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Title: New Insights into the Molecular Mechanism of *Clostridium perfringens* Spore Germination

Abstract approved:

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C. perfringens is a spore-forming, gram-positive, anaerobic pathogenic bacterium capable of causing a wide variety of diseases in both humans and animals. However, the two most common illnesses in humans are *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) illnesses. Interestingly, these two major diseases are caused only by *C. perfringens* Type A isolates that are able to produce the *C. perfringens* enterotoxin (CPE). Importantly, the CPE-encoding gene (*cpe*) can be found in different places in FP and NFB isolates. *C. perfringens* FP isolates carry the *cpe* gene on the chromosome, while NFB isolates

causing GI illnesses (i.e., sporadic diarrhea and antibiotic-associated diarrhea) carry a plasmid-borne copy of the *cpe.*

C. perfringens spores are highly resistant and can survive in the environment for decades. When spores are in a favorable environment, they can initiate germination and return to active growth to cause disease. Spore germination is an early step and essential stage in the progression of *C. perfringens* infection in humans and animals. Recent findings have identified the germinants of spores of *C. perfringens* FP and NFB isolates. A variety of germinants can initiate the process, including nutrients, amino acids, cationic surfactants, and enzymes termed germinant. Further understanding of the germination of *Clostridium* species is needed, since *Bacillus* species spore germination has been studied well.

In the first study, we demonstrated the individual role of each of these germinant receptors (GRs) in *C. perfringens* spore germination. To reach the goal, we constructed mutant strains carrying single mutations in *gerKA* or *gerKC,* and double mutant *gerKB gerAA*, and characterized the germination phenotypes of their spores in the presence of nutrient and non-nutrient germinants. Through Western blot analyses, the precise location of GerKC protein in spores was also determined. This study offers the following findings: (i) Spores of *gerKC* mutant did not germinate with KCl, L-asparagine, a mixture of asparagine and KCl, and NaPi at pH 6.0. (ii) The *gerKC* spores germinated poorly compared to wild-type and other GRs mutant spores with the non-nutrient germinants dodecylamine and a 1:1 chelate of Ca^{2+} and dipicolinic

acid. (iii) The germination defect in *gerKC* spores was restored by complementing the *gerKC* mutant with wild-type *gerK* operon, indicating that GerKC is essential for germination of *C. perfringens* spores. (iv) GerKC is essential for the release of DPA from the spore's core during germination with KCl and dodecylamin. (v) GerKC is also essential for spore's viability; Finally, (vi) GerKC localizes in the spore's inner membrane.

A second study of this work investigated the precise location for the Csp proteases CspB and cortex lytic enzymes SleC in spores of *C. perfringens* FP strain SM101. It was shown that CspB and pro-SleC are present exclusively in the *C. perfringens* SM101 spore coat layer fraction and absent in the lysate from decoated spores and from the purified inner spore membrane. In addition, quantitative Western blot analyses demonstrated that there are approximately 2,000 and 130,000 molecules of CspB and pro-SleC, respectively, per *C. perfringens* SM101 spore.

The third study was to identify and characterize the germinants and receptors involved in *C. perfringens* NFB strain F4969. Results from these studies indicate that NFB strain F4969 germinates with AK in a cooperative manner, while FP strain SM101 mixture of L-Asparagine and KCl (AK) components are capable of triggering spore germination through independent pathways. Spores of *gerKA-KC, gerKA*, *gerKC*, *gerAA*, and *gerKB* knock-out mutants indicate that (i) germination of FP and NFB spores differs significantly in rich media and several defined germinants; (ii) L-asn and KCl induced germination in NFP F4969 spores cooperatively, while either

germinant alone triggers germination of FP spores; (iii) GerKC and GerAA proteins are required for normal germination of NFB spores with AK and L-cys;(iv) The colony-forming ability of *gerKC* or *gerKA-KC* spores was significantly lower than that of wild-type spores; although the ability of *gerAA* spores to outgrow was significantly affected, they gave rise to similar titers as wild-type spores.

Together, this dissertation study will help further understanding of the mechanism of spore germination of *C. perfringens* and the insights into the roles of spore germination for both FP and NFB CPE-producing *C. perfringens* isolates.

New Insights into the Molecular Mechanism of *Clostridium perfringens* Spore Germination

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Saeed S. Banawas, Author

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CONTRIBUTION OF AUTHORS

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Chapter 1

General Introduction and Literature Review

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped, spore-forming bacterium that produces toxins causing gastrointestinal (GI) and histotoxic diseases in humans and animals (1-3). *C. perfringens* is a ubiquitous microorganism found naturally in soil, water, dust, milk, wastewater, and in the intestinal tract of most humans and animals (1, 2). *C. perfringens* can be classified into 5 types (A-E) depending on their abilities to produce four major toxins (Alpha, Beta, Epsilon and Iota) (see section 1.1 for more details). Some *C. perfringens* isolates are able to produce *C. perfringens* enterotoxin (CPE) that is responsible for *C. perfringens* type A-associated food poisoning (FP) and non-food-borne GI disease. The *C. perfrigens* type A FP is currently ranks as the second most commonly reported bacterial food-borne disease in the United States, accounting for more than 1 million cases per year (2, 7). *C. perfringens* is the most common pathogenic member of the *Clostridium* species, which include pathogenic bacteria, such as *Clostridium difficile*, *Clostridium tetani*, *Clostridium botulinum* and the industrially relevant *Clostridium acetobutylicum* (8). The first description of *C. perfringens* was made by Welch and Nuttal, in 1892, and was commonly known as *Clostridium welchii* (9). The optimal temperature for *C. perfringens* growth is between 37 ºC and 45 ºC; however, growth can be at a temperature as low as 20º C and as high as 50º C (10, 11). Other key

factors for growth are water activity, pH level and Oxidation-reduction. *C. perfringens* can grow with a minimum water activity of 0.93 (2, 11). The optimal pH range for growth is 6.0 - 7.0, with poor growth under pH 5.0 and more than pH 8.0 (12). Oxidation reduction potential significantly affects *C. perfringens* growth, and it is found that its growth is initially between -125 and +320 mV (millivolts)(11). It has been suggested that *C. perfringens* is able to cause various diseases because: i) individual *C. perfringens* strains produce a sub-set of 17 different toxins (3); ii) this bacteria is fast growing, doubling time of around 8 min (13); iii) *C. perfringens* spores are highly resistant various environmental stresses (14-17).

1.1. Toxin production

Depending upon which of the four major types of toxins they produce (alpha-, beta-, epsilon-, and iota-), *C. perfringens* isolates are commonly classified into five toxinotypes (A-E) (Table 1.1).

Table 1.1 toxin types of *C. perfringens* (2, 3, 18)

C. perfringens Type	Major toxins[a]			
	Alpha	**Beta**	**Epsilon**	**Iota**
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	+
E	+	-	-	+

[a] +, produced; -, not produced

1.1.1. Alpha toxin

All *C. perfringens* types (A-E) produce alpha toxin; however, type A strain is the highest producer (1, 3, 19). The alpha (α) toxin gene, termed *plc*, is located on the chromosome of *C. perfringens*; the transcription level of this gene is regulated by the two-component regulatory system VirR/VirS (20). The purified molecular mass protein of α toxin is about 43 kDa and contains two domains (C and N –terminal) depending on zinc-containing metalloenzyme phospholipase C with sphingomyelinase and lecithinose enzyme activity (21). The C-terminal is an active site domain with zinc ions and the binding site N-terminal domain that plays a role in binding, degradation and disruption of the blood cells membrane and endothelial cells (18, 21). The α toxin is a phospholipase C, a major toxin responsible for tissue

damage in gas gangrene (22). In diagnosing, the reverse cAMP test is used as a tool to identify *C. perfringens*, based on the ability of the α toxin to lyse blood cells (22).

1.1.2. Beta toxin

Beta (β) toxin is a lethal toxin produced mainly by *C. perfringens* type B and C strains. It is a single polypeptide chain of 336 amino acids that has a molecular weight around 40 kDa (8, 23). The β-toxin is responsible for the major virulence factor causing narcotic enteritis and enterotoxaemia in many livestocks, such as sheep, lambs, fowl, and pigs (8, 24). β-toxin in type B is a causative agent mainly for animals; however, in type C it causes human enteritis necroticans, or pig-bel (5, 23). Pig-bel is caused by the consumption of under-cooked pork contaminated with a high number of *C. perfringens* type C spores and affects mainly immune-compromised individuals (25). Treatment of pig-bel disease consists of administration of β-toxin (26). A study found that the toxin is an oligomerizing, pore-forming toxin that can form cation-dependent channels in susceptible membrane layers (27). Ongoing work is attempting to generate a safer vaccine from β-toxin that can be used for humans and animals (28, 29).

1.1.3. Epsilon toxin

Epsilon (ε) toxin is produced by both type B and D strains of *C. perfringens* (5). The purified protein, termed prototoxin, is about 34.25 kDa (30). The ε-toxin is listed by Centers for Disease Control and Prevention (CDC) as a Category B bioterrorism agent and is ranked as the third most potent clostridial toxin, after botulinum and tetanus neurotoxins (5). The gene of ε-toxin (*etx*) is located on the large plasmid (31). This toxin has a limited host range that includes lambs, sheep, cattle and goats, causing enterotoxaemia and significant economic loss (5, 24). The mode of action of the toxin is still under investigation, but studies found that ε-toxin can lead to increased vascular permeability in the intestines, brains, and kidneys (32). Because of the high mortality rate due to the disease, a vaccine is a wise choice for prevention (28).

1.1.4. Iota toxin

Iota toxin is produced by *C. perfringens* type E isolates. Iota toxin was first isolated from a calf, showing signs of intoxication and enteritis, that died after 12h of illness (33, 34). Iota toxin is a binary toxin that is also found in strain *Clostridium spiroforme* (35). Trypsin is found to be activated in the iota toxin in early stages of growth after ~ 7h (8). Iota toxin consists of two separate noncovalently-linked components that are immunologically and biochemically distinct, named iota-a (Ia) and iota-b (Ib) (33, 34). The two components, Ia and Ib, have a molecular weight of around 47.5 and 71.5 kDa, respectively (34, 35). The subunits for the two components

(*iap* and *ibp*) are located on a large plasmid uniquely present in *C. perfringens* type E isolates (36). The light chain enzyme Ia is ADP-ribosyltransferase, and Ib is responsible for the binding and internalization of the toxin into the cell (35). Several studies agreed that, once the Ia is inside the cell, it catalyzes ADP-ribosylation of the globular skeletal muscle and non-muscle actin of the host; Ib is required for diffusion of the light chain Ia into the cytosol, causing cell death (35). The observation of disease was related to diarrhea in animals, especially domestic livestock (5).

1.1.5. Other toxins

Even though the four main toxins were used to classify *C. perfringens* into five toxinotypes (A-E), there are other toxins that were not used in the classification but have major effects on pathogenesis *C. perfringens*.

1.1.5.1. Necrotic enteritis toxin B (NetB) is found mainly in *C. perfringens* type A (18, 37). NetB causes necrotic enteritis in chickens (18, 37, 38). This fatal disease can affect the gastrointestinal tract in all poultry stock, costing the industry more than $2 billion per year internationally (39). Bioinformatic analyses suggest that ~ 40% of NetB is a beta-pore toxin and appears to show cytotoxic activity against the male leghorn chicken cell line by forming a heptameric pore which leads to damaged membrane ion permeability (39-41). Several studies found that, from *C. perfringens* isolates that were taken from disease lesions, the *netB* gene is chromosomally encoded (39, 41). The purified protein is ~ 33 kDa, and the expression of NetB is

controlled by the VirSR two-component signal-transduction system (39). The null mutation on the *virR* gene secretes less NetB toxin, and this phenotype is fully restored in complemented strain (39). The disease manifests as dehydration, depression, ruffled feathers, acute diarrhea and decreased daily feed intake (9, 40, 42). Recent studies have shown that NetB can be effective as a vaccine antigen (40, 43, 44).

1.1.5.2. Beta (β)-2 toxin. β-2 toxin is a 28 kDa toxin encoded by the *cpb2* gene, located on the plasmid in same location as the *cpe* gene located in most isolates from antibiotic-associated diarrhea (AAD) and sporadic-diarrhea (SD) (18, 45). The β-2 toxin can be found in *C. perfringens* type C (46) and in Type A isolates (47). Studies found that more than 70% of the isolates from patients with AAD or SD, typically caused by *C. perfringens* type A, also had β-2 toxin in association (46, 48). That β-2 toxin plays an important role in GI diseases may be explained by both the *cpb2* and *cpe* genes being located in the same plasmid in *C. perfringens* type A and causing non-food borne (NFB) diseases in humans (48). Additional studies have supported that β-2 toxin can cause necrotizing enterocolitis in domestic livestock and other animals (49, 50).

1.1.5.3. *Clostridium perfringens* enterotoxin (CPE). CPE shows a significant virulence factor for FP and NFB gastrointestinal illnesses in humans. CPE is produced 1-5%, mostly from *C. perfringens* type A isolates (2). Interestingly, CPE is

the only toxin in *C. perfringens* that is produced in high amounts during sporulation (51). CPE can be found in culture media after mother cell lyses and its expression is highly regulated by sporulation-specific sigma factors SigF, SigK and SigE (52-55). A pervious study found that Spo0A, a master regulator for sporulation initiation in *C. perfringens*, is required for sporulation and also for CPE production in *C. perfringens* (56). CPE is crucial for the pathogenesis of *C. perfringens* type A FP and NFB human GI disease isolates (1). The CPE encoding gene (*cpe*) can be located either on the chromosome or on plasmid (18, 57). Most of the *C. perfringens* FP isolates carry *cpe* gene on the chromosome. On the other hand, *cpe* gene is located on the large plasmid in NFB isolates (4, 14, 58, 59). The purified protein of CPE is about 35 kDa with heat liability and pH sensitivity (2, 60). When *C. perfringens* sporulation starts, CPE toxin releases, binds to the claudin and other co-receptors leading to the formation of an approximately 90-kDa CPE small complex. The small complex interacts with other proteins to form a large complex ~ 155 kDa, allowing CPE prepore to induce plasma membrane permeability alteration by N-terminal, then allowing a strong influx of calcium, causing apoptosis downstream. Death results from massive damage to the small intestinal cells, especially the tips of the villi, and electrolyte disregulation (2, 18, 61). The signs of CPE infection include diarrhea, with acute abdominal pain, as well as nausea, vomiting and fever (61).

C. perfringens is able to produce other toxins that play a minor role in pathogenesis of this bacterium:

1- Theta toxin: this toxin can be named perfringolysin O (PFO) and causes histotoxic infections, such as gas gangrene (62). PFO is chromosomally encoded by the *pfoA* gene, and the production of PFO is regulated by the Agr-like quorum sensing (QS) system and the VirS/VirA two-component regulatory system (63-67). This toxin is classified as a member of the cholesterol–dependent cytolysin (CDC) family of pore-forming toxins, so initially the toxin binds to CDC in the membrane of the host cells and causes a change in the conformation that results in oligomerization and begins pore formation (68).

2- Delta toxin: this is an extracellular hemolysin produced by *C. perfringens* type C isolates. Purified protein of delta toxin is about 42 kDa molecular weight. The toxin mainly shows cytotoxic activity against different types of cells, including erythrocytes from even-toed ungulates (pig, sheep, goat), human monocytes, rabbit macrophages, and blood platelets from humans, rabbits, and goats (69, 70). The toxin activity can be inhabited by G_{M2} ganglioside (69).

3- Kappa toxin: this toxin is encoded by the *colA* gene located on the chromosome of *C. perfringens*. Kappa toxin is collagenases, and gelatinase enzymes appear to be involved in tissue necrosis associated with other toxins (71). A study showed that a null mutation on *colA* is able to cause a pathogenic gas gangrene and clostridial myonecrosis in the mouse (72).

1.2. *C. perfringens* type A food poisoning (FP)

C. perfringens type A FP toxicoinfection is currently ranked as the second most frequent cause of bacterial food-borne illness in the USA (18), causing ~ 1 million cases per year with an economic loss of more than US $270 million (53, 73). Most type A FP isolates carry the *cpe* gene on the chromosome (4, 14, 58, 59). Obviously, the FP isolate spores has better adapted for food-borne transmission than NFB isolate spores because FP spores are highly resistant to various environmental stress factors, such as heat, radiation low and high temperature, high hydrostatic pressure, and toxic chemicals (16, 74-77). The high resistance of FP spores is due to the production of a variant small acid-soluble protein 4 (Ssp4) that binds to the spore DNA and gives protection to the spores from killing by various environmental stresses (16, 75). Moreover, *C. perfringens* is a fast growing bacterium with a rapid doubling time of ~ 8 min (2, 78). In addition, *C. perfringens* spores can remain in incompletely cooked food, including meat, and in poorly reheated foods (2), making the FP isolates a causative agent for several human GI diseases (Fig.1.1). Once the condition of food is favorable for spores, they start to germinate in the nutrient rich environment, resulting in a large number of *C. perfringens* type A FP vegetative cells. When the contaminated food is ingested (~10^6 bacteria cells/g of food), most of the vegetative cells will die off in the acidic environment of the stomach, however, some will survive and pass into the intestinal tract (79) (Fig.1.1). In a favorable intestinal environment, *C. perfringens* cells typically multiply undergo sporulation and produce CPE. The CPE is released into the environment when the endospores are fully

matured and then lysed from the mother cells (79) (Fig.1.1). Usually *C. perfringens* type A FP signs are mild and self-limiting within 12-24 h, including abdominal cramping, nausea and diarrhea, beginning 8-18 h after consuming contaminated food (2).

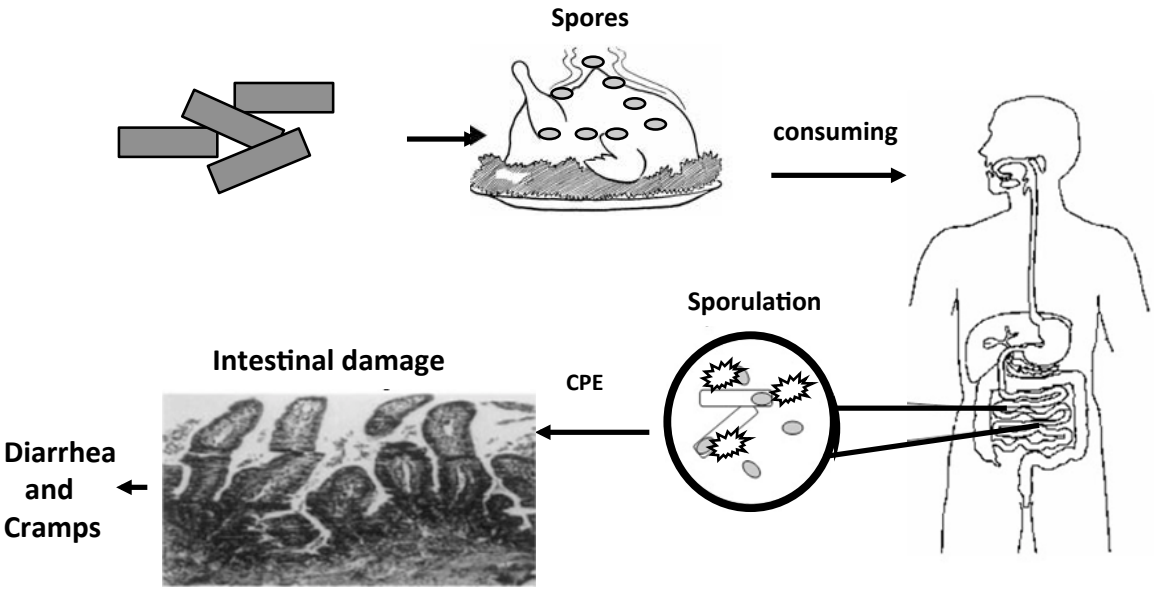

Fig.1.1 Overview of how *C. perfringens* FP infection occurs. When food is improperly cooled or cooked poorly, spores are heat-activated, germinated and grow to high numbers. Then, the contaminated food is ingested with ~10^6 cells /g of food. A small population of the multiplying cells survive and pass into the intestinal tract, where they multiply and sporulate. A high dose of CPE is produced from the sporulated cells, then released from mother cells. After that, CPE binds to the small intestine's epithelial cells, causing massive damage with subsequent cramps and diarrhea (4, 5).

1.3. *C. perfringens* type A non-food-borne (NFB) gastrointestinal disease

C. perfringens type A NFB isolates has been linked to non-food-borne human and animal diseases (59). Resistance for NFB isolates, in general, is less than for FP isolates in wet heat (14), osmotic, nitrite, pH level (60), storage temperature (81), and high hydrostatic pressure (77). Approximately 5-20% of all cases of ADD and SD are caused by *C. perfringens* type A NFB isolates (18, 48, 82). Most of NFB isolates carry the *cpe* gene on the plasmid (83). While studies found that AAD occurs after taking a course of antibiotic treatment, SD is developed without such treatment. Basically, studies suggest that ADD and SD diseases result from infection by a small dose of NFB *C. perfringens* cells that can be transferred to *cpe*-negative cells in the intestinal tract as normal flora conjugatively (82). Cells replicate and spore formation is induced; both CPE and spores are released (Fig. 1.2). CPE is able to damage the small intestinal cells, leading to diarrhea and cramping symptoms associated with disease (Fig. 1.2). However, the symptoms of *C. perfringens* type A NFB GI diseases begin after ~ 1-2 week (48). The method of transmission of *cpe* into the plasmid remains unclear. AAD and SD can occur in older people, who are taking antibiotics, and can be transmitted person-to-person. The necessary medical treatment for the AAD and SD patient is to restore fluids and electrolyte balance in the body (1).

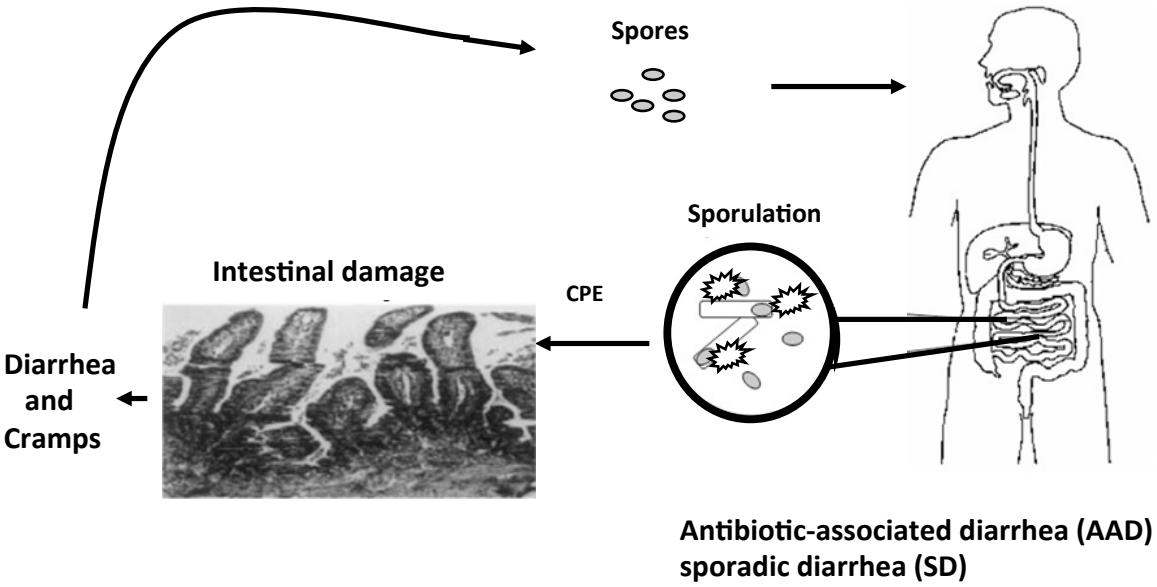

Fig.1.2 Overview of how *C. perfringens* NFB infection occur by ADD and SD. Treatment with/without antibiotic, population of the normal gut *C. perfringens* will decrease, resulting of spores to multiplying in the intestinal track where they can initially multiply, and sporulate. CPE will produced in high dose from sporulate cells, when the endospore become mature and begin autolysis from mother cells. After that, CPE with bind to small intestinal epithelial cells causing massive damage small intestinal epithelial cells, cramps and diarrhea.

1.4. *C. perfringens* spore germination

C. perfringens strain can form dormant and highly resistant spores from vegetative cells when in an unfavorable growth environment (6, 84). Spores can survive for years in a dormant phase. However, they can rapidly lose their dormancy and resistance to a variety of environmental stresses upon germination. Basically, spores can sense a favorable growth condition in their environment and can initiate a germination process that will be followed by an outgrowth that converts the germinated spores into growing active vegetative cells (85-87). Spore germination is initiated by a number of chemicals such as nutrients, cationic surfactants, enzymes, and high pressure (HP) (88, 89). Nutrients can trigger spore germination that occurs naturally in the environment; they are termed as germinants, including amino acids, sugar, and purine nucleosides (90). Extensive studies on spore germination of *Bacillus subtilis* and *C. perfringens* show that nutrient germinants could trigger spore germination within seconds of mixing spores and germinants, for instance, L-alanine, inosine, and a mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (AGFK) (87, 88). The spore germination events start in two stages. The first stage starts at an early event when the nutrient germinants bind and interact with cognate germinate receptors (GRs) located in the inner spore membrane (91-93) (Fig. 1.3), which stimulate and release of a monovalent cation such as H^+ and divalent such as Zn^{2+} from spore core. The release of H^+ elevates the core pH from ~ 6.5 to 7.7; this change is crucial for spore metabolism when spore core`s water level is high enough for enzymes activity (90) (Fig. 1.3). After that, the spore core`s large

deposit of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) associated with divalent cations Ca^{2+} (Ca-DPA) is released as well. Releasing of Ca-DPA from spore core which is replaced by water leads to increase in spore core water content or hydration and a decrease in moist heat resistance (90) (Fig.1.3). At this point, the core hydration level is not enough to start up the protein and enzyme activity (94, 95). Second stage of spore germination begins with degradation of the spore`s peptidoglycan (PG) cortex. The hydrolysis of PG allows the spore core to expand and take up more water until it reaches the same level that is found in vegetative cells. Protein mobility resumes as well as enzyme activity, which allow the process of spore to outgrowth and the conversion into active cells (88, 95) (Fig. 1.3).

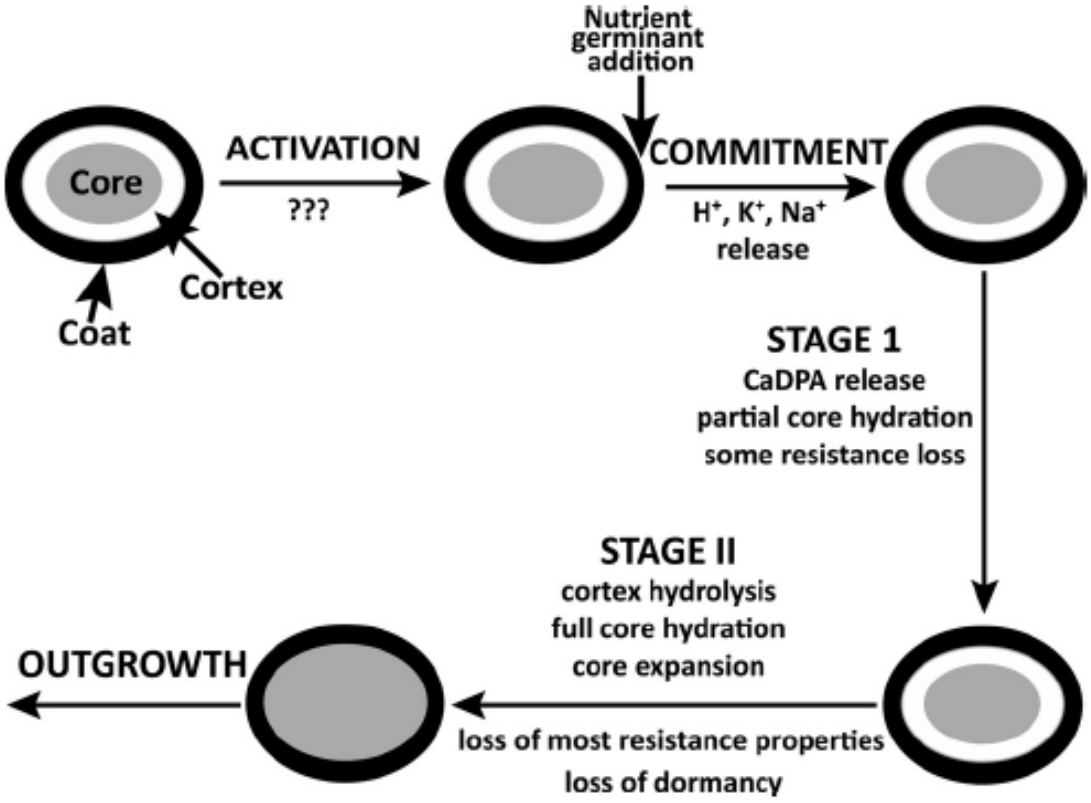

Fig.1.3 Overview of nutrient germination of *Bacillus* species spore. Question marks are an unknown mechanism and under study especially in the activation step. First stage is about activating spore germination by nutrient germinants and followed by release of monovalent cation and Ca-DPA from the spore core and replaced by water. Second stage is about cortex hydrolysis and an expanding spore core and a resumption of metabolisms. Adapted from (6).

1.4.1. **Nutrient and non-nutrient germinants in *C. perfringens*.**

Spore can survive for years in inactive form termed the dormant state; however, when a spore senses a proper stimulus, called a germinant, it can quickly lose its dormancy and resistance during germination. There are different types of germinant compounds that trigger spore germination of *B. subtilis* and *C. perfringens*. However, spore germination has been studied for *B. subtilis* (6, 96, 97), and it was found that spore germination can be triggered by a variety of chemicals, such as nutrients, Ca-DPA and cationic surfactants like dodecylamine. Moreover, high pressure (HP) also can be a germinating agent, especially when HP is combined with relatively high temperatures (98). Several studies are agreed that nutrient germinants bind upon a specific stereotype of spore-specific protein, termed (GRs), which are located in the inner spore membrane (91-93). For instance, spore germination in *B. subtilis* can be triggered by L-valine, L-alanine, and L-asparagine, while D-amino acid inhibits spore germination (99, 100). In addition, *B. subtilis* spores can trigger by a mixture of L-asparagine, glucose, fructose and KCL (AGFK), and inosine (90, 101). The available studies have been focused on the spore germination of *C. perfringens* strains. For example, an early study on *C. perfringens* strain NTCTC3624 showed that spore can germinate with lysozyme (102). Recent studies investigated the spore germination and found specific germinants for specific strains (87, 103, 104). Spores of *C. perfringens* type A FP and NFB strains require different germinants; spores of FP isolates which are able to germinate in the presence of KCl, L-

asparagine, a mixture of KCl and L-asparagine termed AK, L-glutamine, L-cysteine, and the co-germinants Na^{+} and inorganic phosphate (Pi) at pH 6.0 (87, 103, 104). However, spore germination in NFB isolates is triggered by L-cysteine, L-serine, and L-threonine at pH. 6.0 (103).

Ca-DPA and dodecylamine are considered as non-nutrient germinants for *B. subtilis* and *C. perfringens.* However, the mechanisms of germination by these germinants is believed to be different for those bacteria. For instance, a pervious study in *B. subtilis* has shown that Ca-DPA act as a good germinant for the spores that lacked all functional GRs; the reason behind this is that exogenous Ca-DPA work to stimulate cortex hydrolysis by activation of cortex lytic enzyme (CLE) (95). In *C. perfringens* type A FP isolate, Ca-DPA at 50mM is required to induce spore germination through GRs (87) . Dodecylamine, another non-nutrient germinant that is a cationic surfactant (105) is able to trigger spore germination of both *Bacillus* and *Clostridium* species. In *B. subtilis* spores, dodecylamine is thought to open a pore like channel in the spore`s inner membrane and may trigger spore core DPA release (90, 105-107). Certainly, dodecylamine germination can induce germination of spores of *C. perfringens* FP isolate (87)

1.4.2. **Germinant receptors (GRs) and their location.**

Studies on germinant receptors (GRs) of *B. subtilis* spores suggest that GerA family form most of orthologous protein of the GRs (86, 96, 97, 108). In addition, studies have shown that GerA genes are expressed only during sporulation in the developing forespore (92, 109). The GRs in *B. subtilis* contain three germinant receptors that form tricitronic operons named *gerA, gerB,* and *gerK,* and all are located in the spore's inner membrane (91-93). In genome of *C. perfringens* FP SM101 strain, four GR ORFs (CPR0614, CPR0615, CPR0616, and CPR1053) were named as GerKA, GerKC, GerKB, and GerAA, receptively, suggesting that GRs in *C. perfringens* can work as individual GRs (87, 110). All GRs in *C. perfringens* come with ~ 40-57% similarity to the respectively orthologous in *B. subtilis* (86, 87). All GRs in *C. perfringens* belong to A subunit of GerA. (87). The GerK locus is a bicistronic operon of *gerKA* and *gerKC,* where *gerKB*, is a monocictronic gene, located upstream of *gerK* operon in the opposite direction (87, 110). The last one is *gerAA* that is located far from *gerKA, gerKC*, and *gerKB* genes (87). *C. perfringens* type A FP strain SM101 spore germination studies found that nutrient germinants act through the main GR proteins in spore germination GerKA and/or GerKC (87). L-asparagine, KCl, a mixture of L-asparagine and KCl, termed AK, and the co-germinants sodium and inorganic phosphate (NaPi) require GerKA and/or GerKC to complete the spore germination of *C. perfringens* strain SM101(87, 104). Moreover, exogenous Ca-DPA and dodecylamine also have a role in *C. perfringens* strain SM101 spore germination (87). GerKB and GerAA have an auxiliary role for normal

spore germination of the *C. perfringens* strain SM101 (87, 110). However, whether the GerKA or GerKC is required for germination in *C. perfringens* type A FP strain SM101 is still unclear. The roles of GRs in *C. perfringens* NFB strain spore germination also need to be investigated. More studies were determined the location and the protein level of each GR in *B. subtilis* (92, 93, 111). By Western bolt analysis, they found that GerAA and GerAC subunits each ~1,100 molecules/spore and GerBC and GerKA subunits each ~ 700 molecules/spore. In addition, GerBA subunit level was determined previously at 700 molecules/spore (111). GerA was the most abounds protein than the other. However, the ratio between A/C subunit almost 1:1 (111). But, in *C. perfringens* spores, the level of GRs still unknown and need to be determined.

1.4.3. **Ion channels**

Upon binding of the nutrient germinants to their cognate GR, many biophysical and biochemical events take place. One of these events is the huge release of monovalent cation > 75% of the spore`s depot of Na^+, K^+, and H^+ is through a physical transporter called an energy independent process that increases the pH in the spore core (112, 113). Different studies on *Bacillus* spp. have found that genes could be involved somehow on the release mechanism of the monovalent cation, but this is still unclear. GerN, a Na^+/H^+-K^+ anitporter gene, in *B. cereus* show a role in spore germination as a possible block of the release of monovalent cation (113). Similarity, *gerA* mutant in *B. megaterium* also block spore germination with nutrient germinants

(114, 115). Nevertheless, null mutation *gerN* in *B. subtilis* had no major role in normal spore germination (114). In *C. perfringens,* GerO and GerQ are two proteins homologues to monovalent cation transporter (116). *gerO*, a putative Na^+/H^+-K^+ anitporter gene, is essential for spore germination with rich media, L-asparagine, KCl, and Ca^{2+}-DPA (116).

1.4.4. **DPA channels**

Release of the spore`s large depot of dipicolinic acid (DPA), chelated at a 1:1 ratio with divalent cation, Ca^{2+} (Ca-DPA) results in the large efflux (112). These events are thought to happen through a DPA gated channel that is composed from SpoVA protein where it is located on the spore's inner membrane in *B. subtilis* with level of ~10,000 molecule/spore (117). *SpoVA* spores in *B. subtilis* are unstable and lyse during sporulation, perhaps because of their lack of DPA (106, 118, 119). Comparing to *B. subtilis*, a null mutation on *SpoVA* spores in *C. perfringens* was able to germinate with nutrient and non-nutrient germinants (120). Interestingly, GerD is a lipoprotein located in the spore's inner membrane and present in *B. subtilis* spores at level of ~1000 molecule per spore (91, 121). Spores that lack *gerD* show a significantly slower spore germination rate of *B. subtilis,* and this result suggests that the *gerD* gene acts as a signal amplifier between GRs and SpoVA protein (121). However, this gene is not present in all *Clostridium* spp., indicating that the signal transduction system is different from that of *Bacillales* and *Clostridiales* and needs more research (86).

1.4.5. **Cortex hydrolysis**

When DPA is released, the hydrolysis occurs and the spore `s PG cortex allows further uptake of water core leads to expand and resumption of metabolism. In *B. subtilis*, the release of Ca-DPA activates the downstream effecters, which are CLEs (6). CWIJ and SleB are important proteins that are required for full hydrolysis of the spore`s PG cortex (95). A previous study shows that CwlJ is localized in the spore`s outer layers, the spore coat, and SleB is also in the same location, but some part is connected into the spore's inner membrane (122, 123). Both CwlJ and SleB proteins are synthesized as mature forms during the sporulation condition (124); nevertheless, their stimulation signals differ significantly. Ca-DPA, which is released from spore core, is able to activate CwlJ (95); while SleB is activated as the result of a change in the stress of the cortex, when most of the spore core's Ca-DPA depot has been replaced with water during the late event of spore germination (118). Even though, loss of CwlJ or SleB does not eliminate the cortex PG, double mutant CwlJ and SleB spores do not degrade the cortex and so cannot complete spore germination (95, 125). Bioinformatics information identified two CLEs in *C. perfringens* spores, SleC and SleM, and these CLEs appear to have different pathways from CwlJ and SleB In *B. subtilis* (126). Strong evidence suggests that SleC only is essential for cortex PG hydrolysis during *C. perfringens* spore germination, and *B. subtilis,* Ca-DPA release does not activate downstream germination which triggers cortex hydrolysis (126). Inactive zymogen of SleC is present in dormant *C. perfringens* spores as pro-SleC,

and during germination pro-SleC converts into mature active SleC (126, 127). Members of the subtilisin family of proteases, called Csp serine proteases, activate pro-SleC during early germination. In *C. perfringens* FP strain SM101, CspB is the only csp protease that is able to activate pro-SleC into mature SleC that is required for cortex hydrolysis and increase Ca-DPA release during spore germination (128). However, the genome of another strain called *C. perfringens* S40 was found to have Csp protease, CspA, B, and C and any one of them can activate pro-SleC into mature SleC during spore germination (129, 130). Still, the role of CLEs and Csp in *C. perfringens* NFB isolates spore germination and their location in the spore remain unknown.

1.5. Applications of bacterial spore germination

Spore germination of *C. perfringens* type A isolates is the first and critical step to cause FP and NFB human gastrointestinal diseases, such as antibiotic-association and sporadic diarrheas (2, 85-87). It is important to understand the molecular basis of spore germination of *C. perfringens* in order to identify a new drug target, decontamination, therapeutic development, and protection measurement. Also it will prevent spore germination or acceleration, which will kill the newly sensitive germinated spores. For example, when spores start germinating and lose their resistance and dormancy, they become relatively easy to kill. In addition, discovering a compound that will trigger germination may lead to discovering a compound that will block germination, as well as block the release of CPE, which will prevent the disease.

Objective of this study

This dissertation will consist of three studies that will increase our understanding of the GRs of *C. perfringens* FP and NFB spores:

1. In the first study, the specific aims are to evaluate (i) the role of GerKA and GerKC proteins in spore germination of *C. perfringens* FP strain SM101; (ii) the precise location of GerKC protein in the spore; and (ii) the number of GerKC in *C. perfringens* SM101 spores.
2. The second study validates (i) the location of CLE SleC and CspB in the spores of *C. perfringens* type A FP strain SM101; and (ii) levels of SleC and CspB proteins in the spores of *C. perfringens* SM101.
3. The aims of the third study are to (i) evaluate spore germination kinetics of *C. perfringens* FP and NFB isolates in a variety of rich media; and (ii) characterize the GRs in spores of enterotoxigenic *C. perfringens* NFB strain F4969.

CHAPTER 2

The *Clostridium perfringens* germinant receptor protein, GerKC, is located in the spore inner membrane and is crucial for spore germination

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2.1 Abstract

The Gram positive, anaerobic, spore-forming bacterium *Clostridium perfringens* causes a variety of diseases in both humans and animals, and spore germination is thought to be the first stage of *C. perfringens* infection. Previous studies have indicated that the germinant receptor (GR) proteins encoded by the bicistronic *gerKA-KC* operon as well as the proteins encoded by the *gerKB* and *gerAA* genes are required for normal germination of *C. perfringens* spores. We now report the individual role of these GR proteins by analyzing the germination of strains carrying mutations in *gerKA*, *gerKC* or both *gerKB* and *gerAA*. Western blot analysis has also determined the location and numbers of GerKC in spores. Conclusions from this work include the following: 1) *gerKC* mutant spores germinate extremely poorly with KCl, L-asparagine, a mixture of asparagine and KCl, or NaPi; 2) *gerKC* spores germinated significantly slower than wild-type and other GR mutant spores with a 1:1 chelate of Ca^{2+} and dipicolinic acid and very slightly slower with dodecylamine; 3) the germination defects in *gerKC* spores were largely restored by expressing the wild-type *gerKA-gerKC* operon in trans; 4) GerKC was required for spores' viability, almost certainly because of *gerKC* spores' poor germination; and 5) GerKC is located in the spore's inner membrane, with ~ 250 molecules/spore. Collectively, these results indicate that GerKC is the main GR protein required for nutrient and non-nutrient germination of spores of *C. perfringens* food poisoning isolates.

2.2 Introduction

Clostridium perfringens is a Gram positive, anaerobic, spore-forming pathogenic bacterium causing gastrointestinal (GI) diseases in humans and animals (1-3). The most important type that causes *C. perfringens*–associated food poisoning (FP) in humans is *C. perfringens* type A and this illness is the third most commonly reported food-borne disease in the USA (2, 131). *C. perfringens* spores are resistant to many environmental stresses and remain dormant in the environment for a long period of time (16, 74). Once conditions are favorable they can break their dormancy and initiate germination in response to a variety of compounds (called germinants), including amino acids, mixture of a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA), and other non-nutrient compounds like dodecylamine, a cationic surfactant (87, 90).

In *Bacillus subtilis* spores, binding of specific nutrient germinants to their cognate germinant receptors (GRs) (90, 95, 96, 132) located in the spore`s inner membrane triggers the following events: i) release of monovalent cations; ii) release of the spore core`s large depot (~20 % of core dry weight) of DPA; and iii) hydrolysis of the spore's peptidoglycan (PG) cortex by one or more spore cortex lytic enzymes that allows water uptake into the core to the level found in vegetative cells and then resumption of metabolism; spores also lose most of their resistance properties upon completion of germination (133, 134). *B. subtilis* spores have three major GRs, GerA, GerB and GerK, each encoded by tricistronic operons that are expressed only in the

developing forespore late in sporulation (96, 108). Loss of the A, B or C cistrons in these tricistronic GR operons leads to loss of function of that GR (96, 97). Several studies (92, 93, 132) have shown that *B. subtilis* GerAA, GerAC, GerBA and GerKA are located in the spore`s inner membrane, with much of these proteins exposed on the outer surface of the inner membrane (135).

In *C. perfringens,* the organization of GR genes is different than that in *B. subtilis* (87, 110). There is no tricistronic *gerA*-like operon but rather a monocistronic *gerAA* located rather far from a *gerK* locus. The *gerK* locus includes a monocistronic *gerKB* in the opposite orientation to that of a bicistronic *gerKA-KC* (87). Previous work has shown that *gerKA* and/or *gerKC* are required for normal germination of *C. perfringens* spores with germinants such as KCl, L-asparagine, or a L-asparagine-KCl mixture termed AK (87). However, the *gerAA* and *gerKB* genes have at most only a minor role in normal spore germination with KCl (87, 110). In this study, we have evaluated the role of the GerKA and GerKC proteins in the germination of *C. perfringens* spores. The work has demonstrated that GerKC is the most important GR protein in the germination of *C. perfringens* spores, and further that GerKC is located in spores' inner membrane with at least a some of the protein on this membrane's outer surface.

2.3 Material and Methods

Bacterial strains and plasmids. The *C. perfringens* strains and plasmids used in this study are described in Fig. 2.1 and Table 2.2.

Construction of a *gerKC* mutant. Construction of a *gerKC* mutant of *C. perfringens* strain SM101 was based on the modified group II intron (the ClosTron) using www.clostron.com to retarget a suitable insertion at bp 468/469 from the start codon of *gerKC*. PCR was performed on intron DNA to re-target the intron by using *gerKC*-specific primers CPP779, CPP780, CPP781 (Table 2.3) and the LtrBAsEBS2 universal primer (CGAAATTAGAAACTTGCGTTCAGTAAAC) provided with the Targetron gene knockout system (Sigma-Aldrich Corporation, St. Louis, MO). An ~ 350 bp BsrGI-HindIII fragment retargeted to position 468/469 in the sense orientation of *gerKC* was cloned between the BsrGI and HindIII sites of pJIR3566 vector (136), giving plasmid pDP276. Plasmid pDP276 was electroporated (19) into *C. perfringens* strain SM101 and erythromycin-resistant (Em^r) transformants were selected on Brain Heart Infusion (BHI) agar plates supplemented with 30 μg/ml Em. Em^r transformants were screened for insertion of the Targetron by PCR using *gerKC*-specific primers CPP440 and CPP443 (Table 2.3). To cure the Cm^r coding vector, one candidate Targetron-carrying clone was subcultured three times in non-selective fluid thioglycolate (FTG) broth, plated onto BHI agar, and single colonies were patched onto BHI agar with or without Em, giving strain DPS122.

Construction of a *gerKA* mutant. A *C. perfringens* SM101 derivative with an intron inserted into the *gerKA* gene was constructed as follows. To target the *L1.LtrB* intron to *gerKA*, the intron sequence in plasmid pJIR3566 was modified using the InGex intron prediction program (Sigma-Aldrich) and an insertion site at bp 90/91 (score 10.5) from the start codon was chosen. PCR was performed on SM101 DNA to re-target the RNA portion of the intron using *gerKA*-specific primers CPP776, CPP777, CPP778 (Table 2.3) and the LtrBAsEBS2 universal primer (CGAAATTAGAAACTTGCGTTCAGTAAAC) provided with the Targetron gene knockout system (Sigma-Aldrich). An ~ 350 bp BsrGI-HindIII fragment was retargeted to *gerKA* and was cloned between BsrGI and HindIII sites in pJIR3566, giving plasmid pDP300. Plasmid pDP300 was electroporated into *C. perfringens* strain SM101 and the *gerKA* mutant strain was isolated essentially as described above for the *gerKC* mutant.

Construction of a *gerKB gerAA* double mutant. A mutation in the *gerAA* gene of a previously isolated *gerKB* mutant strain DPS108 (*gerKB::catP*) (110) was generated by inserting a Targetron in the antisense strand of *gerAA* between bp 123 and 124 downstream of the *gerAA* start codon as previously described (87). Briefly, the *gerAA* Targetron containing plasmid pDP13 carrying the Targetron insertion between bp 123/124 was introduced into DPS108 by electroporation, and clones were identified with an insertion of the intron into *gerAA*, giving a *C. perfringens gerKB*::*catP gerAA::intron* double mutant strain DPS124.

Construction of a *gerKC* strain complemented with wild-type *gerKA-KC*. To construct a *gerKC* strain complemented with wild-type *gerKA-KC*, we first constructed a shuttle plasmid carrying the wild-type *gerKA-KC* operon. An ~3.2 kb KpnI-SalI fragment carrying the *gerKA-KC* operon with its own promoter and terminator, was excised from plasmid pDP10 (87) by digestion with KpnI and SalI and then ligated between the KpnI and SalI sites of the *E. coli-C. perfringens* shuttle plasmid pJIR750 that encodes Cm^r (137), giving plasmid pSB18. The recombinant plasmid pSB18 was introduced into *C. perfringens gerKC* mutant strain DPS122 by electroporation (19) and Cm^r Em^r transformants were selected. The presence of plasmid pSB18 in strain DPS122 (pSB18) was confirmed by PCR and Southern blot analyses (data not shown).

Spore preparation. Starter *C. perfringens* cultures were prepared by inoculating 0.1 ml of cooked meat stock culture into 10 ml FTG broth (Difco) and incubating at 37°C as described previously (138). 0.4 ml of overnight FTG-grown *C. perfringens* cultures were inoculated into fresh 10 ml FTG broth and incubated at 37°C for 8-12 hours. To prepare sporulating cultures, 0.2 ml of FTG-grown culture was transferred into 10 ml Duncan-Strong (139) broth and incubated at 37°C for 24 h. Spore formation was confirmed by phase-contrast microscopy. Scaling-up this procedure was needed for large scale spore preparation. For purification, spore suspensions were washed with sterile distilled water until they were >98% free of vegetative cells, germinated spores and debris. Purified spores were re-suspended in sterile distilled water at an optical density at 600 nm (OD_{600}) of ~6 and stored at -80 °C.

Spore germination. *C. perfringens* spore germination was as previously described (87, 104). Briefly, heat-activated (80°C; 10 min; and then cooled on ice) spore suspensions (OD_{600} ~1) were incubated with KCl (100 mM KCl in 25 mM Tris-HCl buffer, pH 7.0), L-asparagine (100 mM L-asparagine in 25 mM Tris-HCl buffer, pH 7.0), L-asparagine plus KCl (100 mM L-asparagine-100 KCl in 25 mM Tris-HCl buffer, pH 7.0), $NaPO_4$ buffer (100 mM $NaPO_4$ buffer pH 6.0), or Ca-DPA (50 mM $CaCl_2$, 50 mM DPA, adjusted to pH 8.0 with Tris-HCl) at 40°C and spore germination was measured by monitoring the OD_{600} of germinating spores in a Synergy Mx Multi-Mode Microplate Reader (BioTek® Instruments Inc., Winooski, VT). Levels of spore germination were also routinely confirmed at the end of experiments by phase-contrast microscopy. The extent of spore germination was expressed as a percentage of the initial OD_{600}, with a decrease of 60% seen with ~ 100% spore germination. All values reported are average of three experiments performed with at least three independent spore preparations.

For measuring DPA release during KCl-triggered germination, heat-activated spores (OD_{600} of 1.5) were incubated at 40°C in 100 mM KCl in 25mM Tris-HCl buffer (pH 7.0). DPA release during dodecylamine germination was measured by incubating heat-activated spore suspensions (OD_{600} of 1.5) in 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4) at 60°C. Aliquots (1 ml) of KCl- or dodecylamine germinating spores were centrifuged for 3 min at 13,000 rpm in a microcenterifuge and DPA in the supernatant fluid was measured by monitoring the OD at 270 nm as previously described (87, 105, 140). Initial DPA levels in dormant spores were

measured by boiling 1 ml aliquots for 60 min, centrifugation for 3 min at 13,000 rpm, and DPA content of the supernatant fluid was measured at 270 nm (87, 105, 140).

Preparation of the spore inner membrane (IM) fraction. Spore IM was prepared as described previously (91, 92, 117, 135, 141). Spores (~ 3 ml) at an OD_{600} of 25 were first decoated by incubation at 70°C for 2 h in 0.1 M NaOH, 0.1 M NaCl, 0.5% SDS, 0.1 M dithiothreitol (DTT) and washed 10 times with water as described (142). The washed decoated spores of wild-type strain SM101 and strain DPS122 were suspended at an OD_{600} of 50-70 in 0.5 ml TEP buffer (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (PMSF) with 1 mg lysozyme, 1 μg each of RNase A and DNase I, and 20 μg $MgCl_2$ as described previously (92, 135, 141, 143). The spore suspension was then incubated for 5 min at 37°C. After cooling on ice for 20 min, suspensions were sonicated briefly with 100 mg glass beads and examined microscopically for lysis, centrifuged for 5 min in a microcentrifuge at maximum speed, and the supernatant fluid was saved. The pellet was re-suspended in 0.5 ml TEP buffer and centrifuged again as described above and the supernatant fluids were pooled, and the pellet was also saved. The pooled supernatant fluid was centrifuged at 100,000xg for 1 h at 4°C in an ultracentrifuge TLS 100.2 rotor, giving a soluble fraction (S100), and the IM pellet fraction (P100) that was suspended in 40-80 μl of TEP buffer containing 1% Triton X-100.

GerKC expression, purification and antibody production. The *gerKC* gene was amplified by PCR using genomic DNA from *C. perfringens* strain SM101 as the

template and cloned into a modified pET15b expression vector containing a tobacco etch virus (TEV) protease cleavage site between the N-terminal His6 tag and the target gene. The identity of the resultant plasmid was verified by DNA sequencing. The GerKC protein (residues 28-374) was overexpressed in *Escherichia coli* BL21 cells and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography followed by TEV protease cleavage of the His6 tag and by anion exchange and gel filtration (SD200; GE Healthcare, Piscataway, NJ) chromatography. Purified protein (~2 mg) was used to raise polyclonal antibodies in two New Zealand White female rabbits (Pacific Immunology Group, Ramona, CA). Anti-GerKC antibodies were detected by Western blotting in a bleed 2 months after the initial injection, at which time the animals were exsanguinated. The specificity of the antiserum was confirmed by Western blot analysis using purified GerKC protein (and see Results).

Western blot analysis. Western blot analyses were carried out following SDS-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and transfer of proteins to polyvinylidene difluoride (PVDF) membranes as described previously (134, 135, 143). The GerKC protein was detected by using polyclonal rabbit antisera (1:1000) against GerKC and goat anti-Rabbit IgG-horseradish peroxidase (HRP) conjugate (1:10,000). The activity of HRP was detected using the PicoMax sensitive chemiluminescent HPR substrate (Rockland Immunochemicals, Gilbertsville, PA) with a chemiluminescence detection system (Molecular imagDoc XRS+ system; Bio-Rad, Hercules, CA).

Determination of GerKC level in spores. Dormant spores (~ 20 mg) of *C. perfringens* strains SM101 (wild-type) were decoated, treated with lysozyme, disrupted with glass beads and sonication (~ $8.4x10^9$ spores were disrupted), and then centrifuged at 14,000x*g* for 10 min as described above. The pellet from this initial centrifugation was saved and termed the pellet fraction. The supernatant fraction was further centrifuged as described above giving inner membrane pellet and supernatant fractions, and various percentages of the inner membrane and initial pellet fractions along with known quantities of purified GerKC were subjected to Western blot analysis using anti-GerKC serum. The intensities of the resulting bands were quantitated using ImageJ and the average total number of GerKC molecules per spore was calculated (141, 143).

Determination of GerKC susceptibility to modification by an exogenous biotinylation reagent. Biotinylation was performed as previously described (135) with some modifications. Five ml of dormant SM101 spores at an OD_{600} of ~ 25 were decoated and washed as described above. Buoyant density gradient centrifugation in 50% Histodenz (Sigma Chemical Co, St. Louis, MO) was performed to remove small amounts of germinated spores. The dormant spore pellet from this centrifugation was washed extensively with water, and the resulting decoated dormant spores were suspended in water at an OD_{600} of 100.

Germinated spores were obtained by first heat-shocking intact dormant spores (3 ml at an OD_{600} of 50) at 80°C for 10 min. After cooling to 25°C for 5 min, spores

were heated to 40°C for 10 min and germinated at an OD_{600} of ~1.5 in 50 ml of 100 mM KCl in 25 mM sodium phosphate buffer (pH 7.0) for 2 h, until at least 90% of spores were germinated as determined by phase contrast microscopy. Small amounts of dormant spores were not removed from these germinated spore preparations. However, these dormant spores had not been decoated and were thus not lysed by subsequent lysozyme treatment. Consequently the IM was not isolated from these dormant spores, and only from the spores that had become lysozyme sensitive due to their germination.

Biotinylation of lysyl amino groups in dormant or germinated spore protein was with 5 ml of spores at an OD_{600} of ~15 in 10 mM K-Hepes buffer (pH 7.4) and 150 mM NaCl using 2 mM EZ-Link Sulfo-NHS-SS-Biotin reagent (Pierce Chemical Co., Rockford, IL) for 1 h at room temperature on a rocker. Unreacted reagent was quenched by addition of 1 ml of 2 M glycine and 1 ml of 1 M Tris-HCl buffer (pH 7.4), and incubation for 30 min at 23°C. All labeled spores were washed twice with 20 ml water.

For analysis of GerKC biotinylation, NeutrAvidin Agarose beads (Pierce) were prepared by spinning 100 μl of slurry at 1000xg for 1 min at 23°C, discarding the supernatant fluid, and then washing twice with 100 μL TEP buffer containing 1% Triton X-100. 60 μl of the IM fraction was added to the resulting bead pellet, and the mixture was shaken on a rocker. After shaking at 23°C for 1 h the sample was centrifuged at 1000xg for 1 min and the supernatant fluid containing unbound

proteins (flow through) was saved. The resulting bead pellet was washed three times with 100 μL TEP buffer containing 1% Triton X-100, and washes were also saved. Bound proteins were eluted by incubating the beads with 60 μL Laemmli sample buffer containing 55 mM DTT for 1 h at 23°C. Western blot analyses as described above were performed following SDS-PAGE of equal percentages of the total IM fraction, the flow through fraction and the eluted fraction, all run on the same Western blot, and in some cases with various dilutions of the flow through or eluted fractions.

Other methods. For determination of colony forming efficiencies of spores of various strains, spores at an OD_{600} of 1 (~ 10^8 spores/ml) were heat activated as described above, and aliquots of dilutions were spread on BHI agar with or without lysozyme (1 μg/ml), incubated at 37°C anaerobically for 24 h, and colonies were counted.

The student's *t* test was used for specific comparisons between data sets.

2.4 Results

***gerKA* mutant spores produce lower levels of GerKC compared to wild-type spores.** Previous studies (87, 110) suggested that the main GR proteins involved in germination of *C. perfringens* spores are encoded by the bicistronic *gerKA-KC* operon, while the products of *gerKB* and *gerAA* have auxiliary roles. However, those studies were conducted on a *C. perfringens* strain (DPS101) with an intron insertion into the *gerKA* cistron that likely had a polar effect on expression of the downstream *gerKC*. To dissect the role of the GerKA and GerKC GR proteins individually in *C. perfringens* spore germination, we constructed double *gerAA gerKB*, and single *gerKA* or *gerKC* mutants in strain SM101 (Fig. 2.1A). To evaluate whether insertion of an intron in *gerKA* exerted a polar effect on the downstream *gerKC*, the level of GerKC in spores of wild-type and *gerKA* mutant strains was compared (Fig. 2.1B). Western blotting with antisera against GerKC demonstrated the presence of GerKC in *gerKA* mutant spores. However, quantitative analyses showed there was only ~ 12% of the wild-type GerKC level in *gerKA* mutant spores. These results indicate that the *gerKA* mutation did indeed exert a partial polar effect on the downstream *gerKC* gene.

GerKC, but not GerKA, has a major role in *C. perfringens* spore germination. Spores of the double *gerAA gerKB* strain germinated well in the presence of L-Asn, KCl, AK, or NaPi (Fig. 2.2A-D), confirming the minor roles played by GerKB and

GerAA in spore germination (87, 110), and consistent with GerKA and/or GerKC being the main GR proteins in spores of *C. perfringens* FP strain SM101.

To dissect the precise roles of GerKA and GerKC in *C. perfringens* spore germination, we constructed single *gerKA* or *gerKC* mutants in strain SM101 (Fig. 2.1), and measured the germination of the spores of these various strains. As expected, *gerKA gerKC* spores germinated poorly and to a much lesser extent than wild-type spores with L-Asn, KCl, AK, or NaPi (Fig 2.2A-D). While *gerKA* and wild-type spores germinated similarly, *gerKC* spores germinated much more poorly with these germinants than wild-type and other GR mutant spores ($P < 0.05$) (Fig. 2.2A-D). Most importantly, the germination defects of *gerKC* spores were largely corrected by complementing the *gerKC* mutant with the wild-type *gerKA-KC* operon. Collectively, these results indicate that GerKC is the most important GR protein in *C. perfringens* SM101 spore germination.

After germinants bind their cognate GR, the next easily measurable event in spore germination is the release of DPA from the spore core. Therefore, we also evaluated the effects of mutations in GR protein genes on DPA release during germination with KCl (Fig. 2.3). As expected, wild-type spores released ~ 70% of their DPA within the first 10 min of germination with KCl (Fig. 2.3). Strikingly, the rate and amount of DPA release by *gerKA* spores incubated with KCl were similar to those of wild-type spores, indicating that GerKA is not essential for DPA release during spore germination, at least when GerKB and GerAA are present, and only

slightly less DPA release was obtained when *gerAA gerKB* spores were germinated with KCl. In contrast, *gerKC* spores released ~ 50% less DPA ($P < 0.05$) than wild-type spores during KCl germination (Fig. 2.3). The DPA release-defect in *gerKC* spores was also corrected by complementation with a plasmid carrying wild-type *gerKA-KC*. These results further indicate that GerKC is the main GR protein involved in *C. perfringens* spore germination.

GerKC is also important in germination of *C. perfringens* spores with Ca-DPA and dodecylamine. Germination of bacterial spores can also be triggered by non-nutrient germinants such as the cationic surfactant dodecylamine (105) and exogenous Ca-DPA, and in *B. subtilis* spores, germination with these agents does not involve any GR protein (90, 97). It was shown previously that unlike spores of *Bacillus* species, where Ca-DPA triggers spore germination by direct activation of the cortex-lytic enzyme, CwlJ (95, 144), in *C. perfringens*, Ca-DPA triggers germination in a GerKA and/or GerKC dependent pathway (87). When wild-type, *gerKB gerAA*, and *gerKA* spores were incubated with Ca-DPA, significant germination was observed as measured by the decrease in OD_{600} (Fig. 2.4A). In contrast, there was minimal germination of *gerKC* spores with Ca-DPA (Fig. 2.4A), and phase-contrast microscopy confirmed that ~95% of these spores remained phase bright (data not shown). The Ca-DPA germination defect of the *gerKC* spores was partially corrected by complementation with wild-type *gerKA-KC* (Fig. 2.4A), indicating that the Ca-DPA germination pathway is dependent on GerKC.

Previous work showed that GerKA and/or GerKC are also required for normal spore germination with dodecylamine (87). In the current work *gerKB gerAA*, and *gerKA* spores released almost the same amount of DPA as wild-type spores during germination with dodecylamine (Fig. 2.4B), suggesting that GerAA, GerKB and GerKA might have no role in dodecylamine germination. The *gerKC* spores incubated with dodecylamine released slightly less DPA than wild-type spores (Fig. 2.4B), indicating that GerKC may play a minor role in triggering DPA release during dodecylamine germination.

GerKC is required for normal colony formation in BHI medium. Unlike *B. subtilis*, where spores lacking all GRs retain full viability once these are decoated and rescued in presence of lysozyme, the absence of *gerKA-KC* and *gerKB* does affect the apparent viability of *C. perfringens* spores (87, 110). Therefore, to establish if it is GerKA or GerKC that is involved in *C. perfringens* spores' apparent viability, the colony forming efficiencies of intact and decoated wild-type and mutant *C. perfringens* spores were assessed by plating spores onto BHI agar and incubating them anaerobically for 24 h at 37°C. There was no significant ($P > 0.05$) difference in colony formation efficiency of wild-type and *gerKA* spores, while *gerAA gerKB* spores had an ~ 6-fold lower colony formation efficiency (Table 2.1). However, intact *gerKC* spores exhibited ~ 100-fold lower colony formation efficiency than wild-type and *gerKA* spores (Table 2.1). Decoating of spores and plating on BHI agar containing lysozyme increased colony forming efficiencies of spores of all mutant strains tested, including the *gerKC* spores, to approximately that of the wild-type

spores (Table 2.1). Thus the *gerKC* spores are fully viable and most simply do not germinate well.

To directly examine the possibility that the lower colony formation efficiency of *gerKC* spores was due to their poor germination in BHI broth, we compared germination of spores of all four strains in this medium. As expected, *gerKC* spores exhibited significantly ($P < 0.05$) lower germination in BHI broth compared to that of wild-type spores, and the germination defect in *gerKC* spores was at least partially restored by complementing the mutant with wild-type *gerKA-KC* (Fig. 2.5). The *gerKA* and *gerKB gerAA* spores also exhibited slightly lower germination than wild-type spores (Fig. 2.5). Phase contrast microscopy found that > 65% of wild-type and *gerKA* spores, ~ 50% of *gerKB gerAA* and *gerKC*-complemented spores but only ~ 10% of *gerKC* spores were phase dark after 1 h of incubation in BHI broth (data not shown), in agreement with the results obtained by measurements of germination by OD_{600} (Fig. 2.5). In addition, when the spores were incubated aerobically for 18 h in BHI broth, ~ 95% of wild-type, *gerKA*, *gerKB gerAA*, and *gerKC*-complemented spores were phase dark, and ~ 90% of these phase-dark spores had released the nascent vegetative cell (data not shown). In contrast, only ~ 65% of *gerKC* spores were phase dark and less than 10% of the phase-dark spores had released the nascent vegetative cell (data not shown), again consistent with the lower colony formation efficiency observed with these spores on plates without lysozyme. Collectively, these results indicate that GerKC, but not GerKA, is essential for normal completion of

spore germination and perhaps also outgrowth, and thus for normal colony formation in BHI medium.

GerKC localizes to the IM of *C. perfringens* spores. The GR proteins of *Bacillus* species have been localized in spores' IM (92, 93, 132). Therefore, since GerKC is essential for germination of *C. perfringens* spores we attempted to immunolocalize GerKC in spores using polyclonal rabbit antiserum raised against recombinant *C. perfringens* GerKC, and the recombinant GerKC was detected as a ~ 40 kDa protein by the antiserum (Fig. 2.6). When spore IM fractions obtained from decoated *C. perfringens* wild-type and *gerKC* spores were separated by SDS-PAGE and subjected to Western blot analysis (Fig. 2.6), a dominant 40-kDa protein band was detected in the IM fraction from wild-type but not *gerKC* spores. These results clearly indicate that the GerKC localizes to the IM of *C. perfringens* spore.

***C. perfringens* has ~ 250 GerKC molecules per spore.** In bacterial spores, GRs act as environmental sensors that trigger spore germination when conditions are deemed adequate for growth (86, 90, 96). The germination machinery in *B. subtilis* spores appears to amplify the germination signal through increases in the levels of various germination components, with the GRs being in 100s of molecules per spore, and proteins involved downstream of GRs, the SpoVA proteins that are essential for DPA release in spore germination, being present at 5-10 thousand molecules/spore (92, 93, 117, 120, 143). Consequently, it was of interest to analyze the level of GerKC in *C. perfringens* spores. In order to obtain a complete assessment of GerKC levels in

spores, the IM fraction and the low speed pellet fraction obtained after spore disruption were analyzed for GerKC by Western blot analysis, and the intensities of GerKC bands in these fractions were compared to those of purified GerKC (Fig. 2.7). While ~ 60% of the GerKC was present in the IM fraction, ~ 40% was found in the low speed pellet fraction, presumably reflecting the presence of significant amounts of inner membrane in this fraction as well, and as also found in spores of *B. subtilis* (143). In total, *C. perfringens* spores were calculated to have ~ 250 GerKC molecules/spore, although this number may vary significantly between individual spores in the population.

Topology of GerKC in *C. perfringens* spores' IM. Analysis of the amino acid sequence encoded by the *gerKC* gene indicates that GerKC is likely a peripheral membrane lipoprotein. While most of the protein is relatively hydrophilic, it has a hydrophobic N-terminal region that is likely a signal peptide that is followed by a consensus sequence for covalent addition of a diacylglycerol moiety to an N-terminal cysteine residue formed by signal peptide cleavage. A high-resolution structure of a *B. subtilis* GR C protein has been determined, and is a relatively globular structure (145). Even more importantly, prediction of the structure of other GR C proteins, including those from *Clostridium* species, suggests that all GR C proteins adopt a similar structure. Given this likely structure of *C. perfringens* GerKC, as well as the expression of *gerKA-gerKC* only in the developing forespore, it is thus most likely that GerKC is a peripheral membrane protein on the outer leaflet of the spore IM.

Analysis of the topology of GR C proteins by analysis of these proteins' susceptibility to biotinylation by an exogenous reagent has shown recently that these proteins are well biotinylated in intact *B. subtilis* spores, suggesting that GR C proteins are indeed on the IM outer surface (135). Consequently, we also subjected intact dormant and germinated *C. perfringens* spores to the same analysis, and detecting biotinylation of GerKC by Western blot analysis following affinity purification of biotinylated proteins (Fig. 2.8). ~ 33% of *C. perfringens* GerKC was biotinylated in decoated but otherwise intact *C. perfringens* spores, with this value increasing to ~ 50% in intact germinated spores. This value for dormant spores is almost identical to those found for the biotinylation of two GR-C proteins in dormant *B. subtilis* spores, although these same two GR-C proteins were 74-100% biotinylated in germinated *B. subtilis* spores (18). These results are certainly consistent with the majority of GerKC being located on the outer surface of *C. perfringens* spores' IM, although we cannot be sure that all GerKC is there. We also do not understand why biotinylation of GerKC in dormant and germinated spores is not complete, although this has also been seen with several *B. subtilis* IM proteins, including GR subunits (18).

2.5 Discussion

A first major conclusion from this work is that GerKC is the major GR protein in spores of *C. perfringens* food poisoning strain SM101. Previous work (87) suggested that the products of the bicistronic *gerKA-KC* operon were most important in spore germination; however, those studies were done on a *C. perfringens* strain with a mutation on the *gerKA* cistron, which caused a polar effect on the downstream *gerKC*. Here, we show that a targetron mutant in *gerKA* still allows significant germination of *gerKA* spores, suggesting that GerKC plays a major role in this process. In fact, inactivation of *gerKC* in strain SM101 affected spore germination with all known nutrient germinants. However, it is unclear whether GerKC acts as a receptor itself and contains multiple ligand binding sites, is an essential component of a multiple subunit receptor formed by GerAA, GerKB, and/or GerKA, or even interacts with other unknown proteins. Clearly further studies are needed to answer these questions.

Another major conclusion is that GerKC is required for full apparent viability of *C. perfringens* spores. The lower colony-forming ability of *gerKC* spores in rich BHI medium compared to that of wild-type and *gerKA* spores, was consistent with the significantly slower germination of *gerKC* spores in BHI medium and the slower release of nascent vegetative cells compared to wild-type and *gerKA* spores. The reversion to wild-type levels of colony formation by *gerKC* spores complemented with wild-type *gerKA-KC* is further evidence that GerKC is also important in spore

viability. However, the lower viability of *gerKC* spores is only apparent, since full *gerKC* spore viability was restored when decoated spores were plated in the presence of lysozyme. Perhaps the GerKC protein is involved in the activation of either the CLE SleC to allow cortex hydrolysis or some unknown enzyme that induces the release of nascent vegetative cell from the coat/exosporium. This role of *C. perfringens* GerKC is in contrast with that of *B. subtilis* GRs, where viability of spores lacking one GR is relatively normal, although spores lacking all GRs do exhibit low apparent viability (97).

A third major conclusion of this work is that GerKC localizes to the inner membrane of *C. perfringens* spores. This finding was not unexpected, since GR proteins of *B. subtilis* have been shown to localize in the spore inner membrane (92, 93, 132). The level of GerKC in *C. perfringens* spores was also just 2 to 4-fold lower than GR levels of *B. subtilis* spores. A key difference in the components of the germination machinery between *C. perfringens* and *B. subtilis* spores is that *C. perfringens* spores lack a homologue of the lipoprotein GerD (86) which plays a major role in GR-dependent germination in *B. subtilis* spores (91, 146), perhaps by ensuring that all GRs localize into the germinosome (143).

A minor conclusion from this work is that GerKC is essential for normal DPA release during *C. perfringens* spore germination with many germinants. Previous work (87) suggested that GerKA and/or GerKC were required for normal DPA release with nutrient and non-nutrient germinants. Here, we show that only absence of

GerKC affects DPA release from the spore core during nutrient and non-nutrient germination of *C. perfringens* spores. Further work will be needed to establish the reason for the apparent partial release of DPA during germination of *gerKC C. perfringens* spores. Some possible explanations for these results are that GerKC: i) is required for correct colocalization or stability of other GR proteins and/or to form a functional GR by itself, with other GR subunits or with unknown proteins that will efficiently trigger Ca-DPA release; ii) might be acting as a link between GR subunits (i.e., GerAA, GerKB and GerKA) and proteins that form the Ca-DPA channel; or iii) is required for complete DPA release from individual spores during germination, although this seems unlikely, since DPA release from individual germinating spores of both *C. perfringens* and *Bacillus* species is invariably an all or none phenomenon (38-40).

In conclusion, the results presented in this work should provide a deeper insight into the mechanism of *C. perfringens* spore germination in which KCl, L-Asn, AK, NaPi and the non-nutrient germinants Ca-DPA and dodecylamine act largely or at least in part through the GerKC GR protein. In contrast, GerKA, GerAA and GerKB play at most auxiliary roles, since deletion of either of these GR proteins has no significant effect on *C. perfringens* spore germination.

Acknowledgments

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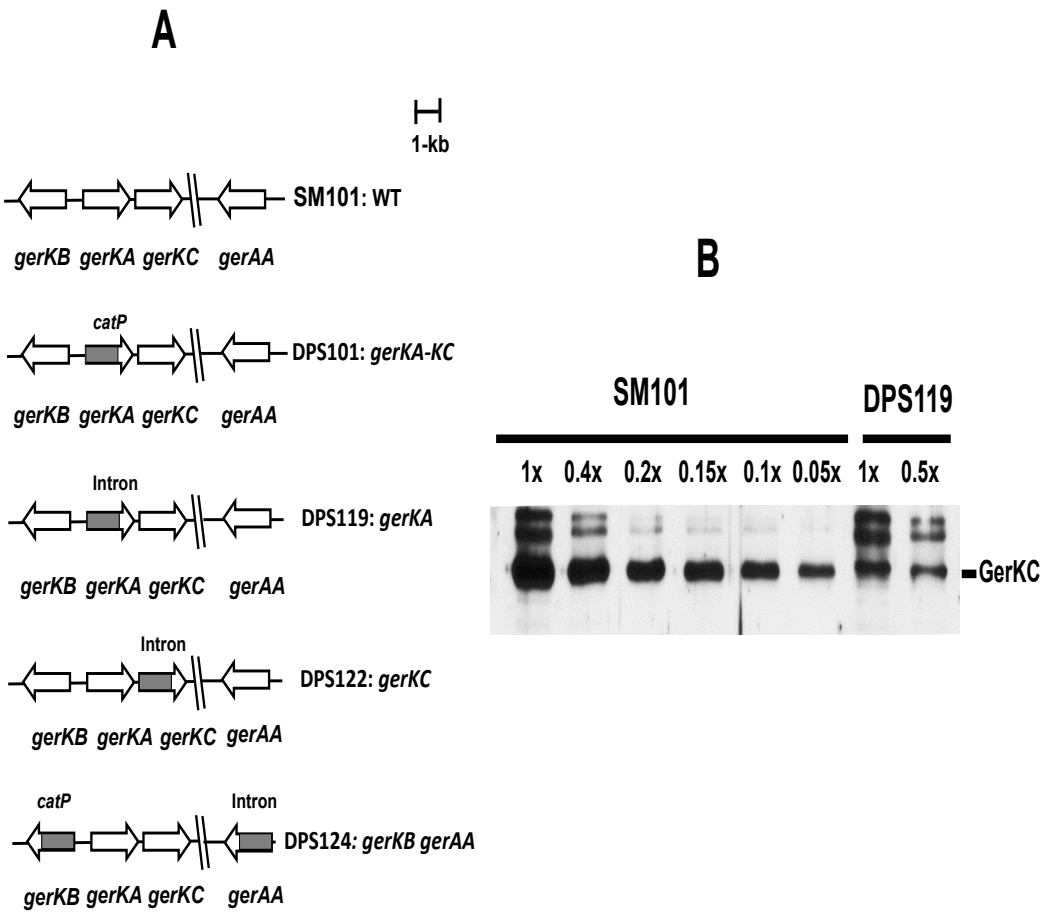

Fig. 2.1A,B. *C. perfringens* GR mutants. A. Arrangement of GR genes in various *C. perfringens* mutant strains. B. Analysis of GerKC levels in total lysates of *C. perfringens* SM101 (wild-type[WT]) and DPS119 *(ΔgerKA*) spores. Spores were decoated and lysed, and various amounts of protein in the total lysates from the spores of the two strains were run on SDS-PAGE followed by western blotting using GerKC antibody as described in Material and Methods. Intensities of the GerKC band in the DPS119 lysate were compared to the intensities of bands given by various amounts of GerKC in the SM101 lysate (1x corresponds to the same amount of spores, ~1.5 x 10^7, in the two fractions). Analysis of the various band intensities by ImageJ gave a value for the GerKC level in DPS119 spores that was 12% of that in SM101 spores. The two bands above the GerKC band are likely bands that reacted non-specifically, since their intensities were greatly increased relative to that of the GerKC band in the DPS119 spores.

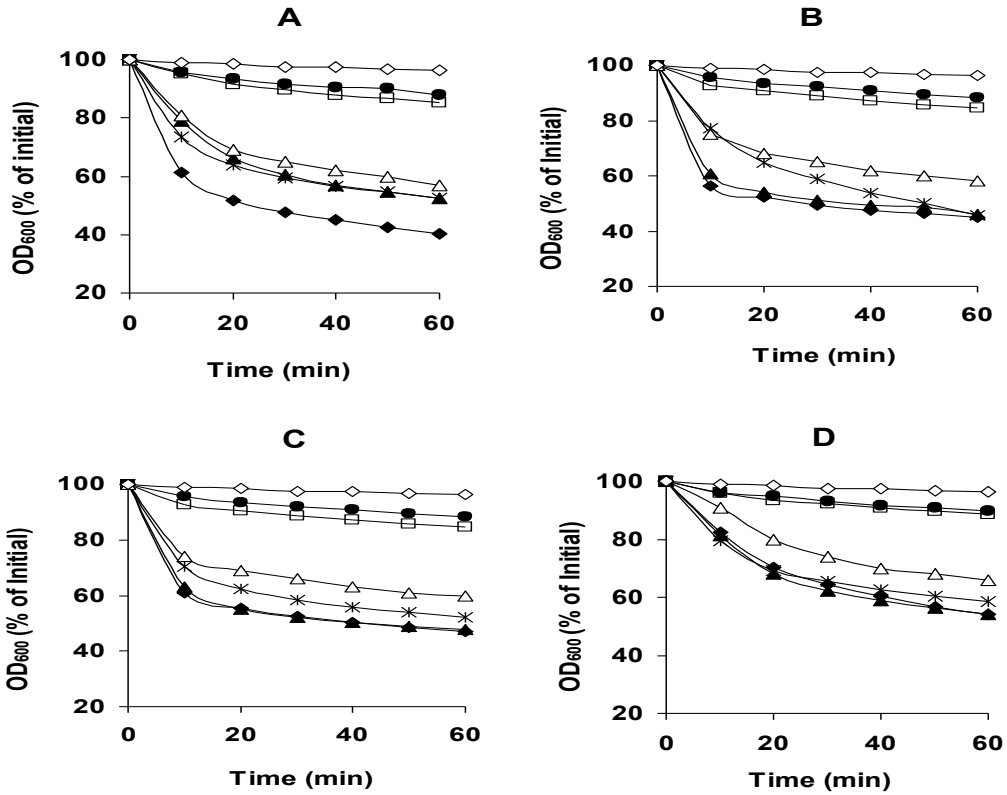

Fig. 2.2A-D. Germination of *C. perfringens* spores with various germinants. Heat-activated spores of strains SM101 (wild-type) (♦), DPS101 (*gerKA-KC*) (●), DPS119 *(gerKA*) (▲),DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (×), and DPS122(pSB18) (*gerKC* mutantcomplemented with wild-type *gerKA-gerKC*) (Δ) were incubated at 40°C with KCl (A), L-Asn (B), AK (C), or NaPi (pH 6.0) (D), and germination was measured by OD_{600} as described in Materials and Methods. The control germination (◊) was heat-activated spores incubated in 25 mM sodium phosphate buffer (pH 7.0) at 40°C, and no difference was observed between spores of SM101 and GR mutant strains.

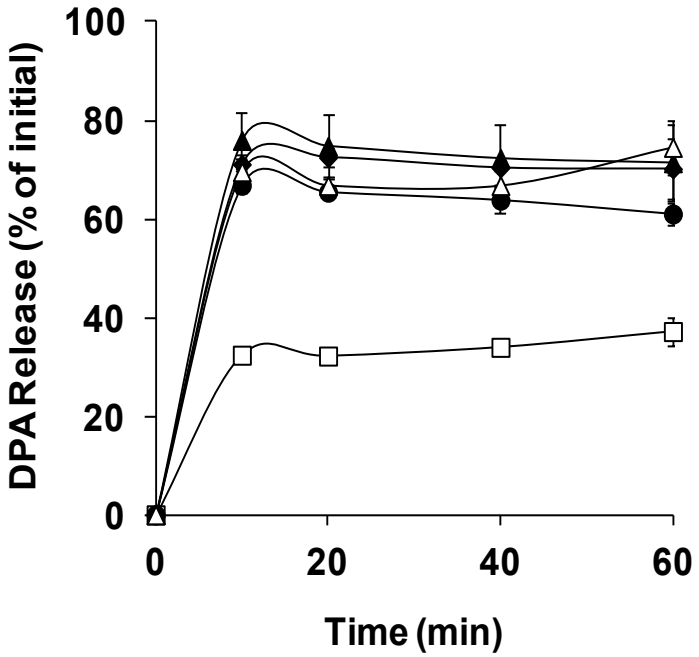

Fig. 2.3. DPA release by spores of *C. perfringens* strains during germination with KCl. Heat-activated spores of strains SM101 (wild-type) (♦), DPS119 (*gerKA*) (▲), DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (●) and DPS122 (pSB18) (*gerKC* mutant complemented with wild-type gerKA-KC) (Δ) were germinated with KCl (pH 7.0) and DPA release was measured as described in Materials and Methods. Error bars represent standard deviations.

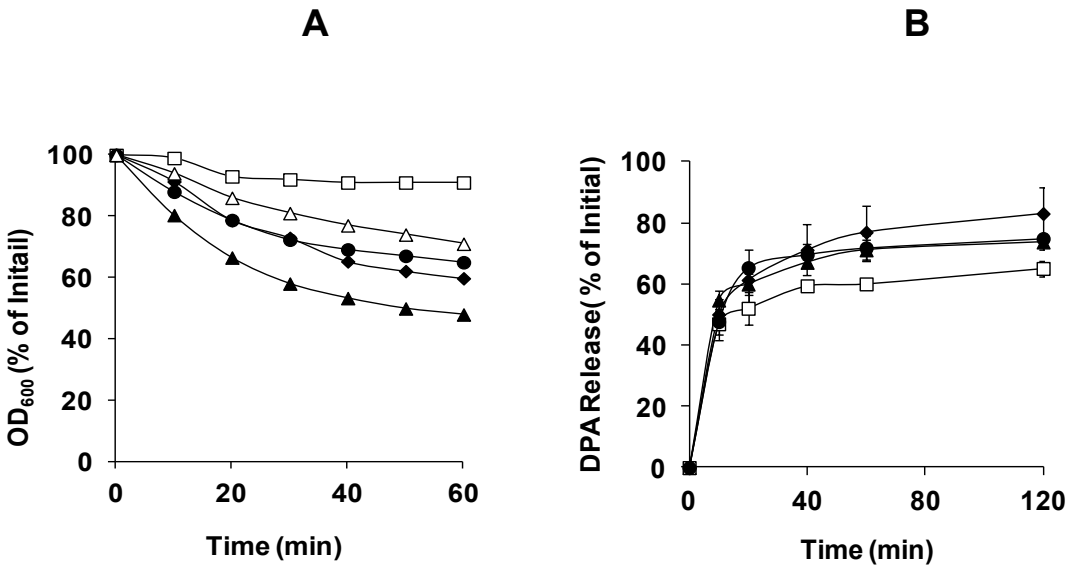

Fig. 2.4A,B. Germination of *C. perfringens* wild-type and mutant spores with Ca-DPA and dodecylamine. Heat-activated spores of strains SM101 (wild-type) (♦), DPS119 (*gerKA*) (▲), DPS122 (*gerKC*) (□), DPS124 (*gerKB gerAA*) (●) and DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-KC*) (Δ) were germinated with (A) Ca-DPA and changes in OD_{600} were measured as described in Methods; and (B) dodecylamine, and germination was monitored by DPA release as described in Methods. Error bars represent standard deviations.

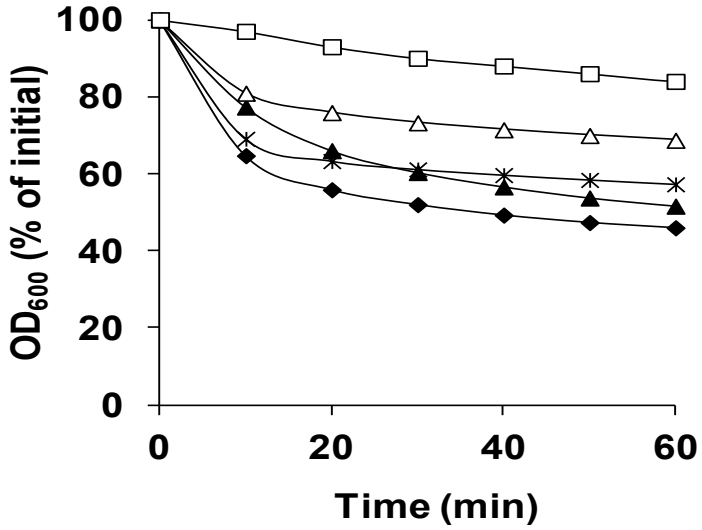

Fig. 2.5. Germination of spores of *C. perfringens* strains in BHI broth. Heat-activated spores of strains SM101 (wild-type) (♦), DPS119 (*gerKA*) (▲), DPS122 *(gerKC*) (□), DPS124 (*gerKB gerAA*) (×), and DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-KC*) (Δ) were germinated at 40°C in BHI broth, and the OD_{600} was measured as described in Methods.

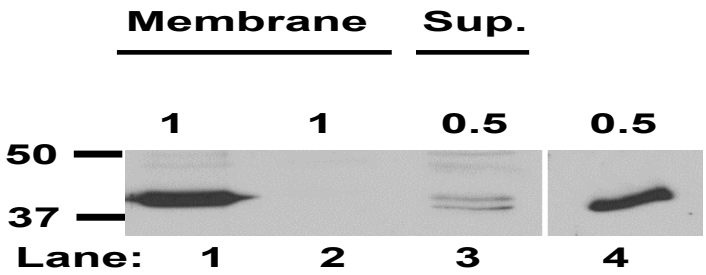

Fig. 2.6. Localization of GerKC in *C. perfringens* spores. Dormant spores of *C. perfringens* strains SM101 (wild-type) and DPS122 (*gerKC*) were decoated, disrupted with glass beads and sonication after lysozyme treatment, centrifuged at 14,000xg for 10 min, and the supernatant fluid was centrifuged at 100,000xg for 1 h as described in Methods. Aliquots of the P100 pellet IM fractions (lanes 1 and 2) from wild-type and *gerKC* spores and the S100 supernatant fraction from wild-type spores (lane 3) were run on SDS-PAGE followed by western blotting using GerKC antibody as described in Material and Methods. The arrow on the left indicates the size of GerKC, ~ 40kDa. The amounts of sample run in lanes 1-3 are given above the lanes as the percentage of the total IM or supernatant fraction. The S100 supernatant fraction from *gerKC* spores had no detectable GerKC band, data not shown. Lane 4 is 0.5 ng purified GerKC antigen. The numbered lines to the left of the figure denote the migration positions of molecular mass markers in kDa. All lanes are from the same gel, but an intervening region between lanes 3 and 4 was removed for clarity.

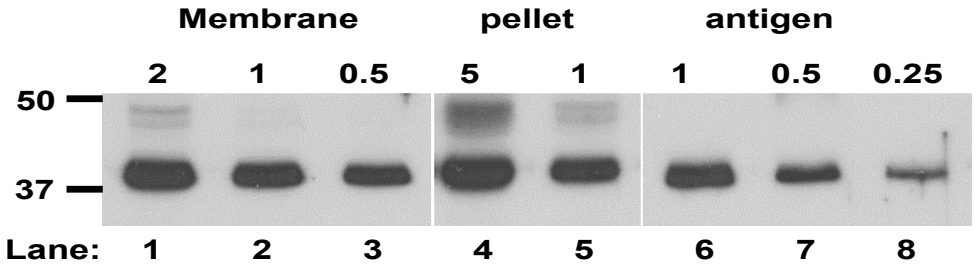

Fig. 2.7. Quantitation of GerKC level in spores of *C. perfringens* strain SM101. Various amounts of P100 pellet IM fractions (lanes 1 to 3) and 14,000xg pellet fractions of decoated, disrupted spores (lanes 4 and 5) and various amounts of purified GerKC antigen (lanes 6 to 8) were run on SDS-PAGE followed by western blot analysis using anti-GerKC antibody. The amounts of samples run are shown above the lanes as either the percentage of the total membrane or pellet fractions from $8.4x10^9$ spores (lanes 1-5), or the ng of GerKC antigen (lanes 6 to 8). The numbered lines to the left of the figure denote the migration positions of molecular mass markers, in kDa. All lanes are from the same gel, but lanes 4 and 5 were moved from their original position to the right of the GerKC antigen lanes.

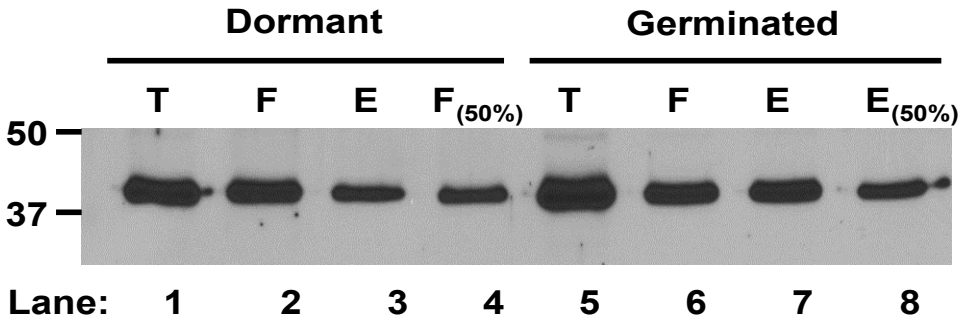

Fig. 2.8. Analysis of the level of biotinylation of GerKC in decoated dormant and germinated spores of *C. perfringens* wild-type strain SM101. Biotinylation was carried out on decoated dormant spores and intact germinated spores as described in Materials and Methods. Spores were disrupted, fractionated, and levels of various germination proteins in: i) the total IM fraction (T); ii) the IM fraction that did not adsorb to the NeutrAvidin beads (F); and iii) the eluate from the NeutrAvidin beads (E) were assayed by Western blotting using anti-GerKC antiserum as described in Materials and Methods. Aliquots in the T, F, and E samples were from equal amounts of spores. The $F_{50\%}$ sample (lane 4) from dormant spores was also the protein that did not adsorb to the NeutrAvidin beads but from 1/2 the amount of spores as in other lanes. The $E_{50\%}$ sample (lane 8) from germinated spores was also the eluate from the NeutrAvidin beads but from 1/2 the amount of spores as in other lanes. Analysis of this western blot by the program ImageJ indicated that ~ 33% of GerKC was biotinylated in dormant spores, with ~ 50% biotinylated in germinated spores.

Table 2.1. Colony formation by spores of *C. perfringens* strains[a]

Strain (genotype)	Spore titer (CFU/ml/OD_{600})[b]	
	BHI	BHI + Lyz[C]
SM101 (wild type)	2.4×10^8	7.1×10^8
DPS 119 (*gerKA)*	1.7×10^8	5.7×10^8
DPS122 (*gerKC*)	2.3×10^6	4.2×10^8
DPS124 (*gerKB gerAA*)	3.8×10^7	6.1×10^8
DPS122(pSB18) (*gerKC* mutant complemented with wild-type *gerKA-KC*)	1.2×10^8	ND[d]

[a] Heat-activated spores of various strains were plated on BHI agar with or without lysozyme, and colonies were counted after anaerobic incubation at 37°C for 24 h as described in Materials and Methods.

[b] Titers are the average number of colony forming units (CFU)/ml/OD_{600} determined in three experiments, and the variation was less than 15%.

[c]Spores were decoated, heat activated, and plated onto BHI agar containing lysozyme (Lyz), and colonies were counted after incubated overnight anaerobically at 37°C.

[d]ND, Not Determined.

Table 2.2. Bacterial strains and plasmid used

Strain or plasmid	Relevant characteristic	Source or reference
C. perfringens strains		
SM101	Electroporatable derivative of food poisoning type A isolate NCTC8798; carries a chromosomal *cpe* gene	(59, 147)
DPS101	*gerKA*::*catP*	(87)
DPS119	*gerKA*::*ermB*	This study
DPS122	*gerKC*::*ermB*	This study
DPS124	*gerKB::catP gerAA::intron*	This study
DPS122(pSB18)	*gerKC* mutant expressing wild-type *gerKA-KC*	This study
Plasmids		
pJIR750ai	*C. perfringens*/*E. coli* shuttle vector containing an *L1.LtrB* intron retargeted to the *plc* gene	(148)
pJIR3566	*C. perfringens/E. coli* shuttle vector; Em^r.	(136)
pJIR750	*C. perfringens*/*E. coli* shuttle vector; Cm^r.	(137)
pDP276	~ 350-bp BsrGI-HinDIII fragment retargeted to *gerKC* cloned between BsrGI-HinDIII sites in pJIR3566	This study
pDP300	~350-bp BsrGI-HinDIII fragment retargeted to *gerKA* cloned between BsrGI-HinDIII sites in pJIR3566	This study
pDP10	~ 3.1- kb *gerKA-KC* operon in pMRS104	(87)
pSB18	~ 3.1-bp KpnI-XhoI fragment carrying *gerKA-KC* operon from pDP10 cloned between KpnI-SalI sites of pJIR750	This study

Table 2.3. Primers used in this study

Primer name	Primer sequence[a]	Gene	Position[b]	Use[c]
CPP443	5' GATGAAAATGAAGTGGGAAATATAGAC 3'	*gerKC*	+120 to +137	MD
CPP440	5'GTTGTGCCATTAATTTCAACATCAACA 3'	*gerKC*	+1076 to +1103	MD
CPP209	5'TATAGTGAAAATCCAAGTATCTC 3'	*gerKA*	-224 to - 201	MD
CPP208	5'ATCATTATTATCACCTCTGCTACTAT 3'	*gerKA*	+980 to +1006	MD
CPP206	5' CAAGTATTAATCCTCCAATAACAG 3'	*gerAA*	+1102 to +1126	MD
CPP211	5' CTTTAATGGGAATTATAGCA 3'	*gerAA*	-264 to -244	MD
CPP876	5'AAAAAAGCTTATAATTATCCTTATTAGGCCCAGCCG TGCGCCCAGATAGGGTG 3'	*gerKA*	IBS 91s	MP
CPP877	5'CAGATTGTACAAATGTGGTGATAACAGATAAGTCC CAGCCACTAACTTACCTTTCTTTGT3'	*gerKA*	EBS1d 91s	MP
CPP878	5'TGAACGCAAGTTTCTAATTTCGGTTCCTAATCGATA GAGGAAAGTGTCT 3'	*gerKA*	EBS2 91s	MP
CPP879	5' AAAAAAGCTTATAATTATCCTTATATTTCGGTGTT GTGCGCCCAGATAGGGTG 3'	*gerKC*	IBS 469s	MP
CPP880	5'CAGATTGTACAAATGTGGTGATAACAGATAAGTCG GTGTTTTTAACTTACCTTTCTTTGT 3'	*gerKC*	EBS1d 469s	MP
CPP881	5'TGAACGCAAGTTTCTAATTTCGGTTAAATATCGATA GAGGAAAGTGTCT 3'	*gerKC*	EBS2 469s	MP

a- The nucleotide numbering begins from the translation start codon and refers to the relevant position within the respective coding sequence.
b- Nucleotide numbering being at the first base of the translation codon of the relevant gene.
c- MD, Mutation detection; MP, Construction of mutator plasmid.

CHAPTER 3

Location and Stoichiometry of the Protease CspB and the Cortex-Lytic Enzyme SleC in *Clostridium perfringens* Spores

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3-1Abstract

The protease CspB and the cortex-lytic enzyme SleC are essential for peptoglycan cortex hydrolysis during germination of spores of the *Clostridium perfringens* food poisoning isolate SM101. In this study, Western blot analyses were used to demonstrate that CspB and SleC are present exclusively in the *C. perfringens* SM101 spore coat layer fraction and absent in the lysate from decoated spores and from the purified inner spore membrane. These results indicate why decoating treatments greatly reduce both germination and apparent viability of *C. perfringens* spores in the absence of an exogenous lytic enzyme. In addition, quantitative Western blot analyses showed that there are approximately 2,000 and 130,000 molecules of CspB and pro-SleC, respectively, per *C. perfringens* SM101 spore, consistent with CspB's role in acting catalytically on pro-SleC to convert this zymogen to the active enzyme.

3-2. Introduction

Clostridium perfringens is an anaerobic, gram-positive, and spore-forming bacterium that can cause gastrointestinal (GI) diseases in humans and animals (1, 2). Depending on the production of various major toxins (alpha, beta, epsilon, and iota), *C. perfringens* can be classified into five types, A through E (3). However, less than 5% of *C. perfringens* type A isolates produce *C. perfringens* enterotoxin, the major virulence factor responsible for *C. perfringens* type A food poisoning (FP) and non-food borne (NFB) GI diseases (2, 149). Spores of *C. perfringens* may remain in a dormant state for long periods of time due to their innate resistance to many environment stresses such as heat, radiation, and toxic chemicals (16, 74, 75, 150). Under favorable conditions in the environment, these spores can return to active growth through the processes of spore germination and outgrowth that lead to the synthesis and release of toxins causing diseases (87, 88).

In *Bacillus* and *Clostridium* species, spore germination is initiated when spores sense compounds called germinants, such as nutrients (i.e., amino acids and salts), a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (Ca-DPA) and cationic surfactants (87, 88, 97). In *Bacillus subtilis,* two spore-specific cortex-lytic enzymes (CLEs), CwlJ and SleB, are responsible for degradation of spore cortex peptidoglycan. Loss of either CwlJ or SleB does not abolish cortex degradation, but spores lacking both enzymes do not degrade their cortex and cannot complete germination (88, 95, 125). CwIJ and SleB are both

synthesized in a mature form during sporulation; CwIJ is activated by Ca-DPA released from the spore core in the initial stage of germination, signaling activation of cortex hydrolysis, while SleB is perhaps activated when the spore cortex undergoes structural deformation during Ca-DPA release (85, 95, 125, 151, 152).

Two CLEs have been identified in *C. perfringens* spores, SleM and SleC, and these enzymes appear to differ significantly from CwIJ and SleB in *Bacilli* (126). SleM seems to be an N-acetylmuramidase, and SleC exhibits transglycosylase and *N*-acetylmuramoyl-Lalanine amidase activity (129). However, only SleC is required for cortex hydrolysis during *C. perfringens* spore germination, and Ca-DPA release does not act as a signaling molecule to trigger cortex hydrolysis. Rather SleC is present in dormant *C. perfringens* spores as an inactive zymogen, pro-SleC, and early in germination the pro-sequence is removed, generating mature active SleC (86, 126, 127). Pro-SleC is activated by the Csp serine proteases that are members of the subtilisin family of proteases, and the *C. perfringens* S40 genome encodes three Csp proteases, CspA, B, and C, any of which can convert pro-SleC into mature SleC *in vitro* (130, 153). The Csp proteases are also made as zymogens (pro-CspA, B or C) that most likely autoprocess to the mature active enzymes during sporulation. However, the *C. perfringens* FP strain SM101 has only one Csp protease, CspB, which is essential for cortex hydrolysis and rapid Ca-DPA release during spore germination (128).

Previous work with *B. subtilis* spores showed that CwIJ is in the spore´s outer layers, most likely the spore coats, as this protein is removed by coat extraction with detergents plus a reducing agent at high pH, and also does not assemble properly on spores of a *cotE* mutant that have very defective coats (122, 144). Some SleB is also in the outer layers of *B. subtilis* spores, but most SleB as well as its partner protein YpeB are found associated with the inner spore membrane that surrounds the spore core, and this latter SleB is not removed by decoating treatments (Chirakkal et al., 2002). In *C. perfringens* S40 spores, the localization of CLEs was determined using immunoelectron microscopy, and pro-SleC was found on the outside of the cortex but was not detected in germinated spores and chemically decoated spores (154). However, pro-SleC's antigenicity might have been destroyed by the chemical decoating procedure used in the latter work. The Csp proteases that activate pro-SleC are made in the mother cell compartment of the sporulating cell, and can also be extracted by mild detergents from germinated *C. perfringens* spores (130, 153). The latter results suggest that Csp proteases are also in *C. perfringens* spores' outer layers, but this has not been shown definitively. Consequently, in this work we have examined the location of both pro-SleC and the Csp protease CspB in spores of the *C. perfringens* FP strain SM101 that contains only one Csp protease. In addition, the numbers of pro-SleC and CspB molecules in spores have been determined.

3-3. Materials and methods

Bacterial strains and spore preparation and purification. The *C. perfringens* strains used in this work were the FP strain SM101 (wild-type) (59, 147), and its derivatives DPS107 lacking *sleC* (126), and DPS117 lacking *cspB* (128). Spores of these strains were prepared and purified as previously described (87, 138, 139), and stored at -20ºC until used. All spores used in this work were free (> 98%) from growing or sporulating cells and germinated spores as assessed by phase contrast microscopy.

Preparation of various extracts from dormant spores. Coat extracts, lysates from decoated spores and the spores' inner membrane were prepared from dormant *C. perfringens* spores essentially as described previously (92, 111, 135, 155). Briefly, purified spores of *C. perfringens* strains, ~ 3 ml at an optical density at 600 nm (OD_{600}) of 25 (~3.2×10^9 spores/ml), were first decoated by incubation at 37°C for 1.5 h in 8 M urea - 1% SDS - 0.05 M dithiothreitol – 0.05 M TrisHCl buffer (pH 8.0), centrifuged at 22°C in a microcentrifuge for 5 min at 14,000 x g, the supernatant fluid saved as the coat extract, and the decoated spores washed 10 times with water as described (156). The washed decoated spores were suspended at an OD_{600} of 50 to 70 in 0.5 ml TEP buffer (50 mM Tris-HCl buffer (pH 7.4) – 5 mM EDTA - 1 mM phenylmethylsulfonylfluoride plus 1 mg of lysozyme, 1 µg of RNase, 1 µg DNase I, and 20 µg of $MgCl_2$. This mixture was incubated for 5 min at 37°C, and then kept on ice for 20 min. Glass beads (100 mg) were added to each sample and spores were

given four bursts of sonication (15 sec pulses with 30 sec pauses on ice in between pulses) to complete spore disruption as assessed by microscopic analysis, and to reduce the viscosity of the extract. Following the final sonication pulse, samples were allowed to settle for 15 sec and 100 uL of fluid was withdrawn and saved as the total decoated spore lysate. The rest of the decoated spore lysate that contained the glass beads was used for preparation of the spore inner membrane fraction. The various spore fractions were stored at -20°C until use.

CspB and SleC expression and purification and preparation of antisera. For CspB and SleC expression the *cspB* and *sleC* genes were PCR amplified from *C. perfringens* strain SM101 DNA and cloned into a modified pET15b expression vector fusing a His_6-tag plus a tobacco etch virus (TEV) protease cleavage site to the N-termini of CspB and SleC protein. In these constructs, the N-terminus of the full length *cspB* coding sequence was fused to the His-tag giving the CspB zymogen pro-CspB, and the *sleC* sequence encoding SleC residues 115-413, equivalent to pro-SleC, was fused to the His-tag. The protein fusions were overexpressed in *Escherichia coli* BL21 cells and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography. Following TEV protease cleavage to remove the His_6-tag, the proteins were further purified by anion (CspB) or cation (SleC) exchange and gel filtration (SD200; GE Healthcare, Piscataway, NJ) chromatography. Purified protein (2 mg) was used to raise polyclonal antibodies in two New Zealand White female rabbits (Pacific Immunology Group, Ramona, CA). Anti-CspB and -SleC antibodies were detected by Western blotting in a blood sample collected 2 months after the

initial injection, at which time the animals were exsanguinated. The specificity of the antisera was confirmed by Western blotting using purified proteins and various spore extracts (see Results).

Western blot analysis. Western blot analysis was carried out following separation of proteins by SDS-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and transferring the proteins to polyvinylidene difluoride (PVDF) membranes as described previously (111). The CspB and SleC proteins were detected on the PVDF membranes essentially as described previously (135, 155) by incubation with 1:1000 dilutions of the polyclonal rabbit antisera against CspB or SleC for 1 h at room temperature, followed by incubation with a 1:10,000 dilution of goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Millipore Corporation, Billerica, MA, USA) for 45 min at room temperature. The binding of the secondary antibody was detected by incubation with a chemiluminescent HRP substrate (Pierce SuperSignal; Thermo Scientific, Rockford, IL, USA) using X-ray film.

Determination of molecules of CspB and SleC per spore. The average number of CspB and SleC molecules per spore was determined by comparing band intensities given by wild-type spore coat extracts with intensities given by known amounts of purified CspB and SleC antigens on the same Western blots. The concentrations of the purified proteins were determined by their absorbance and molecular extinction coefficient at 280 nm. The intensities of all bands on Western blots were quantitated using ImageJ software (National Institutes of Health, Bethesda, MD USA) and the

average numbers of CspB and SleC protein per spore were calculated (111, 135). Briefly, the amount of the purified protein equivalent to the amount of the corresponding protein in the spores' coat extract was divided by this protein's molecular weight giving the moles of the protein, and this value was multiplied by Avogadro's number. The latter number was then divided by the number of spores in the amount of coat extract analyzed to give the number of molecules of the protein per spore. The simplified formula for determining the number of protein molecules per spore:

$$\frac{[(1/\text{MW (mol/g)}) \times (\text{amount of antigen (g)})] \times (6.023*10^{23})}{\text{(number of spores)}}$$

3-4. Results

Localization of CspB and SleC in spores. Using Western blot analysis, CspB and SleC were readily detected in spores using polyclonal rabbit antisera raised against recombinant *C. perfringens* CspB and SleC (Fig. 3.1). However, these proteins were found only in the coat extract of wild-type dormant spores, as no immunoreactive CspB and SleC species were detected in lysates from decoated spores (< 1% of the levels in coat extracts). In addition, Western blot analysis of purified spore inner membrane also detected no CspB or SleC antigen (data not shown). The sizes of the antigens detected in coat extracts, ~ 50 kDa with anti-CspB and ~ 34 kDa with anti-SleC, were in agreement with the expected molecular weights of CspB and pro-SleC. Furthermore, while these antigens were detected in coat extracts of dormant wild-type spores, the appropriate antigens were absent from spores of strains with deletions in either *cspB* or *sleC* (Fig. 3.1). These latter results indicate that the antisera used in these Western blots were highly specific.

The results of Western blot analysis of various spore fractions strongly indicated that CspB and SleC are located exclusively in coat extracts, consistent with these proteins being located outside the spore cortex. It was possible that the coat extraction regimen used inactivates spores, and thus extracts proteins from more interior spore layers. However, this was not the case, as the decoated spores retained > 80% of the viability of intact spores (as shown previously), although required

addition of lysozyme to plating media in order to get efficient colony formation (126, 128).

Levels of CspB and SleC in spores. Work with B. subtilis has indicated that levels of spore proteins involved in initial steps of germination are significantly lower than levels of proteins involved in late germination steps (111, 122). To establish if this is also the case with C. perfringens SM101 spores, the average numbers of CspB and SleC molecules per spore were determined by comparing intensities given by coat extracts from several amounts of spores to the intensities from known amounts of purified CspB and SleC antigen on the same Western blot (Fig. 3.2). These comparisons allowed calculation that there are ~ 2,000 molecules of CspB per spore and ~ 130,000 molecules of SleC per spore (Fig. 3.2), indicating that CspB levels are ~ 65-fold lower than its substrate pro-SleC.

3-5. Discussion

With *B. subtilis* spores, the signaling process in germination is likely amplified by increasing the levels of the proteins involved in sequential germination steps. Cortex peptidoglycan hydrolysis is the culmination of the germination process and in *B. subtilis* spores the level of the CLE CwIJ (the SleB level is not known) is ~ 8 $\times 10^3$ molecules per spore (122), ~ 8-fold higher than the level of all germinant receptors (GRs) that sense nutrient germinants (111). This increase in levels of germination proteins further along in the germination process was also seen with *C. perfringens* spores, as numbers of CspB and SleC molecules in spores were both much higher than the 250 molecules per spore of the GR protein GerKC found previously (155). In addition, in *C. perfringens* spores, levels of CspB were ~ 65-fold lower than its substrate pro-SleC, consistent with signal amplification from CspB to pro-SleC, with CspB catalytic activity generating the active SleC that is responsible for the major final event in spore germination.

The major conclusion from the work in this communication concerns the localization of the Csp protease, CspB, as well as the single CLE, SleC, in *C. perfringens* spores. In contrast to the situation in *B. subtilis* spores where most SleB is associated with the spore's inner membrane (111, 135, 144), neither pro-SleC nor CspB were associated with the inner membrane of *C. perfringens* spores. This latter finding plus the extraction of essentially all SleC and CspB antigen from dormant *C. perfringens* spores by regimens designed to extract coat and outer membrane proteins

strongly suggest that pro-SleC and CspB are on the outer surface of the spore cortex. This further suggests that these two proteins may physically interact in this location in the dormant spore, facilitating CspB action on pro-SleC when CspB enzyme activity is stimulated. However, at present, the mechanism whereby Csp protease is activated during germination of *C. perfringens* spores is not known.

The absence of CspB and SleC from *C. perfringens* spores' inner membrane which is close to the inner edge of the spore cortex, and the likely concentration of CspB and SleC at the cortex's outer edge also suggests that in germination of *C. perfringens* spores, cortex hydrolysis may start from the outer edge of the cortex and proceed inward. In contrast, in spores of *B. subtilis*, where CLEs are located at both the outer and inner edges of the cortex (144, 156), the hydrolysis of the cortex during spore germination could proceed from both the inner and the outer edges simultaneously. Indeed, loss of CwlJ from spores of *Bacillus* species greatly slows rates of Ca-DPA release during germination of individual spores while loss of SleB has no effect (152, 157), suggesting that there is something different in the cortex hydrolysis catalyzed by these two CLEs perhaps due to where the cortex hydrolysis is taking place. It would be interesting to determine exactly where cortex hydrolysis takes place during germination of spores of *C. perfringens* and *B. subtilis*, and of both *cwlJ* and *sleB B. subtilis* spores.

Finally, the extraction of all pro-SleC and CspB from *C. perfringens* SM101 spores by a decoating regimen explains why such treatments greatly decrease the

apparent viability of *C. perfringens* spores, as these spores simply cannot carry out cortex degradation and complete spore germination. However, as shown in several studies, these decoated spores are not really dead, but can readily be revived by inclusion of low concentrations of a lytic enzyme such as lysozyme in plating media (126, 128). The results in this manuscript on CspB and pro-SleC location in spores thus provide new information indicating that: 1) these essential germination proteins are in spores' outer layers, where they may be inactivated by chemical or physical treatments rather readily, probably more readily than irreversible spore inactivation; 2) as a consequence, *C. perfringens* spores may appear inactivated only because they cannot complete spore germination due to CspB or SleC inactivation; but 3) these apparently inactivated spores can be rescued in foods that retain lytic enzymes that could substitute for SleC and germinate the apparently dead *C. perfringens* spores, which then grow and produce toxins in foods.

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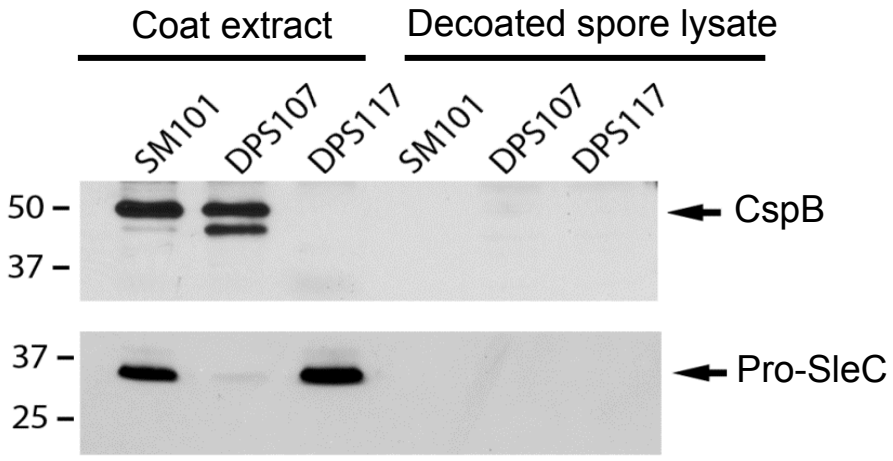

Fig. 3.1. Localization of CspB and SleC in spore fractions**.** Samples from dormant spores of *C. perfringens* strains SM101 (wild type), DPS107 (*sleC*) and DPS117 (*cspB*) were decoated and fractionated as described in Materials and Methods. Samples of either the coat extract or the decoated spore extract from $4.6x10^6$ spores for CspB analysis or $9.2x10^4$ spores for SleC analysis were electrophoresed on SDS-PAGE and analyzed by Western blots using either CspB (upper panel) or SleC (lower panel) antisera. The numbers to the left of the figure denote the migration positions of molecular mass markers in kDa. The arrows to the right of the figure denote the migration positions of pro-SleC and mature CspB.

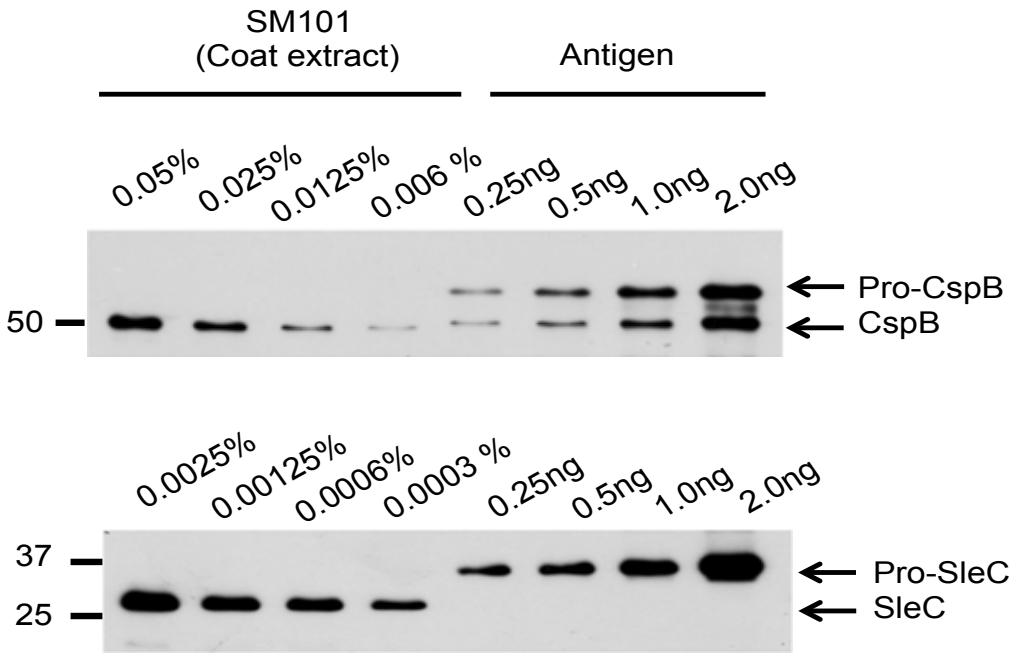

Fig. 3.2. Levels of CspB and SleC in spores of *C. perfringens* strain SM101. Samples of coat extract from wild-type strain SM101 and various amounts of purified pro-CspB and pro-SleC proteins were electrophoresed on SDS-PAGE and Western blot analysis was performed using anti-CspB (upper panel) and anti-SleC (lower panel) sera. The amounts of samples run are shown above the lanes as either the percentage of the total coat extract from 4.6×10^9 spores or the amount (in ng) of pro-CspB antigen with the His_6-tag removed and pro-SleC antigen. Some of the pro-CspB was autoprocessed during purification giving the lower mol wt CspB band. The numbers to the left of the figure denote the migration positions of molecular mass markers in kDa.

CHAPTER 4

Characterization of Germinants and Their Receptors for Spores of Non-Food-Borne *Clostridium perfringens* Strain F4969

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4.1 Abstract

Clostridium perfringens type A isolates carrying a chromosomal enterotoxin gene are associated with food poisoning (FP), while isolates carrying a plasmid-borne enterotoxin gene are associated with non-food-borne (NFB) gastrointestinal diseases. *C. perfringens* spores are the infectious morphotype, and therefore, spore germination is considered to be an essential step for initiation of these diseases. Our previous study reported that a mixture of L-asparagine and KCl (AK) germinated spores of FP and NFB isolates well, but KCl and, to a lesser extent, L-asparagine induced spore germination only in FP isolates. We now report that both L-asparagine and KCl induced germination of NFB isolate F4969 spores in a cooperative manner, while L-asparagine or KCl each trigger germination of spores of FP strain SM101 through independent pathways. Spores of F4969 *gerAA*, *gerKA-KC* or *gerKC* mutants lacking specific germinant receptor proteins germinated slower than wild-type spores with rich media, did not germinate with AK, and germinated poorly compared to wild-type spores with L-cysteine. The germination defects in the *gerKA-KC* spores were largely due loss of GerKC as i) *gerKA* spores germinated significantly with all tested germinants, while *gerKC* spores exhibited poor or no germination; and ii) germination defects in *gerKC* spores were largely restored by expressing the wild-type *gerKA-KC* operon in *trans*. We also found that *gerKA-KC*, *gerAA,* and *gerKC* spores, but not *gerKA* spores, released DPA at a slower rate than wild-type spores with AK. The colony-forming efficiency of F4969 *gerKC* spores was also ~35-fold lower than that of wild-type spores, while *gerAA* and wild-type spores had similar

viability. Collectively, these results suggest that the GerAA and GerKC proteins play roles in normal germination of *C. perfringens* NFB isolates, and that GerKC, but not GerAA, is important in these spores' apparent viability.

4.2 Introduction

C. perfringens is a Gram-positive, spore-forming, anaerobic, pathogenic bacterium capable of causing a wide variety of diseases in humans and animals (1-3). The two most common illnesses in humans are *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) illnesses (2, 48). These two major diseases are caused mainly by *C. perfringens* Type A isolates producing *C. perfringens* enterotoxin (CPE); these isolates account for ~ 5% of Type A isolates (149, 158, 159). Interestingly, differences between FP and NFB isolates not only include the localization of CPE-encoding gene (*cpe*) on the chromosome or plasmid, respectively (160), but also significant differences in their spore properties (14, 17). Numerous studies have highlighted the ability of spores of FP isolates (FP spores) as better able to survive in the harsh conditions encountered in FP environments than spores of NFB isolates (NFB spores) (14, 60, 81, 104, 161, 162). Thus FP spores are more resistant than NFB spores to: i) heat treatments (14); ii) low temperatures (4°C and -20°C) (81); and iii) nitrite induced stress (60). In contrast, NFB spores are more adapted to germinate in host environments, particularly in the presence of epithelial cells and macrophages (86, 163, 164). However, in order to cause disease, both FP and NFB spores must germinate to return to vegetative cell growth (86).

The process of bacterial spore germination is triggered when compounds, called germinants, bind to their cognate germinant receptor (GR) located in spores' inner membrane. Germinant binding to its cognate GR triggers the release of

monovalent cations (i.e., Na^+ and K^+) and the spore core's large depot of dipicolinic acid (DPA) present as a 1:1 chelate with divalent cations, primarily Ca^{2+} (Ca-DPA) (6, 86). The release of these small molecules, at least in *Bacillus* species, activates downstream effectors such as the cortex-lytic enzymes (CLEs) (6, 86, 88, 95, 120), and these initiate the degradation of the spore's peptidoglycan (PG) cortex allowing an increase in water uptake in the spore core to levels similar to that of growing cells (6, 85, 88). Recent studies (86, 87, 104, 110, 116, 120, 126, 128, 165, 166) have dissected the mechanism of germination of *C. perfringens* spores. However, the detailed studies have used primarily a FP isolate, SM101, and have highlighted significant differences in germination of FP versus NFB spores. Notably, FP spores germinate with L-asparagine (L-asn), KCl, a mixture of L-asn and KCl (AK), the co-germinants Na^+ and inorganic phosphate, or the non-nutrient germinant Ca-DPA, all through the main germinant receptor protein GerKC (87, 103, 104, 110, 155). In contrast, NFB spores are able to germinate only with the AK mixture (87, 103, 104, 110). Spores of a NFB GI disease isolate F4969 also germinates to a greater extent than a FP isolate SM101 spores in the presence of cultured intestinal epithelial cells and their growth medium (163). However, the basis for the differential germination response of FP versus NFB spores and the mechanism of spore germination in NFB spores are not known. Therefore, in this study we further compared the germination response between spores from six different FP and NFB strains and examined the role of various GR proteins in germination of NFB spores by constructing GR mutant derivatives of strain F4969 and characterizing the germination phenotypes of the

mutant spores. Our results indicate that the GerKC and GerAA proteins are required for normal germination of NFB spores with rich medium, AK and L-cysteine (L-cys). GerKC also plays a major role in these spores' DPA release triggered by AK and their viability, but the GerAA protein is not required for spore viability.

4.3 Material and Methods

Bacterial strains and plasmids. *C. perfringens* strains and plasmids used in this study are described in (Table 4.1).

Spore preparation. Starter cultures of *C. perfringens* isolates were prepared by overnight growth at 37°C in fluid thyoglycollate (FTG) broth (Difco, BD Diagnostics, USA) as described (138). To prepare sporulation precultures of *C .perfringens,* 0.4 ml of an FTG starter culture was inoculated into 10 ml of Duncan-Strong (DS) sporulation medium (139) . The DS medium was incubated for 24 h at 37°C and the presence of spores was confirmed by phase-contrast microscopy; larger volumes of spores were prepared by scaling-up this procedure. Spores were purified by repeated washing with sterile distilled water until the spores were more than 99% free of sporulating cells, germinated cells, and cell debris. Clean spores were suspended in distilled water at an optical density at 600 nm (OD_{600}) of ~ 6 and stored at -20°C.

Spore germination assay. Spore germination was carried out as previously described (87, 110, 155). Spore suspensions of OD_{600} 6 were heat activated at 75°C for 15 min, cooled on ice and incubated at 37 °C for 10 min prior to germination assays. For germination, heat-activated spores of OD_{600} 1 were incubated with 100 mM each of L-asn or KCl, and 100 mM of both L-asn and KCl (AK) in 25 mM Tris-HCl buffer (pH 7.0), and with 100 mM L-cys in 25 mM Tris-HCl buffer, pH 6.0 at 37°C for 60 min. Spore germination was routinely measured by monitoring the OD_{600} of spore

cultures (Smartspec™ 3000 Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA), which falls ~60 % upon completion of spore germination. The extent of germination was confirmed by phase-contrast microscopy, as fully germinated spores change from phase bright to dark. The extent of spore germination was calculated by measuring the decrease in OD_{600} and expressed as the percentage of the initial value. All values reported are averages of three experiments performed with at least three independent spore preparations. Germination was also carried in TGY (3% Trypticase, 2% Glucose, 1% Yeast extract, and 0.1% L-cys), brain heart infusion broth (BHI), Eagle's Minimum Essential Medium (EMEM), and Dulbecco's Modified Eagle Medium (DMEM).

To determine kinetic parameters of spores' germination with AK, germination was carried out with a fixed concentration of L-asn and different KCl concentrations and vice versa. The rate of germination was expressed as the maximum rate of loss of OD_{600} of the spore suspension. All curves generated by plotting the germinant concentration versus the germination rate were fitted to the Hill equation to determine the maximum initial rate of germination (V_{max}) and the germinant concentration needed to achieve 50% of the maximal rate (K_m).

Construction of a *gerKA-KC* (*gerK* operon) mutant. To construct a derivative of *C. perfringens* strain F4969 with an insertion of *catP* (encoding chloramphenicol resistance) in *gerKA*, the *gerKA-KC* mutator plasmid pDP11 (87) was used. Plasmid

pDP11 contains an inactivated *gerKA-KC* operon and has no origin of replication for *C. perfringens*, and thus is not maintained in *C. perfringens*. We introduced plasmid pDP11 into *C. perfringens* strain F4969 by electroporation (19), and a *gerKA-KC* mutant, strain SB104, was selected by allelic exchange as described previously (149). The replacement of wild-type *gerKA-KC* with the mutant allele in strain SB104 was confirmed by PCR (data not shown).

Construction of a *gerAA* mutant. A derivative of strain F4969 with an intron inserted in the *gerAA* gene was constructed as follows. Plasmid pDP13, which has the L1.LtrB intron retargeted to *gerAA* (87), was introduced into *C. perfringens* strain F4969 by electroporation (19) and chloramphenicol-resistant (Cm^r) colonies were screened for the insertion of the Targetron by PCR using detection primers CPP211 and CPP206 (Table 4.5). The PCR reaction mixture was placed in a thermal cycler (Techne, Bibby Scientific Limited, UK) with a first stage of 1.5 min at 94°C (denaturation), 32 cycles in the second stage each with 1 min at 94°C, 1 min at 47 °C (annealing), and 2 min at 72°C (extension); the final stage was an extension for 5 min at 72°C. To cure the Cm^r encoding vector, one Cm^r Targetron-carrying clone was subcultured for 48 h in FTG medium without Cm, and single colonies were patched onto BHI agar, with or without Cm, giving strain SB103.

Construction of a *gerKC* mutant. Construction of a *gerKC* mutant of F4969 was performed using the modified group II intron (the ClosTron) that inserts the intron into *gerKC* between bp 468 and 469 from the *gerKC* start codon. Plasmid pDP276, which has the L1.LtrB intron retargeted to *gerKC,* was previously constructed (155). This plasmid with the Targetron insertion was introduced into *C. perfringens* F4969 by electroporation (19), and erythromycin-resistant (Em^r) transformants were selected on BHI agar plates with 30 μg/ml Em. The Em^r transformants were screened for insertion of the intron into *gerKC* by PCR using *gerKC*-detection primers CPP440 and CPP443 (Table 4.5). To cure the Em^r-coding vector, one positive intron-inserted clone was sub-cultured three times in FTG broth and then plated onto BHI agar, and single colonies were patching onto BHI agar with or without Em, giving strain SB106.

Construction of *gerKA* mutant. A mutation in the *gerKA* gene in *C. perfringens* strain F4969 by intron insertion into *gerKA* was generated as follows: plasmid pDP300, which carries the L1.LtrB intron retargeted to *gerKA* (155) was introduced into *C. perfringens* strain F4969 by electroporation and Em^r colonies were screened for the insertion of the Targetron by PCR using detection primers CPP208 and CPP209 (Table 4.5). The *gerKA* mutant was isolated in the same way as described above for the *gerKC* mutant, giving strain SB110.

Construction of *gerKB* mutant. To isolate *C. perfringens* F4969 with a deletion in *gerKB,* the *gerKB* mutator plasmid pDP75 (110) (Table 4.1) was used. Plasmid pDP75 (carrying Δ*gerKB::catP)* cannot replicate in *C. perfringens*; thus, we introduced this plasmid into *C. perfringens* F4969 by electroporation, and a Cm^r *gerKB* mutant was isolated as described previously (149). The identity of the *gerKB* strain SB108 was confirmed by PCR (data not shown).

Construction of a *gerKA-KC* complemented strain. A 3.2 kb fragment carrying wild-type *gerKA-KC* was excised from plasmid pDP10 (87) by digestion with KpnI and SalI and then ligated between KpnI and SalI sites of plasmid pJIR751 that contains the *ermB* gene (137), giving plasmid pSB19. This plasmid was introduced into the *C. perfringens gerKA-KC* mutant SB104 by electroporation (19) and Cm^r Em^r clones were selected. The presence of plasmid pSB19 in strain SB104 (pSB19) was confirmed by PCR (data not shown).

Construction of a *gerAA* complemented strain. A 2.5-kb DNA fragment carrying 446-bp upstream of *gerAA*, the *gerAA* ORF and 213-bp downstream was PCR amplified from F4969 DNA with Phusion™ High-Fidelity DNA Polymerase using primers CPP1028/CPP1029 (forward and reverse primers had KpnI and SalI sites at their 5' ends, respectively) (Table 4.5). The KpnI-SalI PCR fragment was isolated,

digested with KpnI and SalI and cloned between KpnI and SalI sites of pJIR751 (137) giving plasmid pSB23. This plasmid was introduced into *C. perfringens gerAA* strain SB103 by electroporation, and Em^r transformants were selected. The presence of both plasmid pSB23 and the original *gerAA* deletion in strain SB103(pSB23) were confirmed by PCR using primers CPP211 and CPP206 (Table 4.5).

Construction of a *gerKC* complemented with wild-type *gerKA-KC*. The recombinant plasmid pSB18 (155), which is a derivative of pJIR750 harboring wild-type *gerKA-KC*, was introduced into *C. perfringens* strain SB106 by electroporation (19), and Cm^r Em^r transformants were selected. The presence of wild-type *gerKA-KC* and the *gerKC* mutation in strain SB106 (pSB18) was confirmed by PCR.

Analysis of *gerKA* gene expression by reverse transcription PCR (RT-PCR). *C. perfringens* wild-type F4969 and *gerKA* mutant SB110 were grown in either TGY medium (vegetative cells), or DS medium (sporulating cells) for 8 h at 37 °C, and total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). The primer pair CPP1259 and CPP1260 (Table 4.5), which amplified a 210-bp internal fragment from *gerKC*, was used to detect *gerKC*-specific mRNA from the RNA preparation by RT-PCR analysis as described previously (56, 167). PCR amplification was performed as follow: an initial denaturation step of 3 min at 98°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C, and finishing

with one cycle of 2 min at 72°C. The primers for 16S rRNA PCR are listed in Table S1. To quantify *gerKC* mRNA levels in the *gerKA* mutant SB110, the *gerKC*-specific band intensities were measured using ImageJ software (NIH) and results were standardized using the 16S rRNA band densities.

DPA release. DPA release during AK-triggered spore germination was measured by incubating heat activated spores (OD_{600} of 1.5) at 37°C with 100 mM AK pH 7.0, to allow adequate measurement of DPA release. Aliquots (1 ml) of germinating cultures were centrifuged for 3 min in a microcentrifuge and the amount of DPA in the supernatant fluid was determined by measuring the OD_{270} as previously described (140). Initial DPA levels in dormant spores were measured by boiling 1 ml aliquots for 60 min, centrifugation, and the DPA content of the supernatant fluid was measured by its OD_{270} (140).

Spore decoating. Spores at an OD_{600} of 20 were decoated in 1 ml of decoating solution (50 mM Tris-HCl (pH 8.0)–8 M urea–1% (wt/vol) sodium dodecyl sulfate–50 mM dithiothreitol) for 90 min at 37°C, and then spores were washed 10 times with distilled water (128). This extraction procedure did not kill the spores, as determined by plating on BHI agar supplemented with lysozyme (1 μg/ml).

Colony formation assay. To evaluate the colony-forming ability of spores of various F4969 strains, spore suspensions at an OD_{600} of 1 (~ 10^8 spores/ml) were heat activated at 75 °C for 15 min, aliquots of various dilutions were plated onto BHI agar +/- lysozyme (1 μg/ml), incubated at 37°C anaerobically for 24 h, and colonies were counted.

Statistical analyses. The Student's *t* test was used for specific comparisons.

4.4 Results

AK-triggered germination of *C. perfringens* spores of FP strain SM101 and NFB strain F4969. Previous studies have shown that spores of *C. perfringens* FP isolates germinate well with either L-asn or KCl, while spores of *C. perfringens* NFB isolates germinate only with the AK mixture (168). This was also the case when germination of spores of FP strain SM101 and NFB strain F4969 were compared (Fig. 4.1 A-C). Plotting the maximum germination rate versus germinant concentration suggested that germination kinetics of F4969 spores with either L-asn or KCl were strongly influenced by the presence of the other component of the AK mixture, indicating that both germinants might be acting in a cooperative manner at different ligand binding sites in the same or different GRs (Fig. 4.2 A,B). In contrast, germination kinetics of SM101 spores suggests that both components of the AK mixture are capable of triggering spore germination through independent pathways (Fig. 4.2 C,D). To analyze spore germination quantitatively, Michaelis-Menten kinetic analysis was applied with fixed concentrations of L-asn and increasing concentrations of KCl and vice versa, to obtain apparent values of maximum germination rates (V_{max}) and germinant binding affinities (K_m) (Fig. 4.2 A-D). Strikingly, germination of F4969 spores with L-asn was strongly dependent on the co-germinant KCl and vice versa. Thus, when the KCl concentration was increased from 0 to 100 mM, F4969 spores had an ~ 40-fold increase in V_{max} and ~ 1000-fold decrease in apparent K_m for L-asn, while when L-asn concentration was increased similarly, V_{max} increased ~ 150-fold and the apparent K_m for KCl decreased ~ 1300-fold (Table 4.2). These results suggest

that GRs' L-asn and KCl binding sites must be saturated to trigger rapid germination of F4969 spores. In contrast, with SM101 spores, the V_{max} values in the presence of either L-asn or KCl were quite high and only increased 1.5- to 3-fold with high levels of both germinants. KCl also only decreased the apparent Km for L-asn ~ 5-fold, although L-asn did decrease the apparent Km for KCl significantly. Collectively, these results suggest that *C. perfringens* GR(s) that respond to AK possess binding sites for both L-asn and KCl, and that these binding sites must cooperate strongly to trigger spore germination in FB4969 spores, while this cooperation is much less strong with SM101 spores.

Comparison of germination of FP and NFB spores in rich media. Previous work also suggested that there are differences in germination of F4969 and SM101 spores in the rich bacterial growth medium BHI (169) and DMEM and EMEM tissue culture media (163). In those studies, germination was quantified by determining loss of spore heat resistance, as heat treatment kills germinated/outgrowing spores, and thus loss of colony forming ability on BHI agar serves as an indication of the relative amounts of spore germination. To validate these results, we first examined germination of F4969 and SM101 spores in DMEM and EMEM by measuring decreases in OD_{600}. With F4969 spores incubated in EMEM or DMEM, a ~50-60% decrease in OD_{600} was observed after 1 h , and ~ >95% of the spores became dark under phase-contrast microscopy, indicating complete germination of these spores

(Fig. 4.3A, and data not shown). In contrast, no OD_{600} decrease was observed with SM101 spores incubated in EMEM or DMEM for 1 h, and >95% of these spores remained phase bright, indicating no significant germination of SM101 spores (Fig. 4.3B, and data not shown). Consistent with these results, spores of two additional *C. perfringens* FP strains (NCTC10239 and E13) also exhibited no significant decrease in OD_{600} after incubation with EMEM or DMEME for 60 min, while spores of two additional NFB strains (NB16 and B40) did (Table 4.3). Collectively, these results further indicate that tissue culture media induces germination of spores of NFB but not of FP isolates (163).

When FP strain SM101 and NFB strain F4969 spores were incubated with the rich bacterial growth medium TGY (pH 6.8) and germination was assessed my measuring OD_{600}, both SM101 and F4969 spores germinated very well (Fig. 4.3 A,B). Similar results were obtained with spores of two additional FP and NFB strains (Table 4.3). However, some of the germination effect of TGY medium appeared to be due to the L-cys (5.7 mM) present, as L-cys (100 mM, pH 6.0) induced significant germination of spores of F4969 (Fig. 4.3A) and other FP and NFB isolates (103). Interestingly, while spores of all tested FP strains germinated significantly ($P < 0.005$) with BHI, spores of NFB strains germinated only slightly after incubation with BHI for 1 h (Fig. 4.3A,B; Table 4.3). F4969 spores incubated with BHI for longer times exhibited significant ($P < 0.05$) germination, but the rate and extent of germination was lower than that of SM101 spores (Fig. 4.3C). Phase contrast microscopy confirmed the slower germination of F4969 spores compared to that of

SM101 spores; while ~60% spores of SM101 became phase dark after incubation in BHI for 1 h, ~56% of F4969 spores became phase dark only after 18 h (Fig 4.3D).

The F4969 spores germinated very poorly with BHI supplemented with 100 mM L-cys (Fig. 4.3A), suggesting that BHI inhibits germination of F4969 spores. Significant inhibition by EMEM or DMEM on BHI germination of SM101 spores was also observed (Fig. 4.3B). Collectively, these results suggest that there is a significant difference in the germination response between *C. perfringens* FP and NFB spores.

GR homologues in *C. perfringens* NFB strain F4969. Having found a significant difference in the germinant requirements for spores of *C. perfringens* FP and NFB isolates, we hypothesized that this diversity might be due to differences in GRs in the FP and NFB strains. To test this hypothesis, we subjected the draft assembled genome sequence of *C. perfringens* NFB strain F4969 to BLASTP analyses to identify GR homologues using *C. perfringens* SM101 GR proteins as baits. Four ORFs (AC5_0662, AC5_0663, AC5_0664, and AC5_1261) encoding proteins with high similarity (> 98%) to GR proteins of *C. perfringens* FP strain SM101 were identified (Fig. 4.4, Fig. 4.8). As in SM101, the F4969 genome encoded a *gerK* locus that contained a bicistronic operon with *gerKA* and *gerKC*, which is flanked by a monocistronic *gerKB* transcribed in the opposite orientation and upstream of the *gerK* operon. Interestingly, the monocistronic *gerAA* gene was found in the opposite orientation relative to *gerAA* gene in SM101. BLASTP analyses revealed that the

GerKC protein sequence was identical in SM101 and F4969, and there was tremendous similarity between the predicted amino acid sequences of GerAA, GerKA and GerKB, although also some differences (Fig. 4.8). Thus GRs could well differ somewhat in their function in F4969 and SM101 spores.

Introducing mutations in GR genes in NFB strain F4969. Previous studies (87, 110, 155) with *C. perfringens* strain SM101 demonstrated that GerKC is the main GR protein for spore germination in *C. perfringens,* while GerKA, GerKB, and GerAA have minor roles. To determine the role of GR proteins in germination of NFB strains of *C. perfringens*, we constructed FB4969 mutants lacking *gerKA* plus *KC* and single *gerKA, gerKB, gerKC* or *gerAA* mutations (Fig. 4.4A) by using mutator plasmids that were used previously to construct these mutants in SM101 (87, 155). All mutant strains grew well in TGY (vegetative growth) and DS (sporulation) medium, and phase-contrast microscopy of DS cultures demonstrated the formation of spores by all mutants except the *gerKB* strain, for reasons that are unknown. Thus, we excluded the *gerKB* mutant strain from our study.

To determine whether insertion of an intron in *gerKA* exerted a polar effect on the downstream *gerKC*, the levels of *gerKC* transcripts in sporulating cells of strains F4969 and its *gerKA* mutant SB110 were compared (Fig. 4.4B). The ~210 bp *gerKC* RT-PCR product was detected in RNA extracted from sporulating F4969 and SB110 cells (Fig. 4.4B). In addition, comparison of levels of *gerKC* transcripts in F4969 and SB110 by semi-quantitative RT-PCR found that the *gerKA* mutant had ~80% of the

wild-type level of the *gerKC* transcript (Fig. 4.4C). Thus the intron-inserted *gerKA* mutation in SB110 had only a minor effect, if any, on the expression of downstream *gerKC* and thus strain SB101 is essentially a *gerKA* single mutant.

Effect of *ger* mutations on germination of F4969 spores in rich media. Not only did wild-type F4969 spores germinate well with TGY, EMEM or DMEM media, but *gerKA* F4969 spores did also (Fig. 4.5). However, germination of *gerKA-KC*, *gerKC* and *gerAA* spores with these media was significantly slower or and/or less complete. Phase contrast microscopy confirmed these latter differences, in particular that after incubation of F4969 (wild-type) or SB104 (*gerKA-KC*) spores in TGY medium for 60 min, >95% of F4969 and at most 5% of SB104 *s*pores became phase dark (data not shown). Interestingly, almost wild-type germination was observed with *gerKC* spores complemented with wild-type *gerKA-KC*, indicating that GerKC is essential for normal germination of spores of F4969 in rich media (Fig. 4.5A-C). However, complementation of *gerAA* mutant spores with wild-type *gerAA* induced slight germination with only EMEM (Fig. 4.5B), suggesting an auxiliary role for GerAA in EMEM-induced germination (see Discussion).

GerKC and GerAA are required for normal germination of F4969 spores with defined germinants. To further define the role of specific GRs in F4969 spore germination, the germination of spores of the wild-type and GR mutant strains with

AK and L-cys were compared. The *gerKA-KC* spores did not germinate with AK (Fig. 4.6A), and germinated very poorly ($P< 0.05$) compared to F4969 spores with L-cys (Fig. 4.6B). Although *gerKA* spores exhibited significant germination in the presence of AK or L-cys (Fig. 4.6 A, B), *gerKC* spores did not (Fig. 4.6 A, B). However, the germination defect in *gerKC* spores could be restored to a nearly wild-type level by complementing the *gerKC* spores with wild-type *gerKA-KC*, indicating that GerKC, but not GerKA, is the most important GR protein in *C. perfringens* F4969 spore germination.

In contrast to results with SM101 spores (87), spores of a *gerAA* mutant of NFB strain F4969 exhibited almost no germination with AK (Fig. 4.6A), and significantly poorer germination ($P< 0.05$) than spores of the wild-type strain with L-cys (Fig. 4.6B). The germination defect of the *gerAA* spores was partially restored by complementation of *gerAA* spores with a plasmid carrying wild-type *gerAA* (Fig. 4.6), indicating that GerAA plays a role in normal germination of F4969 spores in the presence of AK and L-cys.

GerKC and GerAA are required for DPA release during AK germination of F4969 spores. After the germinant binds to its cognate receptor, the next easily measurable event in spore germination is the release of the spore core's large depot of Ca-DPA (88). Therefore, release of DPA during germination of wild-type, *gerKA-KC*, *gerAA*, and *gerKC* spores with AK was assayed. As expected, wild-type spores

released the majority (~80%) of their DPA within the first 10 min of germination with AK (Fig. 4.7). Although *gerKA* spores released less DPA than wild-type spores after 10 min of germination with AK, no significant difference in DPA release was observed between *gerKA* and wild-type spores after 60 (Fig. 4.7). However, *gerAA*, *gerKA-KC* and *gerKC* spores released 70%-80% less DPA ($P < 0.05$) than wild-type spores during AK germination (Fig. 4.7), consistent with almost no germination of these spores with AK (Fig. 4.6A). Importantly, the low DPA release by *gerAA* and *gerKC* spores was restored to nearly wild-type levels by complementation with wild-type genes. Collectively, these results further suggest that GerKC and GerAA are the main GR proteins involved in F4969 spore germination with AK.

GerKC is the major GR protein required for colony formation by F4969 spores on BHI agar. Since GR mutants generally exhibited less germination than wild-type spores, these mutant spores could have lower colony-forming efficiencies than wild-type spores. To evaluate this, the colony-forming efficiencies of intact and decoated wild-type and GR mutant spores were assessed by plating onto BHI agar with or without lysozyme. No significant differences in colony-forming efficiencies on BHI agar were observed between wild-type and *gerKA* spores, while *gerKA-KC* spores exhibited one-log lower colony-forming efficiency than wild-type spores (Table 4.4). The lower colony-forming efficiency of *gerKA-KC* spores was due to specific inactivation of *gerKC* as: i) intact *gerKC* spores also had a one-log lower spore titer

than wild-type spores; and ii) the colony-forming efficiency of *gerKC* spores could be restored to wild-type level by complementation with a plasmid carrying wild-type *gerKA-KC*. Surprisingly, although F4969 *gerAA* spores exhibited significantly lower germination than wild-type spores (Fig. 4.6), intact *gerAA* spores had a colony-forming efficiency on BHI agar similar to that of wild-type spores, suggesting that GerAA is not absolutely required for colony formation by F4969 spores (Table 4.4). Decoating of *gerKA-KC* and *gerKC* spores and plating onto BHI agar supplemented with lysozyme, which can recover spores with germination defects, increased the colony-forming efficiencies of these mutant spores to a level similar to that of wild-type spores (Table 4.4), indicating that *gerKA-KC* and *gerKC* spores are fully viable and simply unable to form colonies because of a defect in completion of germination. Collectively, these results indicate that GerKC is the main GR protein required for normal colony formation by F4969 spores on BHI agar, and that GerAA, although required for normal germination of F4969 spores, does not play any significant role in colony formation.

4.5 Discussion

Several previous studies have found differences between the germinant responses of *C. perfringens* FP and NFB spores (87, 104), suggesting that the GRs of *C. perfringens* spores of these two types of isolates have adapted to different environmental niches. Indeed, the previous work supported this hypothesis by demonstrating that spores of *C. perfringens* FP isolates germinate better with nutrients typically found in meat products (87, 104), while NFB spores germinate well in the presence of intestinal epithelial cells (163). The current work provides further evidence of different germination responses between FP versus NFB spores as follows. 1) The AK-germination pathway is mechanistically different between both types of isolates. In NFB F4969 spores, binding of either L-asn or KCl requires saturation of the other ligand binding site to trigger germination, while either germinant can trigger germination of FP SM101 spores independently, although there is some cooperativity between them. 2) NFB spores germinated more poorly than FP spores in BHI, although did eventually germinate (155) (Table 4.3). One possible reason for the slow germination of NFB spores in BHI medium is that this medium contains some components that inhibited NFB spore germination. This idea was supported in the current work, as spores of NFB strain F4969 germinated poorly in a 1:1 mixture of BHI and L-cys, although germinated well with L-cys alone. However, the identities of inhibitory compounds in BHI medium are not clear. 3) *C. perfringens* NFB spores but not FP spores germinated well with several tissue culture media. The poor germination of FP spores in EMEM or DMEM is intriguing, as these

media contain amino acids that are sufficient to induce germination of *C. perfringens* spores (87, 163). Thus it is indeed possible that some ingredients in rich media might compete with normal germinants in binding to GRs, bind non-productively, or block spore germination in some other way.

The different germination of FP and NFB spores was striking given the fact that both isolates possess nearly identical GR coding genes. This suggests that the few changes in GR sequences between these two strains has drastic effects on GR function. While the products of *gerKA-KC* were required for spore germination in FP strain SM101 (87), recent findings demonstrated that GerKC alone is sufficient to allow GR functions (155). Consistent with previous results with FP strain SM101 (87), the products of *gerKA-KC* were also required for normal germination of NFB strain F4969 spores. However, the inactivation of *gerKC* only in strain F4969 demonstrated that most, if not all, of the germination defect in *gerKA-KC* spores was due to the loss of *gerKC*, suggesting that GerKC is the main GR protein in F4969 spores. This suggestion was further supported in that: 1) *gerKA* spores germinated like wild-type spores with AK or L-cys; and 2) the germination defect in *gerKC* spores was complemented by wild-type *gerKC*. Overall, when our current results are coupled with those obtained in previous work (103, 155), they indicate that GerKC is the main GR protein involved in germination of spores of *C. perfringens*, regardless of the type of strain, either FP or NFB, and the germinant(s) used.

Another striking finding from this study is that in contrast to the results with FP strain SM101 (87), GerAA is required for normal germination of NFB strain F4969 spores with AK or L-cys, presumably by acting as a receptor for both of these germinants. Since disruption of *gerAA* in F4969 significantly affected AK- and L-cys-induced germination and AK-induced DPA release, and some of these defects could be restored at least partially by complementation with wild-type *gerAA*, it appears that GerAA is required for NFB spore germination. The different germination of F4969 *gerAA* and SM101 *gerAA* spores (87) cannot be due to differences in the *gerAA* mutants, as both the SM101 and FB4969 *gerAA* mutants each had the same intron inserted between 123/124 of *gerAA* (87). However, this difference in phenotypic behavior of the *gerAA* mutation in the NFB and FP strains could be due to differences in the aa sequences of GerAA in FP versus NFB isolates (Fig. S1). Indeed, single alterations in any of three aa in either the GerBA (2) or GerBB (1) proteins changed *B. subtilis* spore germination with the L-asn – D-glucose – D-fructose – KCl mixture (AGFK) from absolutely requiring both the GerB and GerK GRs, to allowing good germination with L-asn alone (170). However, the L-asn germination of the spores of the various *gerBA* or *gerBB* mutants was still stimulated significantly by the GFK mixture. This change in *B. subtilis* spore germination phenotype is similar in many respects to the difference in the AK germination phenotype of FP and NFB *C. perfringens* spores with AK. Since GerKA and GerAA aa sequences in FP strain SM101 and NFB strain F4969 do show a few aa differences, despite being >98% identical, it is tempting to speculate that one or more

of these differences, in particular in GerAA, is responsible for the different germination behavior of FP and NFB spores. Structure-function analyses of SM101 and F4969 GR proteins, and comparison with the *B. subtilis* GerBA and GerBB proteins could help illuminate the possible effects of mutations in various regions of these proteins.

Another striking finding in this work is that while GerKC was required for full apparent viability of F4969 spores, GerAA was not. While *gerAA* spores germinated significantly slower than wild-type spores, the colony-forming efficiency of *gerAA* spores was similar to that of wild-type spores. This role of F4969 GerAA is consistent with results with *B. subtilis* spores, where the viability of spores lacking one GR is relatively normal, while spores lacking all GRs exhibit low viability (97). Therefore, seems likely that normal apparent viability of *C. perfringens* F4969 *gerAA* spores is due primarily to GerKC, as this GR protein is required for spore viability (this study). However, contributions of GerKB and other germination proteins with significantly different sequences from those of the GerA of GRs family cannot be excluded. Further experiments with spores lacking both GerAA and GerKB may help clarifying reasons for the low viability of *gerKC* spores.

In conclusion, notable findings in the current study are as follows: (i) germination of FP and NFB spores differs significantly in rich media and several defined germinants; (ii) L-asn and KCl induced germination in NFP F4969 spores cooperatively, while either germinant alone triggers germination of FP spores; (iii)

GerKC and GerAA proteins are required for normal germination of NFB spores with AK and L-cys; (iv) although GerKCs play a major role in DPA release with AK and in spores' viability, GerAA is not required for spore viability. Collectively, these results provide new insight into the determinants of the efficiency of spore germination in *C. perfringens*.

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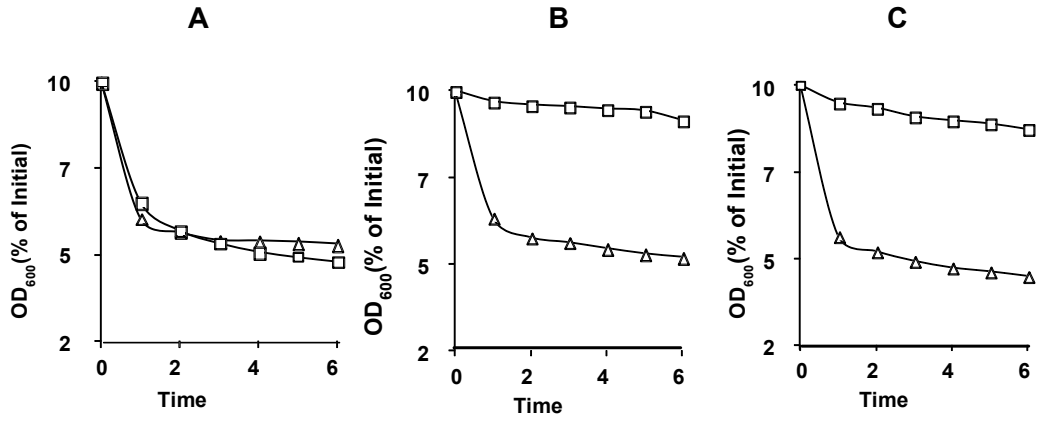


Fig. 4.1 A-C. Germination of *C. perfringens* F4969 and SM101 spores with various germinants. Heat activated spores of strains F4969 (□) and SM101 (Δ) were germinated with: A) 100 mM AK (100 mM L-asn and 100 mM KCl) in 25 mM Tris-HCl; B) 100 mM KCl in 25 mM Tris-HCl; C) 100 mM L-asn in 25 mM Tris-HCl. Germination was followed by measuring the decrease in cultures' OD_{600} as described in Material and Methods.

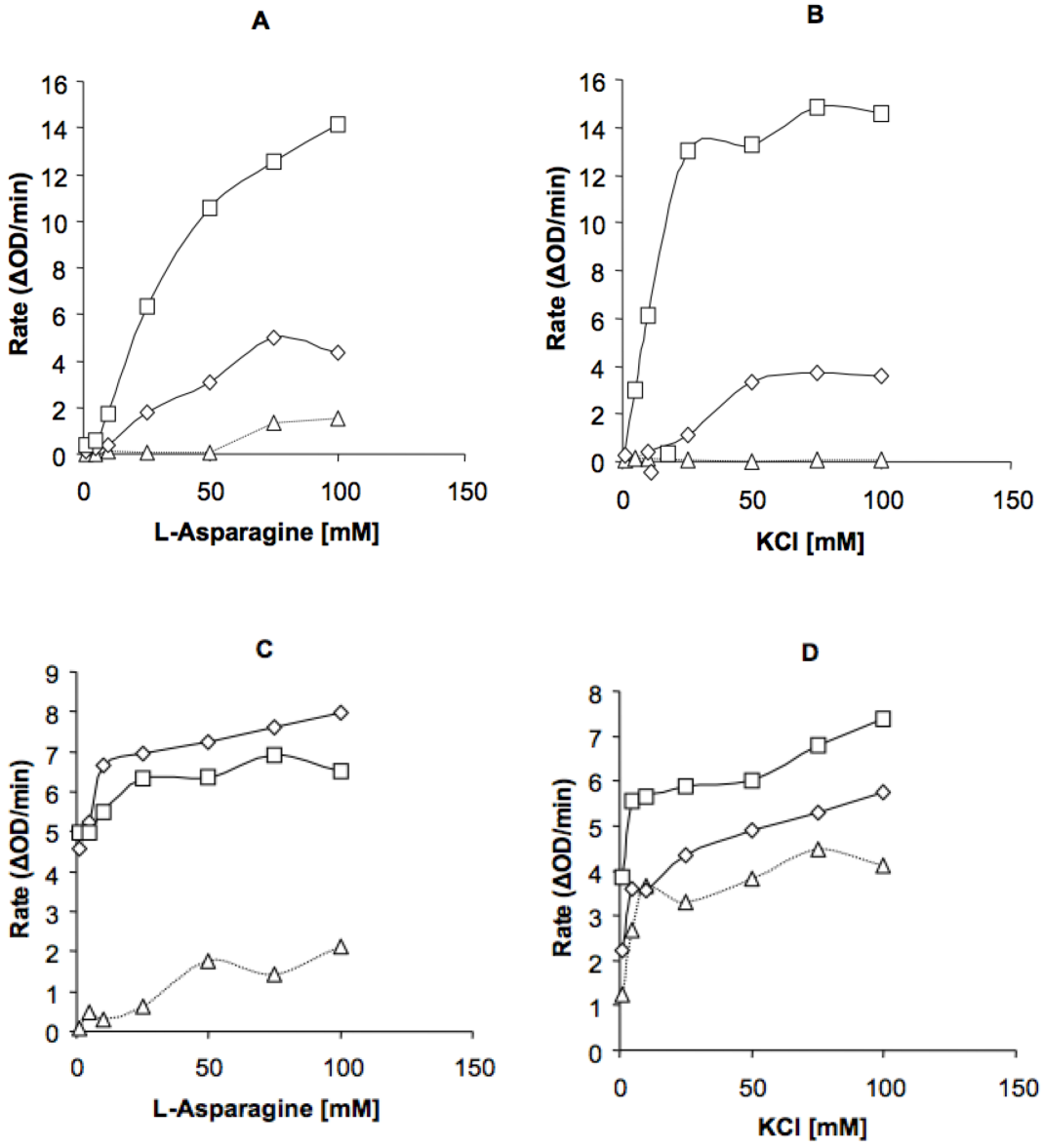
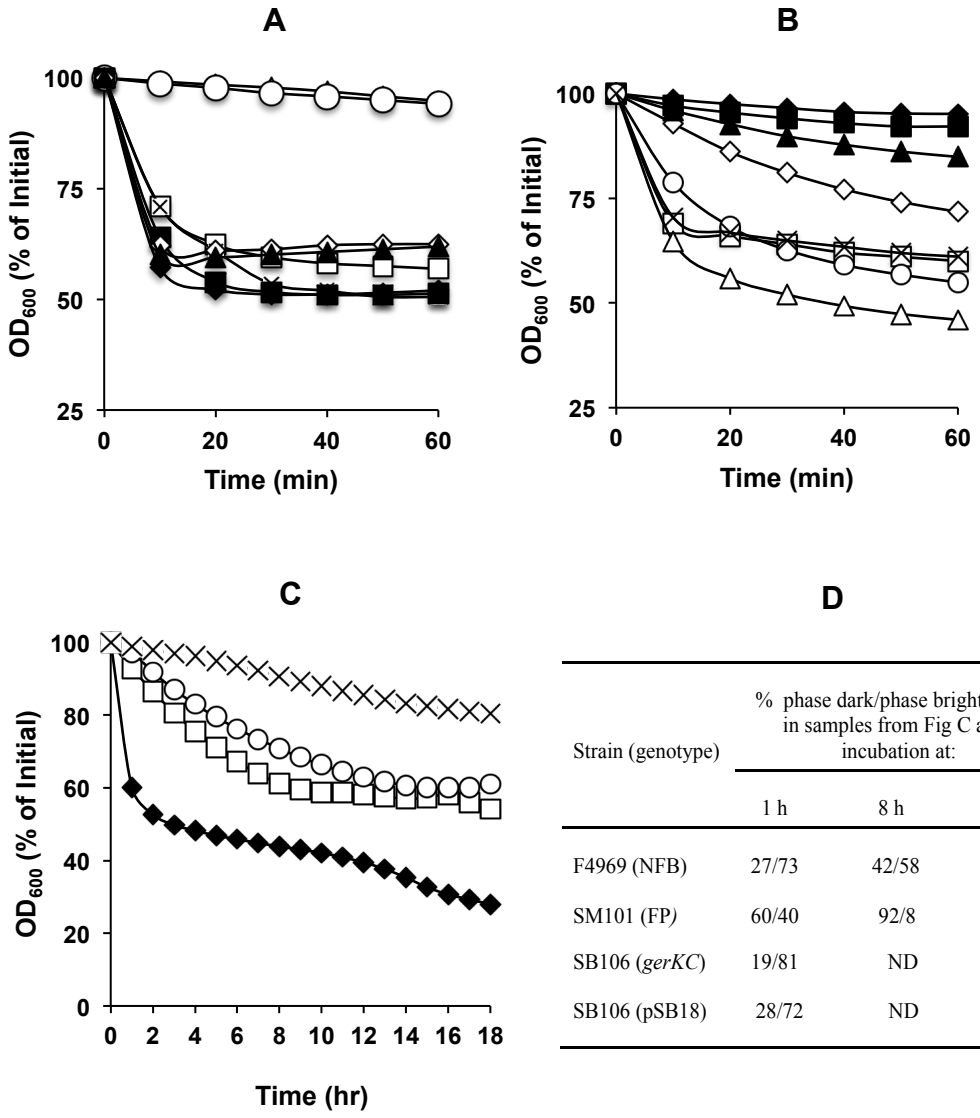


Fig. 4.2 A-D. Germination kinetics of *C. perfringens* NFB F4969 and FP SM101 spores with AK. A, C) Heat-activated spores of strains F4969 (A,B) and SM101 (C,D) were germinated with various concentrations of (A,C) L-asn supplemented with 0 (Δ), 50 (◊) and 100 (□) mM KCl, or (B,D) germinated with various KCl concentrations supplemented with 0 (Δ), 50 (◊) and 100 (□) mM L-asn. The linear portion of germination curves was used to determine maximum rates of decrease in OD_{600} and these values were plotted versus L-asn (A, C) or KCl (B, D) concentrations. Data are the means of two replicates and standard errors of the mean were less than 25% of the mean.



D

Strain (genotype)	% phase dark/phase bright spores in samples from Fig C after incubation at:		
	1 h	8 h	18 h
F4969 (NFB)	27/73	42/58	56/44
SM101 (FP*)*	60/40	92/8	97/3
SB106 (*gerKC*)	19/81	ND	37/63
SB106 (pSB18)	28/72	ND	67/33

Fig. 4.3 A-C. Germination of *C. perfringens* spores in various rich media. A,B) Heat-activated spores of strains F4969 (A) and SM101 (B) were incubated at 37°C with BHI (Δ), TGY (□), EMEM (■), DMEM (◆), BHI supplemented with EMEM (◊), BHI supplemented with DMEM (▲), BHI supplemented with L-cys (○), and 100 mM L-cys, pH 6.0 (×) and germination was monitored by measuring OD_{600} as described in Material and Methods. C) Spores of SM101(◆), F4969 (□), SB106 (*gerKC*)(✕), and SB106(pSB18) (*gerKC* mutant complemented with wild type *gerKA-KC*) (○) were incubated in BHI broth at 37°, and OD_{600} values were measured at 1 h intervals. D) Phase-contrast microscopic counting (%) of phase dark versus phase bright spores in samples from panel C after incubation for 1, 8 and 18 h. ND, not determined.

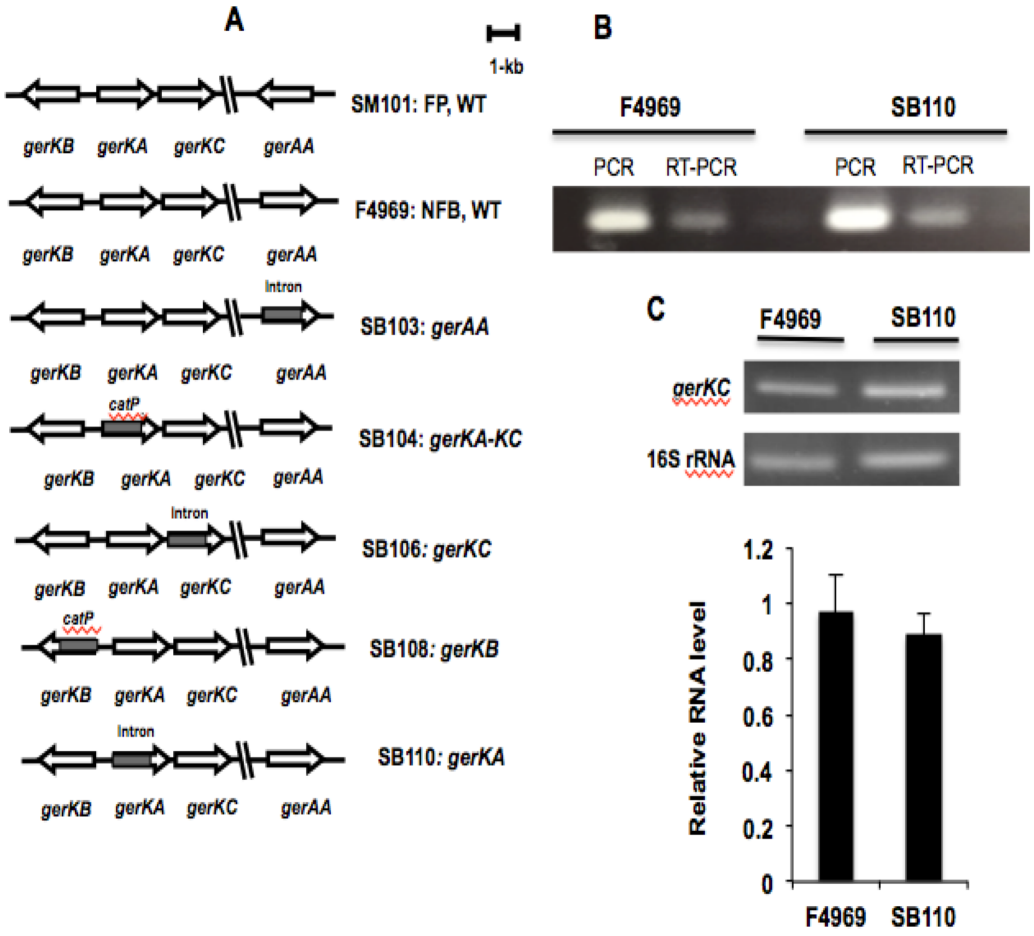


Fig. 4.4 A-C. Genetic organization of GR genes and expression of *gerKC* in wild-type and mutant strains. A) Genetic organization of the *ger* locus of *C. perfringens* SM101 (FP isolate), F4969 (NFB isolate) and F4969 GR mutant derivatives. B) Expression of *gerKC* in strain F4969 and *gerKA* mutant strain SB110. RNAs isolated from sporulating cultures of strains F4969 and SB110 were subjected to RT-PCR using *gerKC*-specific internal primers. Lanes labeled RT-PCR indicate RT-PCR products amplified from RNAs, and lanes labeled PCR indicate PCR products amplified from DNA. The PCR- and RT-PCR-amplified products were analyzed by agarose (1%) gel electrophoresis and photographed under UV light. No PCR amplified product was obtained from RNAs of both strains in the absence of reverse transcriptase (data not shown), indicating that RNAs used were DNA-free. C) RT-PCR products were separated by electrophoresis on a 2% agarose gel and band intensities were compared to those of RT-PCR amplified 16S rRNA by ImageJ software to give a ratio of *gerKC*

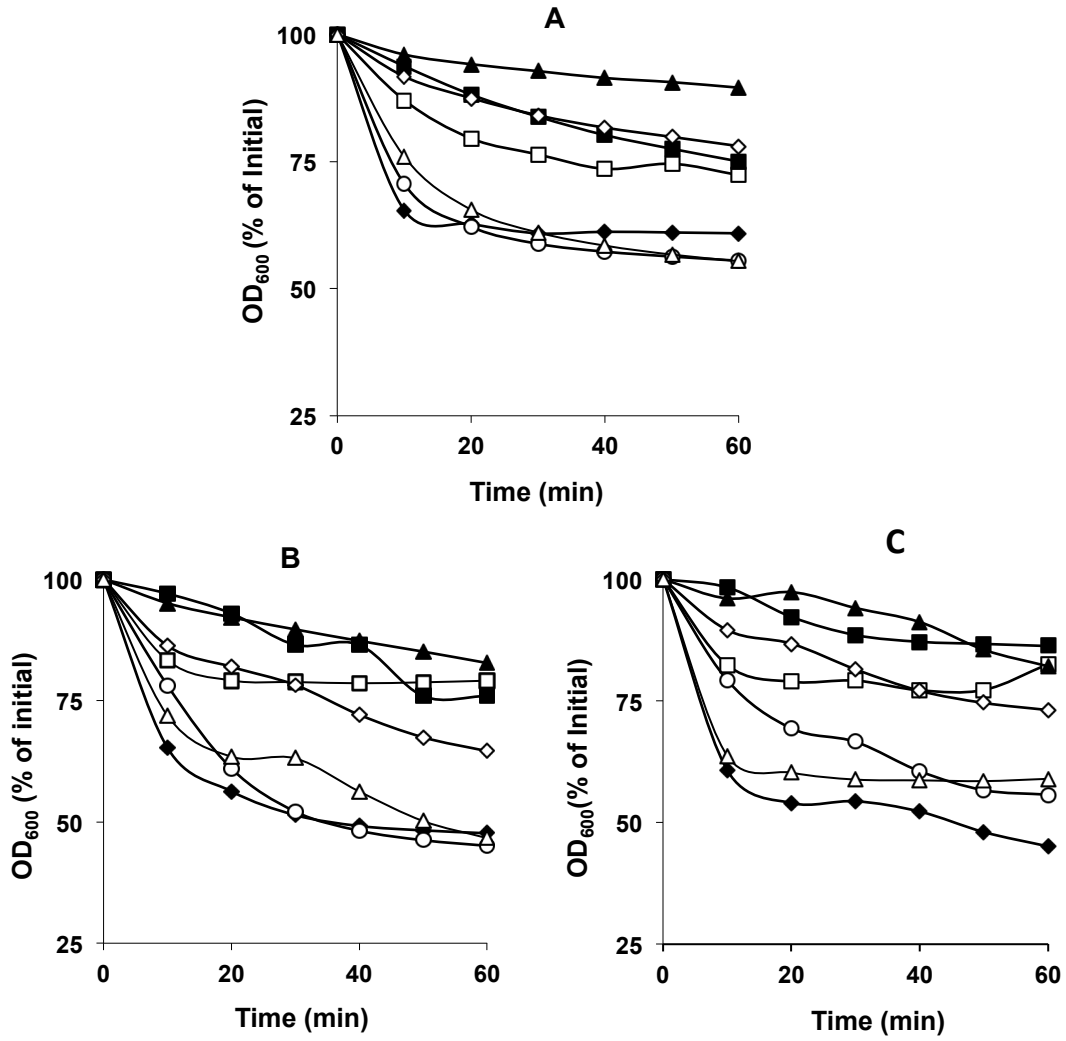


Fig. 4.5 A-C. Germination of *C. perfringens* F4969 spores and its GR mutant derivatives. Heat-activated spores of strains F4969 (wild-type) (♦), SB103 (*gerAA*) (□), SB104 (*gerKA-KC*) (▲), SB106 (*gerKC*) (■), SB110 *(gerKA*) (Δ), SB103(pSB23) (*gerAA* mutant complemented with wild-type *gerAA*) (◊), and SB106(pSB18) (*gerKC* mutant complemented with wild type *gerKA-KC*) (o) were incubated at 37°C with: A) TGY medium; B) EMEM medium; and C) DMEM medium, and spore germination was monitored by measuring OD_{600} as described in Methods.

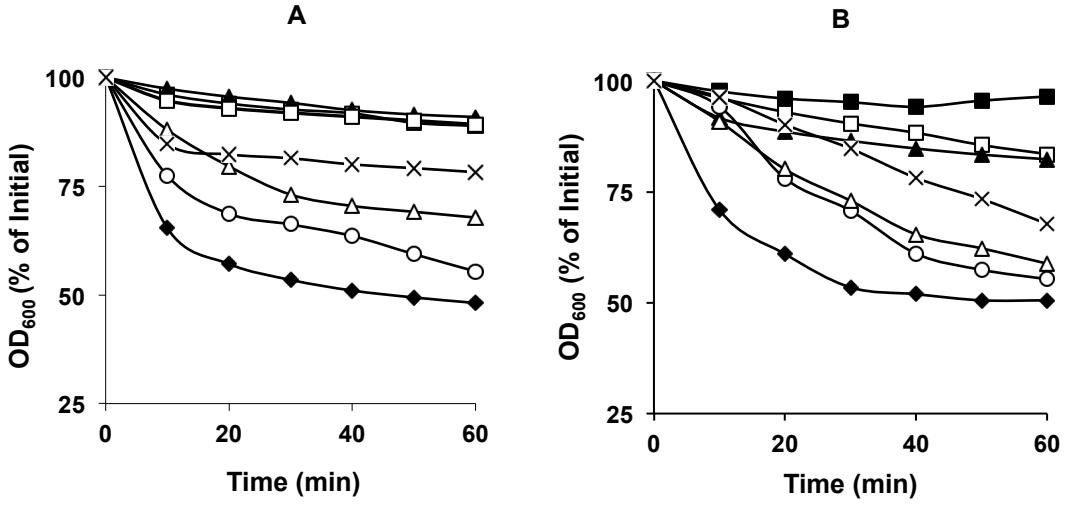


Fig. 4.6 A-B. Germination of *C. perfringens* spores of strain F4969 and its GR mutant derivatives with AK (A) and L-cys (B). Heat activated spores of strains F4969 (wild-type) (♦), SB103 (*gerAA*) (□), SB104 (*gerKA-KC*) (▲), SB106 (*gerKC*) (■), SB110 (*gerKA*) (Δ), SB103(pSB23) (*gerAA* mutant complemented with wild type *gerAA*) (×), and SB106(pSB18) (*gerKC* mutant complemented with wild type *gerKA-KC*) (o) were incubated at 37°C with: A) 100 mM AK (100 mM L-asn plus 100 mM KCl, in 25 mM Tris-HCl buffer); B) 100 mM L-cys in 25 mM Tris-HCl buffer), and germination was monitored by measuring OD_{600} min as described in Methods. Spores of various *C. perfringens* strains incubated in 25 mM Tris-HCl buffer (pH 7.0) at 37°C exhibited less than a 10 % decrease in OD_{600} in 60 min (data not shown). Note that, in panel A data denoted by the symbols □,▲ and ■ overlapped.

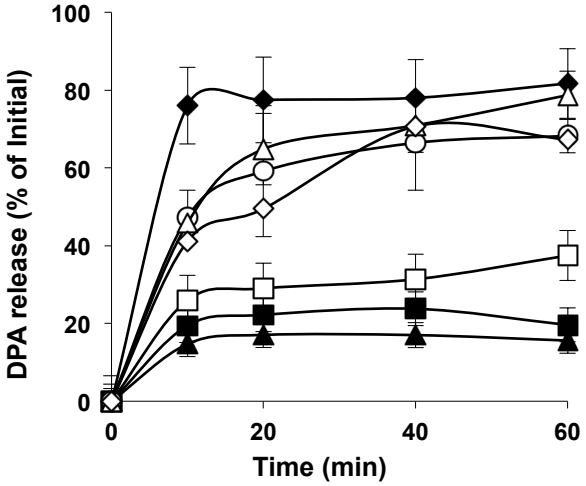


Fig. 4.7 DPA release during AK-triggered germination of spores of F4969 and its mutant strains. Heat-activated spores of F4969 (wild-type) (♦), SB103 (*gerAA*) (□), SB104 (*gerKA-KC*) (▲), SB106 (*gerKC*) (■), SB110 (*gerKA*) (Δ), SB103(pSB23) (*gerAA* mutant complemented with wild-type *gerAA*) (◊), and SB106(pSB18) (*gerKC* mutant complemented with wild type *gerKA-KC*) (o), were incubated with 100 mM AK at 37°C in 25 mM Tris-HCl (pH 7.0) and DPA release was monitored by measuring OD_{270} as described in Methods. Error bars represent standard deviations.

A) GerKA

```
SM101      MEKQLDFLKDKLKDSFDVKYREVDTALGPATIVFMDVLCSTQFISEYIVKPLTLVKDGVK
F4969      MQKQLDFLKDKLKDSFDVKYREVDTALGPATIVFMDVLCSTQFISEYIVKPLTLVKDGIK
           *:************************************************************:*

SM101      DENDIMTKVIDINITNWSKDKNDTLLHVLSGDVVIIFDKFDKVIYCETKGYTRRGVGIPI
F4969      DENDIMTKVIDINITNWSKDKNDTLLHVLSGDVVIIFDKFDKVIYCETKGYTRRGVGIPI
           ************************************************************

SM101      TEAVIKGPREGFNEAFVDNVTLLRRRIKNHNLKFEPLYVGEDTQTVVCISYIKNKAPKEL
F4969      TEAVIKGPREGFNEAFVDNVTLLRRRIKNHNLKFEPLYVGEDTQTVVCISYIKNKAPKEL
           ************************************************************

SM101      IDEIREKIKNLDYKFILDTNYIEAKLREDRTLFDTVGYTEKADEVAAKLLEGRVAIIVDG
F4969      IDEIREKIKNLDYKFILDTNYIEAKLREDRTLFDTVGYTEKADEVAAKLLEGRIAIIVDG
           ****************************************************:*******

SM101      TPFVLTVPFFFIENFQTPDDYYLNRYFTSFTRILRWIAFFIAMFLPGIYVALVTHHFSVL
F4969      TPFVLTVPFFFIENFQTPDDYYLNRYFTSFTRILRWIAFFIAMFLPGIYVALVTHHFSVL
           ************************************************************

SM101      PSLFVFRLAVARAGVPFTIVAEVIIMMLLFQIIKEAGLRLPQPIGTSMSLVSGLILGEAA
F4969      PSLFVFRLAVARAGVPFPIVAEVIIMMLLFQIIKEAGLRLPQPIGTSMSLVSGLILGEAA
           **************** ****************************************

SM101      VGAGIASRITIVVVALSAVCYFLIPKLYGAVSIWSIGIVILAALFGIPGVILISVVLLSH
F4969      VGAGIASRITIVVVALSAVCYFLIPKLYGAVSIWSIGIVITAAFFGIPGVILISVVLLSH
           ************************************ **:****************

SM101      LAHLRSCEYQYLFPLGTLSRYKFKDIIIRGRLEEISKDIVGKDGKNEPKESYS
F4969      LAHLRSCEYQYLFPLGTLNRYKFKDIIMRGRLDEISKDIVGKDGKNEPKESYS
           ******************.********:****:********************
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B) GerKC

```
SM101      MNLKKVIASLLSIIIMSTMLIGCYNYKDINRVTFVTSLIFDENEVGNIDIYLDCVKPYRS
F4969      MNLKKVIASLLSIIIMSTMLIGCYNYKDINRVTFVTSLIFDENEVGNIDIYLDCVKPYRS
           ************************************************************

SM101      SNESSDKGRRVLYKGTGKTVLEAIKDINMYSSSKLDFTQCKGYIFTEKAAKNGIRKYIDI
F4969      SNESSDKGRRVLYKGTGKTVLEAIKDINMYSSSKLDFTQCKGYIFTEKAAKNGIRKYIDI
           ************************************************************

SM101      INKNQEFMIRPYMFVLFGSPEELLNDVTVDEEYLGVFIDDLVQRMNKSPRVIAIDANDYL
F4969      INKNQEFMIRPYMFVLFGSPEELLNDVTVDEEYLGVFIDDLVQRMNKSPRVIAIDANDYL
           ************************************************************

SM101      ELRTNYGNLLVLGALSIRDDMEEKRLELSGGALLKNEVLVRRITAEEGMSYNLLMGDLKR
F4969      ELRTNYGNLLVLGALSIRDDMEEKRLELSGGALLKNEVLVRRITAEEGMSYNLLMGDLKR
           ************************************************************

SM101      GTLEVMNPQDPNTFITLDISNAKTKTSISYDGENITLYKDIKVKCLIGESQSRLIVDEKL
F4969      GTLEVMNPQDPNTFITLDISNAKTKTSISYDGENITLYKDIKVKCLIGESQSRLIVDEKL
           ************************************************************

SM101      LNLLELEEEAVIKQYLELFFESFKESDIDIVNVGNLFYRKYPDEVLEKDPISITNLKINV
F4969      LNLLELEEEAVIKQYLELFFESFKESDIDIVNVGNLFYRKYPDEVLEKDPISITNLKINV
           ************************************************************

SM101      DVEIDGTTITGNTL
F4969      DVEIDGTTITGNTL
           **************
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C) GerAA

```
SM101      MDLTSNFDKNINTLSKALRVGKSFDIIERSIIIGDKKATMYYIDGFVKDDVMELIMSDFF
F4969      MDLTSNFDKNINTLSKALRVGKSFDIIERSIIIGDKKATMYYIDGFVKDDVMELIMSDFF
           ************************************************************

SM101      SLSKTEMNLIKSPKDFMRRQIPYVEVAEEVSIDKIVSQILCGQTALILEGFSSAIMIDLR
F4969      SLSKTEMNLIKSPKDFMRRQIPYVEVAEEVSIDKIVSQVLCGQTALILEGFSSAIMIDLR
           ************************************:***********************

SM101      TYPVRGPQEPETEKVLRGSRDGFVETIVFNTALIRRRIRDPRLTFEMMSIGNVSKTDVVI
F4969      TYPVRGPQEPETEKVLRGSRDGFVETIVFNTALIRRRIRDPRLTFEMMSIGNVSKTDVVI
           ************************************************************

SM101      SFLDEVVDKKTLNLIKKKFENLDIQALTMSEQSLVESLSNASWYNPFPKVRYTERPDVAA
F4969      SFLDEVVDKKTLNLIKKKFENLDIQALTMSEQSLVESLSNASWYNPFPKVRYTERPDVAA
           ************************************************************

SM101      AHITEGKIVIIIDNSPSVIILPTTIFDFIQDVDDYYLPVITGNFIRLIRNFILISTIFIT
F4969      AHITEGKIVIIIDNSPSVIILPTTIFDFIQDVDDYYLPVITGNFIRLIRNFILISTIFIT
           ************************************************************

SM101      PLYLLLIQNAYRIPDYLKFLLPVDGYKVPLIVQFLLLEVAVDGLKLASLNTPNALGMSLS
F4969      PLYLLLIQNAYRIPDYLKFLLPVDGYKVPLIVQFLLLEVAVDGLKLASLNTPNALGMSLS
           ************************************************************

SM101      VIGGLILGQFAVDTGLFLPQTILYMAIVTLGSFSQPSLELSYALKFWRTLLLILTSLFNV
F4969      VIGGLILGQFAVDTGWFLPQTILYMAIVTLGSFSQPSLELSYALKFWRTLLLILTSLFNV
           ************** ************************************************

SM101      IGFFVGLIIGIVLIASNKTLTGDSYLYPLIPLDWKALKALIFRVRLVPKQKEE
F4969      IGFFVGLIIGIVLIASNKTLTGDSYLYPLIPLDWKALKALIFRVRLVPKQKEE
           ***************************************************
```

D) GerKB

```
SM101      MSLGKLNTRHFIFFIIAAISTSLVNYTSLFIKIGGRDTWIFTIISGIIFFFVTSFIFSVI
F4969      --MGKLTARHFIFFIIAAISTSLVNYTSLFIKIGGRDTWIFTIISGIIFFFVTSFIFSVI
             :***.:*****************************************************

SM101      SKIEYYDFKETCYLVLGKSLGNIYILIFSLTLLLMCIESASVSSSSINVNIFAESPIWYC
F4969      SKIEYYDFKETCYIVLGKPLGNIYILIFSLTLVLMCIESASVSSSSINVNIFAESPIWYC
           *************:**** *************:**************************

SM101      LLFFVITVFLIGKNRFNSILIICIVCISIVLAINLLLALLNIRYIDYTLLLPIFKDRRIS
F4969      LLFFIITVFLIGKNRFNSILIICIVCVSIVLAINLLLALLNIRYIDYTLLLPIFKDRRIS
           ****:*********************:**********************************

SM101      EYILCALTQLGSLSSLAIVLPILPRIDDKKNLKKISINTILLTSIFCTLCIVILISTLGS
F4969      EYILCALTQLGSLSSLAIVLPILPRIDDKKNLKKISITTILLTSIFCALCVVILISTLGS
           ************************************.*********:**:**********

SM101      LRSANVFYPQFIQNQRIYFGGFVENGNIFVMISSTLSWIVKYLITIFSLYTIWKDRVKYK
F4969      LRSANVFYPQFVQTQRIYFGGFVENGNVFVMISSTLSWVVKYLITIFSLYTIWKDRVKYK
           ************:*.*************:***********:**********************

SM101      RNFIALISVIVYVFSYLAAKNAYTLFILLRYYQYILLIVLFICPIIIYTLAYFKSHKFKY
F4969      RNFIALISVIVYAFSYLAAKNAYTLFILLRYYQYILLIVLFICPIIIYTLAYFKRQKFKY
           ************.****************************************** :****

SM101      LK
F4969      LK
           **
```

Fig.4.8 A-D. Alignment of aa sequences of germinant receptor (GR) proteins from *C. perfringens* strains SM101 and F4969. Asterisks below sequences indicate aa that are identical in SM101 and F4969. A colon indicates a difference between two aa of strongly similar properties, a period indicates a difference between aa of weakly similar properties and a gap indicates different aa with dissimilar properties. The deduced aa sequences of GR proteins from SM101 and F4969 strains are available on the National Center for Biotechnology Information (NCBI) website at (https://www.ncbi.nlm.nih.gov/genome/term=Clostridiumperfringens). Aa sequence alignment was performed using the ClustalW program and modified with Microsoft word.

TABLE 4.1. Bacterial strains and plasmid used

Strain or plasmid	Relevant characteristics	Source or reference
C. perfringens strains		
F4969	Non-Food-borne GI diseas isolate; carries *cpe* gene on plasmid	(59)
SM101	Electroporatable derivative of food poisoning type A isolate NCTC8798; carries a chromosomal *cpe* gene	(59, 147)
E13	Food poisoning type A isolate; carries chromosomal *cpe* gene	(14)
NCTC8239	Food poisoning type A isolate; carries chromosomal *cpe* gene	(14)
NCTC10239	Food poisoning type A isolate; carries chromosomal *cpe* gene	(14)
NB16	Non-food-borne GI disease isolate; carries *cpe* gene on plasmid	(14)
B40	Non-food-borne GI disease isolate; carries *cpe* gene on plasmid	(14)
SB103	*gerAA*::intron	This study
SB104	*gerKA-KC*::*cat*P	This study
SB106	*gerKC*::*ermB*	This study
SB108	*gerKB*::*cat*P	This study
SB110	*gerKA*::*ermB*	This study
SB103(pSB23)	*gerAA* mutant expressing wild-type *gerAA*	This study
SB104(pSB19)	*gerK* mutant expressing wild-type *gerK* operon	This study
SB106(pSB18)	*gerKC* mutant expressing wild-type *gerK* operon	This study
Plasmid		
pJIR750ai	*C. perfringens*/*E. coli* shuttle vector containing an *L1.LtrB* intron retargeted to the *plc* gene	(148)
pJIR3566	Clostridial targetron vector derived from pJIR750ai, contains *ermB*-RAM and *lacZ*α, Cm^r	(39)
pJIR751	*C. perfringens/E. coli* shuttle vector; Em^r.	(137)
pJIR750	*C. perfringens/E. coli* shuttle vector; Cm^r.	(137)
pDP10	~ 3.2- kb *gerKA-KC* operon in pMRS104	(87)
pDP11	~1.3-kb NaeI-SmaI *catP* fragment from pJIR418, in the SpeI site in the *gerKA* ORF in pDP10	(87)
pDP13	pJIR750ai with IBS, EBS1d, and EBS2 retargeted to insert in *gerAA*	(87)
pDP75	~ 4.8-kb *gerKB* fragment cloned between the KpnI and SalI sites of pMRS104	(110)
pDP276	~ 350-bp BsrGI-HinDIII fragment retargeted to *gerKC* cloned between BsrGI-HinDIII sites in pJIR3566	(155)
pDP300	~350-bp BsrGI-HinDIII fragment retargeted to *gerKA* cloned between BsrGI-HinDIII sites in pJIR3566	(155)
pSB18	~ 3.2-bp *gerKA-KC* operon cloned between KpnI-SalI sites of pJIR750	(155)
pSB19	~ 3.2-kb *gerKA-KC* operon fragment from pDP10 cloned into KpnI-SalI sites of pJIR751	(155)
pSB23	~ 2.5-kb *gerAA* fragment cloned between KpnI/SalI sites of pJIR751	This study

TABLE 4.2. Kinetic parameters for L-asn, KCl and AK germination of FP and NFB *C. perfringens* spores.

F4969 (NFB) spores					
	L-asn[a]			KCl	
[KCl] mM	V_{max}[ΔOD/min]	K_m [mM]	[L-asn] mM	V_{max}[ΔOD/min]	K_m [mM]
0	0.1[b]	750	0	0.1	85
50	1.1	5.6	50	0.8	4.1
100	3.8	0.7	100	14.6	0.06
SM101 (FP) spores					
	L-asn			KCl	
[KCl] mM	V_{max}[ΔOD/min]	K_m [mM]	[L-asn] mM	V_{max}[ΔOD/min]	K_m [mM]
0	4.4	0.14	0	2.3	6.6
50	5.2	0.05	50	7.3	0.01
100	6.1	0.03	100	6.0	0.01

[a] L-asn and KCl were used at 1, 5, 10, 25, 50, 75 and 100 mM

[b] Standard deviations of all values shown were less than 25 % of the mean.

TABLE 4.3 Germination of *C. perfringens* FP and NFB spores with different rich media.

Germinants	Decrease in OD_{600} (% Mean ± SD) in 60 min with [a]:					
	FP [b]			NFB [c]		
	SM101	NCTC10239	E13	F4969	NB16	B40
TGY	46 ± 6.9	44 ± 0.7	47 ± 0.2	43 ± 4.3	41 ± 3.4	41 ± 10
BHI	40 ± 1.2	39 ± 3.6	47 ± 1.6	4 ± 1.2	26 ± 6.8	14 ± 4.6
EMEM	7 ± 0.3	5 ± 3.0	10 ± 2.4	49 ± 0.8	46 ± 2.1	48 ± 4.3
DMEM	6 ± 0.2	6 ± 2.2	8 ± 1.5	45 ± 3.5	42 ± 6.5	46 ± 1.0

[a] Values are averages for duplicate experiments with two different spore preparations.

[b] Food Poisoning isolates

[C] Non Food Borne isolates

TABLE 4.4. Colony formation by spores of *C. perfringens* NFB strains[a]

Strain (genotype)	Spore titer (CFU/ml/OD_{600}) [b]	
	BHI	BHI + Lyz[C]
F4969 (wild-type)	3.88×10^7	7.15×10^8
SB103 (*gerAA)*	3.83×10^7	6.43×10^8
SB104 (*gerKA-KC*)	2.11×10^6	1.77×10^8
SB106 (*gerKC)*	1.11×10^6	7.83×10^8
SB110 (*gerKA)*	4.06×10^7	1.20×10^8
SB106 (pSB18) (*gerKC* mutant complemented with wild-type *gerKA-KC*)	2.68×10^7	ND[d]

[a] Heat-activated spores of various strains were plated on BHI agar with or without lysozyme (1 μg/ml), and colonies were counted after anaerobic incubation at 37°C for 24 h as described in Methods.

[b] Titers are the average number of colony forming units (CFU)/ml/OD_{600}) determined in three experiments, and the variation was less than 15%.

[c]Spores were decoated, heat activated, and plated onto BHI agar containing lysozyme (Lyz), and colonies were counted after overnight incubation anaerobically at 37°C.

[d]ND, Not Determined.

TABLE 4.5. Primers used in this study

Primer name	Primer sequence[a]	Gene	Position[b]	Use[c]
CPP206	5' CAAGTATTAATCCTCCAATAACAG 3'	*gerAA*	+1102 to +1126	MD
CPP211	5' CTTTAATGGGAATTATAGCA 3'	*gerAA*	-264 to -244	MD
CPP443	5' GATGAAAATGAAGTGGGAAATATAGAC 3'	*gerKC*	+120 to +137	MD
CPP440	5'GTTGTGCCATTAATTTCAACATCAACA 3'	*gerKC*	+1076 to +1103	MD
CPP208	5'ATCATTATTATCACCTCTGCTACTAT 3'	*gerKA*	+980 to +1006	MD
CPP209	5'TATAGTGAAAATCCAAGTATCTC 3'	*gerKA*	-224 to - 201	MD
CPP394	5`GGCGGAAGAGATACTTGGATTTTC 3`	*gerKB*	-54 to -25	MD
CPP395	5`GTATAAAAAGGGTATAGGCATTCTTAG 3`	*gerKB*	+998 to +955	MD
CPP857	5`GGTACCGCTACC CTT GCT ATG GTT GAT GT-3`	*gerAA*	+469 to +447	CP
CPP858	5`GTCGACTTG AGC TGC TTC CAT GAG AGC-3`	*gerAA*	-1625 to -1604	CP
CPP1259	GAGAAGGCTGCTAAAAATGGAA	*gerKC*	+294 to +316	RT-PCR
CPP1260	TGGCATCAATAGCTATAACTCTAGGAC	*gerKC*	-502 to - 478	RT-PCR
CPP1278	CGCATAATGTTGAAAGATGG	*16s rRNA*	+176 to +195	RT-PCR
CPP1279	CCTTGGTAGGCCGTTACCC	*16s rRNA*	-258 to -276	RT-PCR

a-Restriction sites are marked by underlining.

b-The nucleotide numbering begins from the translation start codon and refers to the relevant position within the respective coding sequence.

c- MD, Mutation detection; CP, construction of complementing plasmid.

CHAPTER 5

General Conclusion

Enterotoxigenic *Clostridium perfringens* type A isolates are capable of causing a wide spectrum of diseases in both humans and animals, including food poisoning (FP) and non-food-borne (NFB) gastrointestinal diseases. Spore germination of *C. perfringens* is considered the earliest and most essential step for initiation of the disease. Thus, spores must germinant when they sense the nutrient with rapid enzymatic and biochemist changes follow with release the nascent cell, outgrow, multiply to increase numbers and then release the toxins which lead to acute diseases. Our lab has been studied spore germination in *C. perfringens* but still much unknown mechanisms during germination machinery needs further understanding. In these three studies I tried with available molecular analyses to gain more information in order to identify new insights into the mechanism of *C. perfringens* spore germination.

In the first study, we identified the major GR in *C. perfringens* spore germination. This study offers the following findings: (i) Spores of *gerKC* mutant did not germinate with KCl, L-asparagine, a mixture of asparagine and KCl, and NaPi at pH 6.0. (ii) The *gerKC* spores germinated poorly compared to wild-type and other GRs mutant spores with the non-nutrient germinants dodecylamine and a 1:1 chelate of Ca^{2+} and dipicolinic acid. (iii) The germination defect in *gerKC* spores was restored by complementing the *gerKC* mutant with wild-type *gerK* operon, indicating

that GerKC is essential for germination of *C. perfringens* spores. (iv) GerKC is essential for the release of DPA from the spore's core during germination with KCl and dodecylamin. (v) GerKC is also essential for spore's viability; Finally, (vi) GerKC localizes in the spore's inner membrane.

The second study examined the precise location for the Csp proteases CspB and cortex lytic enzymes SleC in spores of *C. perfringens* FP strain SM101. Results from this study show that CspB and pro-SleC are present exclusively in the *C. perfringens* SM101 spore coat layer fraction and absent in the lysate from decoated spores and from the purified inner spore membrane. In addition, quantitative Western blot analyses show that there are approximately 2,000 and 130,000 molecules of CspB and pro-SleC, respectively, per spore.

The third study was to identify and characterize the germinants and their GRs involved in germination of spores of *C. perfringens* NFB strain F4969. Results from this study indicate that NFB strain F4969 germinates with AK in a cooperative manner, while FP strain SM101 trigged by both L-asn and KCl (AK) components are capable of triggering spore germination through independent pathways. Spores of *gerKA-KC, gerKA*, gerKC, *gerAA*, and *gerKB* knock-out mutants suggest that (i) germination of FP and NFB spores differs significantly in rich media and several defined germinants; (ii) L-asn and KCl induced germination in NFP F4969 spores cooperatively, while either germinant alone triggers germination of FP spores; (iii) GerKC and GerAA proteins are required for normal germination of NFB spores with

AK and L-cys; (iv) although GerKCs play a major role in DPA release with AK and in spores' viability, GerAA is not required for spore viability

Collectively, this dissertation study will give new insights into the understanding of the molecular mechanism of *C. perfringens* spore germination. Moreover, these findings indeed will further enhance our understanding in the differential role of GRs in spore germination in FP versus NFB *C. perfringens* isolates. Our continuing efforts in understanding the mechanism of spore germination should lead to develop new approaches to block or trigger spores germination in *C. perfringens*.

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Appendices

Appendix A: Scientific work published or in preparation during Mr. Saeed S. Banawas's Ph.D. degree.

1- Udompijitkul P, Alnoman M, Banawas S, Paredes-Sabja D, Sarker MR. 2014. New amino acid germinants for spores of the enterotoxigenic *Clostridium perfringens* type A isolates. Food Microbiol. **44:**24-33.
2- Olguin-Araneda V, Banawas S, Sarker MR, Paredes-Sabja D. 2015. Recent advances in germination of *Clostridium* spores. Res. Microbiol. **166:**236-243.
3- Alnoman M, Banawas S, Sarker MR. 2016. Bicarbonate is a Co-germinant for *Clostridium perfringens* type A Non-Food-Born Isolates. Manuscript on preparation.