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 involve their production of a variant small acid-soluble protein-4 that binds more tightly to spore DNA than 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 United States. During this foodborne disease, C. perfringens is ingested with food and then, by 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 review, 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 to E) based on 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-toxins, 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.
Like some spore-forming 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 as follows: (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 by 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 non-intestinal 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 United States 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 foodborne 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 Darminbrand (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 foodborne 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 (as discussed later in this review); those spores later germinate and cause illness when the food is ingested.

CPE-producing type A strains carrying a plasmid cpe gene are responsible for 2 to 15% of all cases of non-foodborne 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 to 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 review 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 have not yet been carefully studied; however, in other Gram-positive sporeformers, the spore coat is thought to comprise >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 sporeformers, including *C. perfringens*. Beneath the coat is the outer membrane, which does not provide protection to dormant spores, but it is essential for spore formation and is presumably lost by shearing forces after short periods. The spore peptidoglycan cortex underlies 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 sporeformers, 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-acetyl muramic acid (NAM) residues are substituted with short peptides, giving the cortex a lower degree of cross-linking 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 peptidoglycan is converted to muramic-δ-lactam (MAL) (17, 18), which seems to be the recognition substrate element for the cortex lytic enzymes (CLEs) which uniquely hydrolyze the peptidoglycan cortex, but not the germ cell wall, during spore germination (19, 20). The germ cell wall underlies the spore peptidoglycan 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), which results in a low permeability to small molecules including water and DNA-damaging chemicals (12, 21).

The core is the innermost 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 to 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 *C. 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 (P_i) in the environment (26). In contrast, in sporulation medium containing P_i, *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, P_i can neutralize the inhibitory effect of glucose at the onset of sporulation and induces spo0A expression, indicating that P_i acts as a key signal triggering sporulation in *C. perfringens* (26). As introduced earlier, *C. perfringens* sporulation directly

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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, the cortex region, and the core with ribosomes giving a granular appearance. The bar represents 1.0 μM. Reproduced with permission from reference 9. doi:10.1128/microbiolspec.TBS-0022-2015.f1
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 P1 is normally present in the GI tract of humans and animals, it appears that C. perfringens has efficiently adapted to sporulating in the GI tract. By extension, it can be speculated that P1 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, Bacillus 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 knockout 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 downregulated, 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 on Spo0A (37, 42).

In C. perfringens, the morphological events during sporulation are divided into seven stages (I to VII), resembling those of sporulating B. subtilis cells. In B. subtilis, four major sporulation-specific sigma (σ) factors (σF, σE, σG, and σK) regulate this sporulation process. The homologues 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 the following: (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 the production of all four sporulation-associated alternative sigma factors. Differences 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 σE-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, spoIIAA, and spoIAB 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 with 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 homologues 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 upregulation between 3 and 5 log2-fold increases and 451 genes were upregulated 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 foodborne 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-foodborne human GI diseases, since these illnesses are thought to be transmitted by the 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-foodborne GI disease isolates. Spores of *C. perfringens* food poisoning isolates can germinate in the presence of KCl, NaPi (pH 6.0), L-asparagine, or the exogenous 1:1 chelate of Ca²⁺ and Ca-DPA, while spores of non-foodborne GI disease isolates initiate germination in the presence of L-alanine, L-valine, and with the mixture of KCl and L-asparagine (51, 52). Also, spores of a non-foodborne 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-foodborne GI disease isolates are better

**FIGURE 3** Sporulation in *C. perfringens*. Working through unidentified intermediates, the Agr QS 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 references 28, 29, 31, and 44. Not shown in this drawing, SigE (and possibly SigK) can also regulate production of TpeL toxin (97). doi:10.1128/microbiolspec.TBS-0022-2015.f3
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 composes 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* account 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 knockout 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 knockout 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 *Bacillus 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 mutants 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 water content (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 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-foodborne 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-foodborne 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-foodborne 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-foodborne 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 pre-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 foodborne 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 review, 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 (D100 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 D100 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 D100 values exceeding 2 h (69).

Of epidemiologic significance, a survey detected both spores and vegetative cells of cpe-positive type A isolates in nonoutbreak raw meats and seafood sold retail in the United States (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 3- to 4-fold greater, respectively, compared with 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 with 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 on a synergistic interplay of multiple factors, which include sporulation temperature, mineralization of the core with DPA and its
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cations, binding of α/β-type small acid-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 2-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 peptidoglycan cortex also plays a major role in C. perfringens sporulation 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, SASP2, and SASP3 levels in sporulating cells were reduced by >90% using antisense-RNA-mediated downregulation approaches, a 5-fold reduction in spore heat resistance was observed (73, 74, 76). Those spores also showed greater sensitivity to chemicals (i.e., nitrous acid, hydrogen peroxide, formaldehyde, and HCl) and UV radiation. However, SASP1, SASP2, and SASP3 levels could not explain the resistance differences observed between spores of type A chromosomal cpe isolates versus spores of other C. perfringens since all strains produce similar amounts of SASP1, SASP2, and SASP3 and no consistent sequence variation in these proteins occurs among C. perfringens strains (73, 74, 76, 77).

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 Asp36 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 (EMSAs) and DNA binding studies showed that SASP4 variants with an Asp at residue 36 bind 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 ultrastructural features of C. perfringens spores are similar to those of other sporeformer 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 eight 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 apparently have now evolved to excel at causing foodborne 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 (2) of these Darmbrand isolates showed they carry both beta (cpb) 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., they both produce spores that are highly resistant to food environment stresses—which allows their spores to germinate for entry of vegetative cells into the human GI tract by ingestion in food—and they both produce toxins that are highly active in the intestines. 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**

*C. perfringens* Enterotoxin

As already introduced, CPE-producing type A strains of *C. perfringens* cause the second most common bacterial foodborne illness in the United States, along with many cases of non-foodborne 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 \(\sim 10\) min). 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 \textit{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-foodborne 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 Koch’s molecular postulates demonstrated that CPE production is essential for the GI pathogenicity of CPE-positive type A human food poisoning and non-foodborne 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 nonreceptor (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.

**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 reference 1. doi:10.1128/microbiolspec.TBS-0022-2015.f5
CPE pore formation increases the permeability of mammalian cells for the second messenger calcium, among 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 and further damage to the tight junction by sequestering even more tight-junction proteins in CPE complexes and inducing the internalization of 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 to 5 h postinoculation 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 QS 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 open reading frame (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 inositol hexaphosphate (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 low-density lipoprotein receptor-related protein 1 (LRP1) as a receptor (93).

The tpeL gene is present ∼3 kb downstream of the beta-toxin-encoding gene (cph) 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 versus chromosomal) has not been determined in those strains. Studies have shown that tpeL is present (i) in ∼18% of type A necrotic enteritic 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 σK- and σK-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 E. coli gusA reporter gene; when that construct was introduced into C. perfringens strain SM101, no significant beta-glucuronidase (GUS) activity was observed in 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 an 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 σK-dependent expression of tpeL came from experiments that measured GUS specific activity in vegetative and sporulation cultures of an 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 σK-dependent promoter upstream of tpeL, it is possible that other σ factors such as σK might also be involved in regulating tpeL expression (97). Further detailed studies on tpeL promoter binding with Spo0A, σK, and σE 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. sordelli TcsR.

**SUMMARY**

The important pathogen C. perfringens utilizes its spores to survive in harsh environments. Spores are also important for transmission of this foodborne 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 foodborne disease in the United States. Interestingly, another
Gram-positive sporeformer, Bacillus 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 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
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