release the mature spore (2).

Regardless of whether an isolate carries a chromosomal *cpe* gene or a plasmid-borne *cpe* gene, CPE production is strictly sporulation-associated. Therefore, it is not surprising that a *spo0A* null mutant of SM101, which cannot sporulate, is unable to produce CPE (37). Similarly, the Agr-like quorum sensing system, which regulates sporulation in *C. perfringens* by reducing production of Spo0A, is also required for wild-type production levels of CPE (44). In contrast, *virX* RNA inhibits CPE production by repressing sporulation (47).

One reason for the strong, sporulation-associated expression of CPE by *cpe*-positive type A strains is the presence of three promoters upstream of the *cpe* ORF (Fig. 3). Two of those *cpe* promoters (named P2 and P3) are similar to consensus SigE dependent promoters that drive mother cell gene expression, while the other *cpe* promoter (named P1) resembles a consensus SigK-dependent promoter. Consistent with *cpe* transcription being dependent on these two sporulation-associated sigma factors, *sigK*- and *sigE*-null mutants of strain SM101 failed to drive beta-glucuronidase production when transformed with a plasmid carrying the *cpe* promoter region fused to the *Escherichia coli* reporter gene *gusA* (29). Another study (28) investigated the role of SigF and SigG in CPE production and reported that *cpe* transcription is also blocked in an SM101 *sigF* null mutant, but not in a SM101 *sigG* null mutant. The role of SigF in controlling CPE production can be explained by the dependence of SigE and SigK upon SigF expression. The ability of a *sigG*-null mutant to produce CPE indicates that, while all four sporulation-associated sigma factors are needed for *C. perfringens* sporulation, those sigma factors are not all necessary for *cpe* transcription and CPE production (28, 29).

TpeL toxin

Many *C. perfringens* isolates encode a novel toxin named TpeL. TpeL was initially identified in the supernatant of *C. perfringens* strain CP4 and shown to be cytotoxic to Vero cells by causing cell rounding (4). TpeL has a molecular mass of ~205 kDa (88) and belongs to the family of large clostridial toxins (LCTs) that encompass *Clostridium difficile* toxin A (TcdA) and B (TcdB), along with similar toxins such as *Clostridium sordellii* lethal toxin (TcsL) made by other clostridial species (89). TpeL has no signal peptide region within the open reading frame (4).

Classically, large clostridial toxins were thought to contain four domains: i) A-domain: involved in N-terminal biological activity; ii) B-domain: C-terminal carbohydrate-binding

repeats, often assumed to be involved in receptor binding; iii) C-domain: autoproteolytic cleavage during toxin-processing; and iv) D-domain: delivery of the A-domain into cytosol (89). LCTs enter host cells via endocytosis and insert via the D-domain into the endosome membrane. The protease C-domain is activated by intracellular inositolhexaphosphate (InsP₆), resulting in toxin-cleavage and release of the A domain to the cytosol, where it glycosylates small GTPases inactivating their cellular functions.

Notably, TpeL has a shorter amino acid sequence than other LCTs, with homology encompassing to the N-terminal domain, including the DXD motif (essential for glycosyltransferase activity) and a conserved W102 (essential for enzymatic activity) (4, 89-91). Interestingly, despite the dogma that LCTs use their B-domain to bind to host cell membrane receptor(s) (89, 92), the carbohydrate binding repeats present in the C-terminal domain of other LCTs are absent from TpeL (88). Recently, this puzzle was resolved when a receptor binding domain was identified in the C-terminal half of the TpeL D domain (93). This region allows TpeL to bind to LDL receptor-related protein 1 (LRP1) as a receptor (93).

The *tpeL* gene is present ~3 kb downstream of the beta toxin-encoding gene (*cpb*) on large plasmids in many *C. perfringens* type B and C strains (94, 95). The *tpeL* gene is also present in some type A isolates (96), although its location (plasmid vs. chromosomal) has not been determined in those strains. Studies have shown that *tpeL* is present, i) in ~18% of type A necrotic enteritis outbreak isolates (96); ii) in ~2% of *C. perfringens* isolates from retail chicken samples (96); and iii) in 100% and 75%, respectively, of type B and type C isolates (94, 95). The contribution of TpeL, when produced, to *C. perfringens* pathogenesis remains unclear.

Bioinformatic analysis of the *tpeL* promoter region on the 65-kb plasmid of *C. perfringens* type B strain ATCC 3626 revealed the presence of σ^E - and σ^K -dependent promoter sequences (97). In contrast, no sequence with similarity to the consensus OA box was found (97). Evidence that *tpeL* can be expressed during sporulation came from fusion of the *tpeL* promoter region with the *Escherichia coli gusA* reporter gene; when that construct was introduced into *C. perfringens* strain SM101, no significant beta-glucuronidase (GUS) activity was observed in the vegetative growth of SM101 transformants. However, GUS activity became significant in sporulating cultures of those SM101 transformants within as little as 4 h after initiation of sporulation (97). Sporulation-specific expression of *tpeL* was confirmed by introducing plasmids carrying the *tpeL-gusA* fusions into a SM101 *spo0A* mutant (37); no GUS specific activity was observed when this *spo0A* mutant carrying the *tpeL-gusA* fusion construct was grown under sporulation conditions, indicating that sporulation-regulated *tpeL* expression is dependent upon *spo0A* expression.

Evidence of σ^E -dependent expression of *tpeL* came from experiments that measured GUS specific activity in vegetative and sporulation cultures of a SM101 *sigE* mutant carrying the *tpeL-gusA* fusion construct (97). No significant GUS specific activity was observed during vegetative or sporulation growth of *sigE* mutant carrying the *tpeL-gusA* fusion construct (97). The expression of *tpeL* was only detectable in the mother cell compartment and at ~80 to 150 fold lower levels than *cpe* expression (97). Given the presence of the putative σ^K -

dependent promoter upstream of *tpeL*, it is possible that other σ factors such as σ^K might also be involved in regulating *tpeL* expression (97). Further detailed studies on *tpeL* promoter binding with Spo0A, σ^E and σ^K should clarify the mechanism of sporulation-regulated *tpeL* expression (97).

While the above evidence supports *tpeL* expression during sporulation, TpeL production by *C. perfringens* isolates during vegetative growth has also been reported (98-99). Furthermore, those studies found repression of TpeL production mediated by glucose in a similar manner to LCT production by *C. difficile*. During vegetative growth, the regulator TpeR is critical for TpeL production, similar to the cases of *C. difficile* TcdR and *C. sordellii* TcsR.

Summary

The important pathogen *C. perfringens* utilizes its spores to survive in harsh environments. Spores are also important for transmission of this food-borne disease pathogen, particularly where type A chromosomal *cpe* food poisoning isolates often produce highly resistant spores that can survive in temperature-abused foods. Another linkage between sporulation and virulence for *C. perfringens* is the expression of two *C. perfringens* toxins from promoters recognized by sporulation-associated sigma factors. The production of one of those toxins, CPE, is essential for the pathogenesis of the second most common bacterial food-borne disease in the USA. Interestingly, another Gram-positive spore-former, *B. thuringiensis*, also produces sporulation-associated, pore-forming toxins that affect the gastrointestinal tract, albeit in insects rather than humans. The similarities between the pathogenesis of *C. perfringens* and *B. thuringiensis* suggest that these bacteria show a common strategy of producing toxins when sporulating in the GI tract of their host in order to induce diarrhea, which may then facilitate transmission of their spores back into the environment so they can be picked up by additional hosts. The prevalence of *C. perfringens* type A food poisoning suggests this strategy has been highly successful.

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References

1. McClane, BA.; Robertson, SL.; Li, J. *Clostridium perfringens*. In: Doyle, MP.; Buchanan, RL., editors. Food Microbiology: Fundamentals and Frontiers. 4th. ASM press; Washington, D.C.: 2013. p. 465-489.
2. McClane, BA.; Uzal, FA.; Miyakawa, MF.; Lyster, D.; Wilkins, TD. The Enterotoxigenic Clostridia. In: Dworkin, M.; Falkow, S.; Rosenberg, E.; Schleifer, H.; Stackebrandt, E., editors. The Prokaryotes. 3rd. Springer NY press; New York: 2006. p. 688-752.
3. Mallozzi M, Viswanathan VK, Vedantam G. Spore-forming Bacilli and Clostridia in human disease. Future Microbiol. 2010; 5:1109-1123. [PubMed: 20632809]

4. Amimoto K, Noro T, Oishi E, Shimizu M. A novel toxin homologous to large clostridial cytotoxins found in culture supernatant of *Clostridium perfringens* type C. Microbiology. 2007; 153:1198–1206. [PubMed: 17379729]
5. Stevens, DL.; Rood, JI. Histotoxic Clostridia. In: Fischetti, VA.; Novick, RP.; Ferretti, JJ.; Portnoy, DA.; Rood, JI., editors. Gram-positive pathogens. 2nd. ASM press; Washington, DC: 2006.
6. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011; 17:7–15. [PubMed: 21192848]
7. Ma M, Li J, McClane BA. Genotypic and phenotypic characterization of *Clostridium perfringens* isolates from Darmbrand cases in post-World War II Germany. Infect Immun. 2012; 80:4354–4363. [PubMed: 23027533]
8. Carman RJ. *Clostridium perfringens* in spontaneous and antibiotic-associated diarrhoea of man and other animals. Rev Med Microbiol. 1997; 8(supplement 1):S43–S5.
9. Novak JS, Juneja VK, McClane BA. An ultrastructural comparison of spores from various strains of *Clostridium perfringens* and correlations with heat resistance parameters. Int J Food Microbiol. 2003; 86:239–247. [PubMed: 12915035]
10. Orsburn B, Melville SB, Popham DL. Factors contributing to heat resistance of *Clostridium perfringens* endospores. Appl Environ Microbiol. 2008; 74:3328–3335. [PubMed: 18378644]
11. Henriques AO, Moran CP Jr. Structure, assembly, and function of the spore surface layers. Annu Rev Microbiol. 2007; 61:555–588. [PubMed: 18035610]
12. Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol. 2006; 101:514–525. [PubMed: 16907802]
13. Klobutcher LA, Ragkousi K, Setlow P. The *Bacillus subtilis* spore coat provides “eat resistance” during phagocytic predation by the protozoan *Tetrahymena thermophila*. Proc Natl Acad Sci USA. 2006; 103:165–170. [PubMed: 16371471]
14. Laaberki MH, Dworkin J. Role of spore coat proteins in the resistance of *Bacillus subtilis* spores to *Caenorhabditis elegans* predation. J Bacteriol. 2008; 190:6197–6203. [PubMed: 18586932]
15. Paredes-Sabja D, Sarker MR. Host serum factor triggers germination of *Clostridium perfringens* spores lacking the cortex hydrolysis machinery. J Med Microbiol. 2011; 60:1734–1741. [PubMed: 21799201]
16. Popham, DB.; Bernhards, CB. Spore peptidoglycan. In: Eichenberger, P.; Driks, A., editors. The Bacterial Spore. ASM Press; Washington, DC: in press
17. Warth AD, Strominger JL. Structure of the peptidoglycan of bacterial spores: occurrence of the lactam of muramic acid. Proc Natl Acad Sci USA. 1969; 64:528–535. [PubMed: 4982357]
18. Warth AD, Strominger JL. Structure of the peptidoglycan from spores of *Bacillus subtilis*. Biochemistry. 1972; 11:1389–1396. [PubMed: 4259915]
19. Setlow P. Spore germination. Curr Opin Microbiol. 2003; 6:550–556. [PubMed: 14662349]
20. Makino S, Moriyama R. Hydrolysis of cortex peptidoglycan during bacterial spore germination. Med Sci Monit. 2002; 8:RA119–RA127. [PubMed: 12070452]
21. Moir A, Corfe BM, Behravan J. Spore germination. Cell Mol Life Sci. 2002; 59:403–409. [PubMed: 11964118]
22. Setlow P. I will survive: DNA protection in bacterial spores. Trends Microbiol. 2007; 15:172–180. [PubMed: 17336071]
23. Setlow P. Spore resistance properties. Microbiol Spectrum. 2014; 2(4):TBS-0003-2012.doi: 10.1128/microbiolspec.TBS-0003-2012
24. Labbe, RG. Sporulation (Morphology) of *Clostridium*. In: Durre, P., editor. Handbook on Clostridia. CRC Press; Boca Raton: 2005. p. 647–658.
25. Stragier, P. A gene odyssey: exploring the genomes of endospore-forming bacteria. In: Sonenshein, AL.; Hoch, JA.; Losick, R., editors. *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press; Washington, D.C.: 2002. p. 519–525.
26. Philippe VA, Méndez MB, Huang IH, Orsaria LM, Sarker MR, Grau RR. Inorganic phosphate induces spore morphogenesis and enterotoxin production in the intestinal pathogen *Clostridium perfringens*. Infect Immun. 2006; 74:3651–3656. [PubMed: 16714597]

27. Sarker MR, Carman RJ, McClane BA. Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Mol Microbiol.* 1999; 33:946–958. [PubMed: 10476029]
28. Li J, McClane BA. Evaluating the involvement of alternative sigma factors SigF and SigG in *Clostridium perfringens* sporulation and enterotoxin synthesis. *Infect Immun.* 2010; 78:4286–4293. [PubMed: 20643850]
29. Harry KH, Zhou R, Kroos L, Melville SB. Sporulation and enterotoxin (CPE) synthesis are controlled by the sporulation-specific sigma factors SigE and SigK in *Clostridium perfringens*. *J Bacteriol.* 2009; 191:2728–2742. [PubMed: 19201796]
30. Shih NJ, Labbe RG. Effect of glucose on sporulation and extracellular amylase production by *Clostridium perfringens* type A in defined medium. *Curr Microbiol.* 1994; 29:163–169.
31. Varga J, Stirewalt VL, Melville SB. The CcpA protein is necessary for efficient sporulation and enterotoxin gene (*cpe*) regulation in *Clostridium perfringens*. *J Bacteriol.* 2004; 186:5221–5229. [PubMed: 15292123]
32. Bettegowda C, Huang X, Lin J, Cheong I, Kohli M, Szabo SA, Zhang X, Diaz LA Jr, Velculescu VE, Parmigiani G, Kinzler KW, Vogelstein B, Zhou S. The genome and transcriptomes of the anti-tumor agent *Clostridium novyi*-NT. *Nat Biotechnol.* 2006; 24:1573–1580. [PubMed: 17115055]
33. Bruggemann H, Baumer S, Fricke WF, Wiezer A, Liesegang H, Decker I, Herzberg C, Martinez-Arias R, Merkl R, Henne A, Gottschalk G. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proc Natl Acad Sci USA.* 2003; 100:1316–1321. [PubMed: 12552129]
34. Myers GS, Rasko DA, Cheung JK, Ravel J, Seshadri R, DeBoy RT, Ren Q, Varga J, Awad MM, Brinkac LM, Daugherty SC, Haft DH, Dodson RJ, Madupu R, Nelson WC, Rosovitz MJ, Sullivan SA, Khouri H, Dimitrov GI, Watkins KL, Mulligan S, Benton J, Radune D, Fisher DJ, Atkins HS, Hiscox T, Jost BH, Billington SJ, Songer JG, McClane BA, Titball RW, Rood JI, Melville SB, Paulsen IT. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Res.* 2006; 16:1031–1040. [PubMed: 16825665]
35. Nölling J, Breton G, Omelchenko MV, Makarova KS, Zeng Q, Gibson R, Lee HM, Dubois J, Qiu D, Hitti J, Wolf YI, Tatusov RL, Sabathe F, Doucette-Stamm L, Soucaille P, Daly MJ, Bennett GN, Koonin EV, Smith DR. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J Bacteriol.* 2001; 183:4823–4838. [PubMed: 11466286]
36. Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, Ogasawara N, Hattori M, Kuhara S, Hayashi H. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc Natl Acad Sci USA.* 2002; 99:996–1001. [PubMed: 11792842]
37. Huang IH, Waters M, Grau RR, Sarker MR. Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiol Lett.* 2004; 233:233–240. [PubMed: 15063491]
38. Huang IH, Sarker MR. Complementation of a *Clostridium perfringens* *spo0A* mutant with wild-type *spo0A* from other *Clostridium* species. *Appl Environ Microbiol.* 2006; 72:6388–6393. [PubMed: 16957268]
39. Sonenshein AL. Control of sporulation initiation in *Bacillus subtilis*. *Curr Opin Microbiol.* 2000; 3:561–566. [PubMed: 11121774]
40. Kroos L. The *Bacillus* and *Myxococcus* developmental networks and their transcriptional regulators. *Annu Rev Genet.* 2007; 41:13–39. [PubMed: 18076324]
41. Molle V, Fujita M, Jensen ST, Eichenberger P, González-Pastor JE, Liu JS, Losick R. The Spo0A regulon of *Bacillus subtilis*. *Mol Microbiol.* 2003; 50:1683–1701. [PubMed: 14651647]
42. Li J, Paredes-Sabja D, Sarker MR, McClane BA. Further characterization of *Clostridium perfringens* small acid soluble protein-4 (Ssp4) properties and expression. *PLoS One.* 2009; 4:e6249. [PubMed: 19609432]
43. Vidal JE, Chen J, Li J, McClane BA. Use of an EZ-Tn5-based random mutagenesis system to identify a novel toxin regulatory locus in *Clostridium perfringens* strain 13. *PLoS One.* 2009; 4:e6232. [PubMed: 19597556]

44. Li J, Chen J, Vidal JE, McClane BA. The Agr-like quorum-sensing system regulates sporulation and production of enterotoxin and beta2 toxin by *Clostridium perfringens* type A non-food-borne human gastrointestinal disease strain F5603. *Infect Immun*. 2011; 79:2451–2459. [PubMed: 21464088]
45. Ohtani K, Yuan Y, Hassan S, Wang R, Wang Y, Shimizu T. Virulence gene regulation by the *agr* system in *Clostridium perfringens*. *J Bacteriol*. 2009; 191:3919–3927. [PubMed: 19363118]
46. Li J, Ma M, Sarker MR, McClane BA. CodY is a global regulator of virulence-associated properties for *Clostridium perfringens* type D strain CN3718. *MBio*. 2013; 4:e00770–e13. [PubMed: 24105766]
47. Ohtani K, Hirakawa H, Paredes-Sabja D, Tashiro K, Kuhara S, Sarker MR, Shimizu T. Unique regulatory mechanism of sporulation and enterotoxin production in *Clostridium perfringens*. *J Bacteriol*. 2013; 195:2931–2936. [PubMed: 23585540]
48. Xiao Y, van Hijum SA, Abee T, Wells-Bennik MH. Genome-wide transcriptional profiling of *Clostridium perfringens* SM101 during sporulation extends the core of putative sporulation genes and genes determining spore properties and germination characteristics. *PLoS One*. 2015; 10:e0127036. [PubMed: 25978838]
49. Paredes-Sabja D, Setlow P, Sarker MR. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. *Trends Microbiol*. 2011; 19:85–94. [PubMed: 21112786]
50. Moir A, Cooper G. Spore germination. *Microbiol Spectrum*. 2014; 3(5):TBS-0014-2012.doi: 10.1128/microbiolspec.TBS-0014-2012
51. Paredes-Sabja D, Torres JA, Setlow P, Sarker MR. *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *J Bacteriol*. 2008; 190:1190–1201. [PubMed: 18083820]
52. Paredes-Sabja D, Udombijitkul P, Sarker MR. Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning-related isolates. *Appl Environ Microbiol*. 2009; 75:6299–6305. [PubMed: 19666724]
53. Paredes-Sabja D, Sarker MR. Germination response of spores of the pathogenic bacterium *Clostridium perfringens* and *Clostridium difficile* to cultured human epithelial cells. *Anaerobe*. 2011; 17:78–84. [PubMed: 21315167]
54. Mercado-Lubo R, McCormick BA. A unique subset of Peyer's patches express lysozyme. *Gastroenterology*. 2010; 138:36–39. [PubMed: 19932663]
55. Reitamo S, Klockars M, Adinolfi M, Osserman EF. Human lysozyme (origin and distribution in health and disease). *Ric Clin Lab*. 1978; 8:211–231. [PubMed: 366724]
56. Ghosh S, Zhang P, Li YQ, Setlow P. Superdormant spores of *Bacillus* species have elevated wet-heat resistance and temperature requirements for heat activation. *J Bacteriol*. 2009; 191:5584–5591. [PubMed: 19592590]
57. Moir A, Smith DA. The genetics of bacterial spore germination. *Annu Rev Microbiol*. 1990; 44:531–553. [PubMed: 2252393]
58. Banawas S, Paredes-Sabja D, Korza G, Li Y, Hao B, Setlow P, Sarker MR. The *Clostridium perfringens* germinant receptor protein GerKC is located in the spore inner membrane and is crucial for spore germination. *J Bacteriol*. 2013; 195:5084–5091. [PubMed: 24013629]
59. Udombijitkul P, Alnoman M, Banawas S, Paredes-Sabja D, Sarker MR. New amino acid germinants for spores of the enterotoxigenic *Clostridium perfringens* type A isolates. *Food Microbiol*. 2014; 44:24–33. [PubMed: 25084641]
60. Paredes-Sabja D, Setlow P, Sarker MR. Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Appl Environ Microbiol*. 2009; 75:3813–3817. [PubMed: 19363077]
61. Paredes-Sabja D, Setlow P, Sarker MR. GerO, a putative Na⁺/H⁺-K⁺ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens*. *J Bacteriol*. 2009; 191:3822–3831. [PubMed: 19363115]
62. Kumazawa T, Masayama A, Fukuoka S, Makino S, Yoshimura T, Moriyama R. Mode of action of a germination-specific cortex-lytic enzyme, SleC, of *Clostridium perfringens* S40. *Biosci Biotechnol Biochem*. 2007; 71:884–892. [PubMed: 17420590]

63. Paidhungat M, Ragkousi K, Setlow P. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca(2+)-dipicolinate. J Bacteriol. 2001; 183:4886–4893. [PubMed: 11466292]
64. Paredes-Sabja D, Setlow P, Sarker MR. The protease CspB is essential for initiation of cortex hydrolysis and dipicolinic acid (DPA) release during germination of spores of *Clostridium perfringens* type A food poisoning isolates. Microbiology. 2009; 155:3464–3472. [PubMed: 19628563]
65. Chen Y, Miyata S, Makino S, Moriyama R. Molecular characterization of a germination-specific muramidase from *Clostridium perfringens* S40 spores and nucleotide sequence of the corresponding gene. J Bacteriol. 1997; 179:3181–3187. [PubMed: 9150212]
66. Paredes-Sabja D, Setlow P, Sarker MR. SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. J Bacteriol. 2009; 191:2711–2720. [PubMed: 19218389]
67. Paredes-Sabja D, Sarker MR. Effect of the cortex-lytic enzyme SleC from non-food-borne *Clostridium perfringens* on the germination properties of SleC-lacking spores of a food poisoning isolate. Can J Microbiol. 2010; 56:952–958. [PubMed: 21076486]
68. Shimamoto S, Moriyama R, Sugimoto K, Miyata S, Makino S. Partial characterization of an enzyme fraction with protease activity which converts the spore peptidoglycan hydrolase (SleC) precursor to an active enzyme during germination of *Clostridium perfringens* S40 spores and analysis of a gene cluster involved in the activity. J Bacteriol. 2001; 183:3742–3751. [PubMed: 11371539]
69. Sarker MR, Shivers RP, Sparks SG, Juneja VK, McClane BA. Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. Appl Environ Microbiol. 2000; 66:3234–3240. [PubMed: 10919775]
70. Li J, McClane BA. Comparative effects of osmotic, sodium nitrite-induced, and pH-induced stress on growth and survival of *Clostridium perfringens* type A isolates carrying chromosomal or plasmid-borne enterotoxin genes. Appl Environ Microbiol. 2006; 72:7620–7625. [PubMed: 17041163]
71. Li J, McClane BA. Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. Appl Environ Microbiol. 2006; 72:4561–4568. [PubMed: 16820444]
72. Deguchi A, Miyamoto K, Kuwahara T, Miki Y, Kaneko I, Li J, McClane BA, Akimoto S. Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. PLoS One. 2009; 4:e5598. [PubMed: 19479065]
73. Wen Q, McClane BA. Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. Appl Environ Microbiol. 2004; 70:2685–2691. [PubMed: 15128519]
74. Paredes-Sabja D, Sarker N, Setlow B, Setlow P, Sarker MR. Roles of DacB and Spm proteins in *Clostridium perfringens* spore resistance to moist heat, chemicals, and UV radiation. Appl Environ Microbiol. 2008; 74:3730–3738. [PubMed: 18441110]
75. Paredes-Sabja D, Setlow B, Setlow P, Sarker MR. Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid. J Bacteriol. 2008; 190:4648–4659. [PubMed: 18469104]
76. Raju D, Setlow P, Sarker MR. Antisense-RNA-mediated decreased synthesis of small, acid-soluble spore proteins leads to decreased resistance of *Clostridium perfringens* spores to moist heat and UV radiation. Appl Environ Microbiol. 2007; 73:2048–2053. [PubMed: 17259355]
77. Raju D, Waters M, Setlow P, Sarker MR. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiol. 2006; 6:50. [PubMed: 16759397]
78. Li J, McClane BA. A novel small acid soluble protein variant is important for spore resistance of most *Clostridium perfringens* food poisoning isolates. PLoS Pathog. 2008; 4:e1000056. [PubMed: 18451983]
79. Miyamoto K, Li J, Akimoto S, McClane BA. Molecular approaches for detecting enterotoxigenic *Clostridium perfringens* Res Adv Appl Environ Microbiol. 2009; 2:1–7.

80. Caserta JA, Robertson SL, Saputo J, Shrestha A, McClane BA, Uzal FA. Development and application of a mouse intestinal loop model to study the in vivo action of *Clostridium perfringens* enterotoxin. *Infect Immun*. 2011; 79:3020–3027. [PubMed: 21628512]
81. Robertson SL, Smedley JG III, McClane BA. Identification of a claudin-4 residue important for mediating the host cell binding and action of *Clostridium perfringens* enterotoxin. *Infect Immun*. 2010; 78:505–517. [PubMed: 19884339]
82. Shrestha A, McClane BA. Human claudin-8 and -14 are receptors capable of conveying the cytotoxic effects of *Clostridium perfringens* enterotoxin. *MBio*. 2013; 4:e00594–e12. [PubMed: 23322640]
83. Robertson SL, Smedley JG III, Singh U, Chakrabarti G, Van Itallie CM, Anderson JM, McClane BA. Compositional and stoichiometric analysis of *Clostridium perfringens* enterotoxin complexes in Caco-2 cells and claudin 4 fibroblast transfectants. *Cell Microbiol*. 2007; 9:2734–2755. [PubMed: 17587331]
84. Chakrabarti G, McClane BA. The importance of calcium influx, calpain and calmodulin for the activation of CaCo-2 cell death pathways by *Clostridium perfringens* enterotoxin. *Cell Microbiol*. 2005; 7:129–146. [PubMed: 15617529]
85. Kitadokoro K, Nishimura K, Kamitani S, Fukui-Miyazaki A, Toshima H, Abe H, Kamata Y, Sugita-Konishi Y, Yamamoto S, Karatani H, Horiguchi Y. Crystal structure of *Clostridium perfringens* enterotoxin displays features of beta-pore-forming toxins. *J Biol Chem*. 2011; 286:19549–19555. [PubMed: 21489981]
86. Briggs DC, Naylor CE, Smedley JG III, Lukoyanova N, Robertson S, Moss DS, McClane BA, Basak AK. Structure of the food-poisoning *Clostridium perfringens* enterotoxin reveals similarity to the aerolysin-like pore-forming toxins. *J Mol Biol*. 2011; 413:138–149. [PubMed: 21839091]
87. Gao Z, McClane BA. Use of *Clostridium perfringens* enterotoxin and the enterotoxin receptor-binding domain (C-CPE) for cancer treatment: opportunities and challenges. *J Toxicol*. 2012; 2012:981626. [PubMed: 21941545]
88. Guttenberg G, Hornei S, Jank T, Schwan C, Lü W, Einsle O, Papatheodorou P, Aktories K. Molecular characteristics of *Clostridium perfringens* TpeL toxin and consequences of mono-O-GlcNAcylation of Ras in living cells. *J Biol Chem*. 2012; 287:24929–24940. [PubMed: 22665487]
89. Belyi Y, Aktories K. Bacterial toxin and effector glycosyltransferases. *Biochim Biophys Acta*. 2010; 1800:134–143. [PubMed: 19647041]
90. Busch C, Hofmann F, Gerhard R, Aktories K. Involvement of a conserved tryptophan residue in the UDP-glucose binding of large clostridial cytotoxin glycosyltransferases. *J Biol Chem*. 2000; 275:13228–13234. [PubMed: 10788427]
91. Busch C, Hofmann F, Selzer J, Munro S, Jeckel D, Aktories K. A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial cytotoxins. *J Biol Chem*. 1998; 273:19566–19572. [PubMed: 9677381]
92. Voth DE, Ballard JD. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev*. 2005; 18:247–263. [PubMed: 15831824]
93. Schorch B, Song S, van Diemen FR, Bock HH, May P, Herz J, Brummelkamp TR, Papatheodorou P, Aktories K. LRP1 is a receptor for *Clostridium perfringens* TpeL toxin indicating a two-receptor model of clostridial glycosylating toxins. *Proc Natl Acad Sci USA*. 2014; 111:6431–6436. [PubMed: 24737893]
94. Gurjar A, Li J, McClane BA. Characterization of toxin plasmids in *Clostridium perfringens* type C isolates. *Infect Immun*. 2010; 78:4860–4869. [PubMed: 20823204]
95. Sayeed S, Li J, McClane BA. Characterization of virulence plasmid diversity among *Clostridium perfringens* type B isolates. *Infect Immun*. 2010; 78:495–504. [PubMed: 19858300]
96. Chalmers G, Bruce HL, Hunter DB, Parreira VR, Kulkarni RR, Jiang YF, Prescott JF, Boerlin P. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. *J Clin Microbiol*. 2008; 46:3957–3964. [PubMed: 18945840]
97. Paredes-Sabja D, Sarker N, Sarker MR. *Clostridium perfringens* tpeL is expressed during sporulation. *Microbial pathogen*. 2011; 51:384–8.

98. Chen J, McClane BA. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. *Infect Immun*. 2015; 83:2369–2381. [PubMed: 25824828]
99. Carter GP, Larcombe S, Li L, Jayawardena D, Awad MM, Songer JG, Lyras D. Expression of the large clostridial toxins is controlled by conserved regulatory mechanisms. *Int J Med Microbiol*. 2014; 304:1147–1159. [PubMed: 25190355]

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Figure 1.

Ultrastructure of *C. perfringens* spores. Transmission electron micrograph of a spore from *C. perfringens* strain H-6, a food poisoning strain. Components of spore shown include: proteinaceous spore coat layers; cortex region; and the core with ribosomes giving a granular appearance. The bar represents 1.0 μM. Reproduced with permission from (9).

Figure 2.

Sporulation-associated sigma factors are required for *C. perfringens* sporulation. Shown are photomicrographs of sporulating cultures of SM101, a transformable derivative of a food poisoning strain, after growth for 8 h in Duncan-Strong sporulation medium. Also shown is the absence of sporulating cells in similar Duncan-Strong cultures of a *sigF* or *sigG* null mutant of SM101 (SM101::*sigF* or SM101::*sigG*). This loss of sporulation was specifically due to inactivation of the *sigF* or *sigG* genes in those mutants since the effect was reversible by complementation, i.e., by adding back a wild-type *sigF* or *sigG* gene, respectively, to those mutants (SM101::*sigF*Comp or SM101::*sigG*Comp). Reproduced with permission from (28). Similar loss of sporulation was observed with *sigE*^{or} *sigK* mutants of SM101 (29).

Figure 3.

Sporulation in *C. perfringens*. Working through unidentified intermediates, the Agr quorum sensing system and CcpA affect Spo0A expression or, possibly, phosphorylation to initiate sporulation. This triggers a cascade of sigma factors where SigF controls production of the three other sporulation-associated sigma factors. Two of these sigma factors (SigE and SigK) then regulate CPE production during sporulation. Compiled from (28, 29, 31, 44). Not shown in this drawing, SigE (and possibly SigK) can also regulate production of TpeL toxin (97).

Figure 4.

DNA binding properties of recombinant His6-tagged SASP4. A) Electromobility shift assays (EMSA) showing binding to biotin-labelled *C. perfringens* DNA by purified rSASP4 from F4969 (a CPE-positive nonfood-borne human GI disease strain that forms sensitive spores and produces an SASP4 variant with a Gly at residue 36), SM101 or 01E809 (two CPE-positive food poisoning isolates that form resistant spores and produce an SASP4 variant with Asp at residue 36). B) EMSA showing binding by purified SM101 rSASP4 or rSASP2 to (left) *C. perfringens* AT-rich biotin-labelled DNA or (right) biotin-labeled *C. perfringens* GC-rich DNA. Reproduced with permission from (42: 78).

Figure 5.

Current model for the mechanism of action of CPE. CPE binds to claudin receptors to form small complexes. Those small complexes then oligomerize on the host cell surface to form an ~450 kDa prepore known as CH-1. The prepore inserts into the membrane to form an active pore that alters host plasma membrane permeability for small molecules. As a result, calcium enters the cytoplasm and triggers either apoptosis (caused by low CPE doses, where there is a modest calcium influx) or oncosis (caused by high CPE doses, where there is a strong calcium influx). Reproduced with permission from (1).