AN ABSTRACT OF THE THESIS OF

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Title: The Signaling Role of Divalent Cations in *Clostridium perfringens* Spore Germination

Abstract approved:

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Mahfuzur R. Sarker

Clostridium perfringens is an anaerobic, gram-positive, rod-shaped, spore-forming bacterium that leads to a broad range of diseases in humans and animals [23, 27, 50]. Among seven *C. perfringens* types (type A-G), type F is known to be the most common bacteria that is responsible for causing human food-borne disease outbreaks worldwide [23, 27, 50]. This pathogen becomes a problem for human health because of its ability to form metabolically dormant spores that can tolerate environmental stresses such as radiation, pH, osmotic stress, desiccation, and temperature [62, 63, 73, 84]. As a result, spore resistance causes a broad range of harmful effects including food-poisoning (FP), food spoilage, and gastrointestinal diseases [50, 80]. However, in order to have these negative effects, dormant spores must undergo germination to become metabolically active cells [36, 50, 59]. Therefore, the germination process starts when the spores sense germinants through specific receptors located in the spore's inner membrane [4, 28, 61, 86]. The germination process can be started through

a different type of germinants including, cationic surfactants, amino acids, and enzymes [30, 66, 67, 83, 91].

The previous study has shown that exogenous dipicolinic acid (DPA) chelated with calcium (Ca^{2+}) (Ca-DPA) can significantly enhance spore germination in *C. perfringens* [20, 60, 66, 83]. However, it is unclear whether Ca^{2+} or DPA alone is needed to enhance spore germination. Therefore, in the current study, we aimed to evaluate the possible role of Ca^{2+} and other divalent cations present in the spore's core (Mn^{+2} and Mg^{+2}) in germination of *C. perfringens* spores. To accomplish this, our study consists of three parts.

The first part of this study evaluates the role of Ca^{2+} and DPA in the spore germination process. We found that Ca^{2+}, but not DPA, is sufficient to trigger spore germination in *C. perfringens* FP isolates. All tested calcium salts (calcium-chloride, calcium-carbonate, or calcium-nitrate) induced germination of spores of *C. perfringens* FP isolates, indicating that exogenous Ca^{2+} ion is significant for spore germination.

The second part of this study evaluates whether spore-specific divalent cations (Mn^{2+}, and Mg^{+2}) can induce spore germination. Our result suggested that all spore core-specific divalent cations (Ca^{2+}, Mn^{2+} and Mg^{+2}) contribute to spore germination in *C. perfringens* FP isolates with slight variations in the percentage of germination. In contrast, non-core-specific divalent cation Zn^{+2} did not induce germination of spores of *C. perfringens* FP isolates.

The third part of this study evaluates whether the exogenous or endogenous spore-specific divalent cations (Ca^{2+}, Mn^{2+} and Mg^{2+}) are needed to induce *C.*

perfringens spore germination. Our results indicated that endogenous Ca^{2+} and Mg^{2+} are not necessary to initiate the spore germination process. While exogenous and endogenous Mn^{+2} are needed to enhance spore germination.

In conclusion, our results indicated that spore-specific divalent cations play a signaling role in *C. perfringens* (FP) spore germination. Further germination assay on spores of germinant receptor mutants and cortex-lytic enzyme mutants in the presence of spore-specific divalent cations should clarify the possible mechanism of divalent cation mediated spore germination. In addition, further experiments in food products is needed to evaluate whether this specific concentration and pH of divalent cations can also trigger the spore germination in food products.

The Signaling Role of Divalent Cations in *Clostridium perfringens* Spore Germination

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Roua G. Almatrafi

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APPROVED:

Major Professor, representing Comparative Health Science

Dean of the College of Veterinary Medicine

Dean of the Graduate School

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Roua G. Almatrafi, Author

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Dr. Mahfuzur R. Sarker as a major professor provided the laboratory facilities needed for the research done on this study.

Dr. Mahfuzur R. Sarker and Dr. Saeed Banwas contributed to the experimental design, data analysis, and preparation of the manuscript in chapter 2.

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Chapter 1

Introduction and Literature Review

Clostridium perfringens is a gram-positive, anaerobic, spores forming, and rod-shaped bacterium that causes a broad range of diseases to humans and animals [23, 27, 50]. Current studies have identified approximately 152 species of the genus *Clostridium*. Some of these species are pathogenic including *C. perfringens*, *C. difficile*, *C. tetani*, *C. botulinum, C. chauvoei* and *C. septicum* [26]. The *Clostridium* genus leads to various diseases in humans such as food poisoning [8, 26]. In contrast, others have been used industrially such as *Clostridium thermocellum*, and *Clostridium acetobutylicum* [26].

C. perfringens is distributed environmentally and can be found in soil, water, food, spices and wastewater, on the other hand, it can be present naturally within both humans and animals intestinal normal flora [31, 48, 90]. The optimal conditions (temperature and pH) for *C. perfringens* growth have been demonstrated in studies. The optimal conditions for growth include temperature ranging from 35 ^{o}C to 40 ^{o}C, and pH ranging between 6.0 to 7.0, which reflects the optimum condition in the food such as cooked meat and other meat products [12, 47, 55]. However, *C. perfringens* can be found in pH as low as 5.0 and as high as 8.0 [31,

47, 55]. *C. perfringens* growth requires lower water activity, ranging between 0.93 and 0.97, depending on the solute [31, 47, 55].

The first identification of *C. perfringens* as a causative agent of food poisoning was in the 1940s in the UK [33]. While the British scientist knew this organism as a cause of food poisoning, there were few incidents before 1960 in the United States [33, 57]. The symptoms of *C. perfringens* food poisoning are abdominal cramping, vomiting, fever, symptoms which begin 8 to 18 hours after ingestion of contaminated food and continuing for 12 to 24 hours [31, 49, 50, 93]. Anyone is susceptible to *C. perfringens* food poisoning, but the most severe infection will be in elderly or sick individuals [31, 49]. This bacterium is the most common bacteria that cause food poisoning in humans by ingestion contaminated food with vegetative followed by the sporulation of cells in the intestines and releasing *Clostridium perfringens* enterotoxin (CPE) [31, 50]. CPE toxin is rapidly released into the intestines and attaches to the epithelial cells receptors which lead to cause of food poisoning symptoms such as common diarrhea and abdominal cramping [31, 47].

The pathogenicity of *C. perfringens* is dependent on three important properties of the organism. Firstly, *C. perfringens* can produce 17 toxins, and a specific subset for each toxin produced by individual bacteria allows it to cause

disease [90, 102]. Based on the production of six major toxins; alpha-, beta-, iota-, epsilon-, CPE- and NetB-toxins, *C. perfringens* strains are classified as seven toxino-types (Type A-G) (Rood et al 2018). Secondly, this bacteria can multiply in less than 10 minutes, leading to bacterial loads that are responsible for causing disease [45, 99]. Finally, *C. perfringens* has the unique feature of producing dormant spores that tolerate environmental stress and survive for hundreds of years [40, 50, 77].

1.1. *C. perfringens* food poisoning

C. perfringens is classified as the second most frequent bacteria responsible to cause food poisoning (FP) illnesses in the United States [34, 78]. The percentage of cases of food poisoning is estimated to be 70%, and *C. perfringens* is the most causative bacteria mostly type F, which is previously known as type A [34]. The shift of the strain type based on toxin productivity [34]. Type F has alpha- and CPE toxins, where type A has alpha-toxin only [34]. The infection has considerable economic consequences including the loss of hundreds of million dollars per year as a result of medical care requirements and reductions in productivity [27, 50, 94].

Most *C. perfringens* strains associated with human diseases such as, food poisoning and non-foodborne (NFB) gastrointestinal diseases (GI) are *C.*

perfringens type F [12, 48, 76]. *C. perfringens* FP isolates spores are more resistant to different environmental factors such as osmotic stress, nitrite, pH, frozen storage and pressure-assisted thermal processing than are *C. perfringens* NFB isolates' spores [77]. The high resistance of FP isolates spores is due to the ability to produce a different small acid-soluble protein (Ssp4), which binds to the DNA of the spores and protects them from being killed by various environmental stresses [42, 80, 87]. The resistance characteristics of spores of FP isolates help them to survive in meat products that are mostly involved in *C. perfringens* type F food poisoning outbreaks [50].

Once these resistance spores sense a suitable environment, they become metabolically active and go through germination stages, outgrowth, and vegetative cells [50–52]. When the spores are germinated, they lose their resistance properties and start making harmful effects such as food spoilage and diseases [43, 83]. Therefore, after ingestion of contaminated food with infection dose of ~10^6 -10^7 CFU/g of vegetative cells, the surviving cell within the stomach's acidity will start sporulating in the intestinal tract and release CPE toxin, which leads to GI disease [50]. Additionally, CPE toxin has common association with different types of NFB humans and animal diarrhea, like antibiotic-associated diarrhea and chronic NFB diarrhea [46, 47]. The *Clostridium perfringens* enterotoxin gene (*cpe*) can be

located either on the chromosome or plasmid; however, most FP isolates carry *cpe* on the chromosome and NFB isolates carry on the plasmid [11].

1.2. *C. perfringens* spore resistance

C. perfringens can produce dormant spores that are resistant to environmental stress such as temperature treatment, UV radiation, or desiccation [62, 63, 73, 84, 100]. However, *C. perfringens* strains show a significant difference in their heat-resistance depending on the isolate's source. Studies have found that spores of *C. perfringens* FP isolates are more resistant to heat than spores of NFB isolates [73, 77]. This higher heat resistance helps *C. perfringens* FP isolates to survive in inappropriately cooked meat products, which are usually associated with food-borne illness in developed countries [25, 50, 57, 75].

C. perfringens spores have different structural layers and each of them contributes to spore resistance properties [43, 54, 58]. The function and structure of the *C. perfringens* spore coat has not been clearly identified. However, in the gram-positive spore-forming bacteria, the spore coat suggests having more than fifty proteins that can protect the spores from different lysis chemicals and enzymes [39, 43, 84]. The spore cortex plays a significant role in the primary dehydration of the spore core, indicating a direct contribution to the environmental resistance [43]. The spore inner membrane is crucially pressed, which leads to highly immobile

lipids followed by low permeability of water, DNA damaging chemicals and other small molecules [43, 84, 103]. In addition, the spore core inner layer contains nucleic acids and most enzymes and has three elements, (a) the low level of water content (20% wet weight) of the core, (b) high rate of Ca-DPA (25% dry weight) in spore's core and (c) the saturated DNA with small acid-soluble proteins (Ssp). These three elements together contribute to the spores' resistance properties [43, 80, 87].

Studies have analyzed each of spore core, cortex, coat and size, which allow them to identify the most important connection between the structure of *C. perfringens* spores and their heat resistance. The low percentage of core volume, and peptidoglycan layer assist spores to be more heat resistance [43, 54, 58]. However, the ultrastructural features of other spore-forming bacteria have similar structure to *C. perfringens* spores [43]. Therefore, the main differences between *C. perfringens* spore's resistance will be at the spore's molecular action level rather than spore's structural level [43].

1.3. Bacterial spore germination

C. perfringens is able to produce metabolically dormant spores that are significantly more resistant to environmental stress than are vegetative cells [70,

87, 88]. The spores of *C. perfringens* can remain dormant for hundreds of years in different harsh environments [56, 66, 87]. However, spores can be activated in a suitable environmental condition and grow in less than 10-20 minutes [19, 31, 49]. Spores are able to sense suitable germinants (such as amino acids or salts) through specific receptors located in the spore inner membrane, which later initiate the spore germination process and outgrowth stage that turns the germinated spores to the vegetative cells [28, 83, 88, 103].

Spores germination is stimulated by different chemicals including nutrients, cationic surfactants and enzymes [66, 83]. Several studies show that nutrient germinants could trigger spore germination of *Bacillus subtilis* and *C. perfringens* [61, 66, 67]. The germinants that can trigger spore germination are purine nucleosides, amino acids, the combination of specific nutrients including mixing of L-asparagine, D-glucose, D-fructose, inosine and KCL (AGFK) triggers *B. subtilis* spore germination [51, 69, 83, 89]. In addition, water content is significant to the spore germination movements. The volume of the spores core of *B. subtilis* increases from 2-fold to 2.5-fold through germination by water uptake [83].

Once spores sense the germinants, they commit to germinate in less than a minute. There are five series of biophysical and biochemical events happening during spore germination:

I. Release of monovalent ions (H^+, $K,^+$ and Na^+) from the spore's core through an energy-independent mechanism. The release of H^+ alters pH from 6.5 to 7.7, this change is crucial for spore metabolism [83, 88, 92].

II. Once the spore core is highly hydrated, spores release (10% of the spore's dry wt) large depot of pyridine-2, 6-dicarboxylic acid (dipicolonic acid [DPA]) as 1:1 chelated with divalent cations, predominantly Ca^{2+} (Ca-DPA) followed by ion release [83, 88, 92].

III. The release of Ca-DPA is substituted by water resulting in increased hydration of the spore core, although it is not sufficient for protein mobility that causes some decrease in wet-heat resistance [13, 81, 83, 88].

IV. The releasing of Ca-DPA causes some hydrolysis in the spore cortex peptidoglycan in *B. subtilis* [61, 83].

V. Breaking down of the spore cortex peptidoglycan removes the physical restrictions, which enable the core to expand and allow the absorbing water, similarly to the level of water that found in the vegetative cells and the recovery of metabolism [71, 83, 88].

1.4. Monovalent ions roles

The role of the monovalent cations in spore germination was examined by various investigators. In *Bacillus* species, most commonly potassium ion (K^+) is required as a co-germinant for nutrient germinant with different monovalent

cations, and only in a few cases (K^+) salts alone were identified as cause of inducing spore germination [66, 74, 85]. In *B. subtilis* all monovalent cations can enhance spore germination at a lower concentration, however, higher concentration can have an opposite effect [53]. The specific commitment that leads to initiation of bacterial spore germination process has not been clearly identified. Although, the release of (H^+, K^+ and Na^+) are related to a significant change in spore's inner membrane permeability and possibly membrane structure [87]. Therefore, in *Bacillus megaterium* spore's monovalent released through the germination process, and this release perhaps happened before the release of the large amount of Ca-DPA [82, 92]. In the Table 1.1 below an approximate amount of monovalent ion that is present in the spore's core.

Table 1.1 Monovalent ion contents of *Bacillus* species (μM/mg of spores dry weight) [7].

Strain	K^+	Na^+
B. megaterium	0.10	0.15
B. subtilis niger	0.28	0.18
B. stearothermopilhus	0.02	0.05

Once spores sense a germinants via germinant receptors located in the inner membrane, the release of monovalent ions (H^+, K^+ and Na^+) from the spore's core

through an energy-independent mechanism takes place and lead to change of the pH from 6.5 to 7.7 due to the release of H^+ [83, 88, 92].

1.5. The role of divalent cations

Studies have shown the link between divalent cations and the effectiveness of bacterial spore germination [18, 24, 32]. Generally, spores contain a higher concentration of cations relative to vegetative cells, especially in Ca^{2+} [14, 24]. In a study of *Bacillus* species shows different levels of divalent cations between spores and vegetative cells (Table 1.2) [15]. In *Bacillus* species, ions can inhibit spore germination, but divalent ions specially DPA-chelated ions such as Mg^{2+} and Ca^{2+} can increase spore germination [17, 24]. In addition, in *Bacillus* species such as *B. stearothermophilus*, and *B. cereus,* Mg^{2+} led to significant enhancement in spore germination, while Cu^{2+} , Mn^{2+} and Fe^{2+} inhibited the outgrowth of these spores [18, 24]. Another study in *B. subtilis* demonstrated that Mg^{2+} and Ca^{2+} have been involved in the stimulation of cortex-lytic enzymes through the process of spore germination [29]. Mn^{2+} also has some role in the growth of bacterial spores by enhancing a variety of enzymes that are essential for *B. megatherium* spores germination [41]. Similarly, in *Clostridium difficile* bacteria, divalent cations have an essential signaling role in initiating spore germination [35, 36, 91].

In *C. perfringens* S40 spores, the lytic enzymes needed 1mM of divalent cations including, Ca^{2+}, Mg^{2+} and Mn^{2+}, to be activated but 1mM of Zn^{2+} and Hg^{+} have the ability to inactivate the enzyme [9]. The lytic enzyme could be activated without adding divalent cations, due to the remaining amounts of endogenous divalent ions that are sufficient to sustain the activity of the enzyme [9]. Some studies have shown that nutrients such as alanine, inosine, and glucose compounds help to enhance ions, which result in effecting the spore germination [17, 74]. The other possible function of divalent cations probably changing coat proteins in the bacterial spore [24].

However, some studies demonstrated an increase in spore thermal resistance due to adding of extra Ca^{2+} or Mn^{2+} in the sporulation medium [1]. Other studies also showed that different metal ions are capable of providing different protections to the spores from wet heat, such as Ca^{2+} providing significant spores protection or resistance than other divalent ions or monovalent ions [6, 7, 79, 104]. In Table 1.3 studies show that, the concentrations of divalent ions and DPA for different strains of *C. perfringens* bacteria are varied, whereas the higher concentration contributes to enhancing spores' heat resistance [58].

Overall, the spore core has high levels of divalent ions, including Ca^{2+} , Mn^{2+}, and Mg^{2+}, which are mostly chelated with DPA, and each level of particular

cations or ions can significantly impact spore resistance or in other cases enhance spore germination [7, 9, 21, 29].

Table 1.2 Divalent ion contents of *Bacillus* species μmol/g spores dry weight, (-) not specify volume [15].

Strain	DPA	Ca^{2+}	Mg^{2+}	Mn^{2+}
Bacillus Species spores	410-470	380-916	86-120	27-56
Bacillus Species Cells	< 0.1	-	-	-

Table 1.3 Divalent ion content of *C. perfringens* spore expressed in fmol/μm^3 all values are averages ± SDs [58].

Strain	DPA	Ca^{2+}	Fe^{2+}	Mg^{2+}
NCTC 8239	49.7 ± 1.3	41.8 ± 5.0	1.8 ± 0.2	6.1 ± 2.3
NCTC 8679	196.6 ± 3.0	130.6 ± 15.6	2.31 ± 0.1	18.8 ± 1.1
SM101	18.9 ± 1.1	23.5 ± 1.9	0.9 ± 0.1	2.1 ±1.0
NCTC10240	28.9 ±1.0	22.5 ±0.9	0.6 ±0.1	4.3 ±1.3
3663	33.1 ±1.4	25.8 ±1.1	0.8 ±0.1	3.7 ±0.9
FD1	9.0 ±0.9	10.5 ±1.3	0.3 ±0.1	1.2 ±0.1

1.6. Ca-DPA release

Spores of *Clostridium* and *Bacillus* species have a unique feature by containing a major level of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), which approximately contain ~20% of the spores dry weight [15, 61, 92]. In *B. subtilis* spores within a minute of initiating the spore germination process, a major abundance of dipicolinic acid 1:1 chelated with Ca^{2+} are released which leads to a significant flaw through SpoVA proteins located in the inner membrane [22, 66, 92].

SpoVA genes are present in most spore-forming *Bacillus* and *Clostridium* species, but only a few were preserved in all species [88]. Remarkably, SpoVA proteins are much superior to the germinant receptors, which reflect only ~25 molecules out of ~15,000 molecules of SpoVAD [61, 97]. There are higher percentages of SpoVA proteins than germinant receptors indicating possible significant increase of the germinant signal to activate the rare germinant receptors, which later unlock a DPA flow channel consisting of numerous SpoVA proteins [97].

There is evidence that shows SpoVA proteins contributed to releasing Ca-DPA through spore germination process [88]. First, mutant spoVA spores (heat sensitive) can take up Ca-DPA at affordable temperature during sporulation, but (Ca-DPA) do not release when the spores cannot tolerate temperature [88, 96];

second, spores which have high SpoVA protein levels release Ca-DPA quicker during germination with various germinants [88, 98]; third, in *E. coli,* dodecylamine turns on the SpoVAC channel in lipid vesicles or makes changes in membrane tension [88, 95]; fourth, spores of *B. subtilis* which have no SpoVAEa take up Ca-DPA as usual, but release Ca-DPA slowly during germination due to dependent in germinant receptors [68, 88]; lastly, the convertible factor containing *spoVA* homologs and other protein inner membrane result in reducing germination percentage with nutrients and dodecylamine [38, 88].

1.7. Applications of bacterial spore germination

Controlling bacterial spore germination is an effective method in order to reduce the harmful effect of them. It has been known that *C. perfringens* spore's germination has a significant role in causing illness and food spoilage. Therefore, identifying germinants that are efficient to trigger *C. perfringens* spore germination is highly needed to develop strategies such as enhancing spore germination, then inactivating germinated spores followed by using conventional treatments. Furthermore, understanding the mechanism of spore germination should help identifying food-garde chemicals/compounds that would prohibit spore germination completely, which would prevent the progression of the disease.

The objective of this study

C. perfringens have the ability to produce metabolically dormant spores. These dormant spores must germinate through germination process to become metabolically active in order to cause diseases. Therefore, identifying germinants that effectively trigger spore germination in a wide range of FP isolates should lead to developing novel strategies to inactivate spores. Such as, turning dormant spores into vegetative cells and then easily inactivate them by using mild treatments. Therefore, knowing the mechanisms of bacterial spore germination is highly needed to eliminate harmful effects. In this study, we will assist to underline the significant role of divalent cations during spore germination of *C. perfringens* FP isolates. The specific aims of this research are:

1- Evaluating spore germination from different *C. perfringens* FP isolates with $CaCl_2$, DPA, or Ca-DPA at optimum pH and concentration.

2- Evaluating the germination of *C. perfringens* FP isolates with other divalent cations within the spore's core (Mg^{2+}, Mn^{2+}) at optimum pH and concentration.

3- Identifying the essential signaling of exogenous or endogenous divalent cations in spore germination.

Chapter 2

The signaling role of divalent cations in *Clostridium perfringens* spore germination

2.1Abstract:

Clostridium perfringens is an anaerobic bacterium that is able to produce metabolically dormant spores, which can tolerate environmental stresses and survive for many years. When the environment is favorable, *C. perfringens* spores germinate and cause the disease. The germination process is initiated when bacterial spores sense a variety of chemicals, including salts, amino acids, cations, and enzymes. Previous study has shown that dipicolinic acid (DPA) chelated with calcium (Ca-DPA) can stimulate significantly spore germination in *C. perfringens*. However, it is unclear whether Ca^{2+} or DPA alone can induce spore germination. Therefore, in this study we aimed to evaluate the possible role of Ca^{2+} and other divalent cations present in spore core (Mn^{2+} and Mg^{2+}) in germination for *C. perfringens* spores. Our study demonstrates that, i) Ca-DPA, but DPA alone, induced germination of spores of *C. perfringens*, suggesting that Ca^{2+} might have signaling role in spore germination; ii) all tested calcium salts (calcium-chloride, -carbonate, or -nitrate) induced spore germination, indicating that Ca^{2+} ion is critical for spore germination; (iii) other spore-specific divalent cations (Mn^{2+}, and Mg^{2+}), but not Zn^{+2}, could induce spore germination, suggesting that all spore core-specific divalent cations are involved in *C. perfringens* spore germination; (iv) endogenous Ca^{2+} and Mg^{2+} is not needed it to induce *C. perfringens* spore germination; surprisingly, exogenous and partly endogenous Mn^{+2} is needed to induce spore germination.

2.2. Introduction:

C. perfringens is obligate anaerobe, Gram-positive, encapsulated, spore-forming bacteria that can cause significant diseases to humans and animals [23, 27, 50]. The virulence of this bacterium is due to its ability to produce 17 toxins, which lead to the cause of serious diseases [90, 102]. The most common diseases associated with *C. perfringens* type A are; food poisoning (FP), antibiotic-associated diarrhea, and gas gangrene in humans, and necrotic enteritis, and enterotoxemia in animals [8]. *C. perfringens* FP is the second most commonly reported bacterial food-borne disease in USA [49, 50]. The Symptoms of *C. perfringens* FP are abdominal cramping, vomiting, fever, and diarrhea start after 8 to 18 hours of ingestion of contaminated food and continue for 12 to 24 hours [49, 93]. Anyone can get infected with *C. perfringens* FP, but the most severe infection occurs in elderly or sick individuals [49]. However, during spore's dormancy, spores are not able to cause these diseases, but they can sense their environmental signals and opportunistically take a chance to grow [85]. Therefore, spores should go through the germination process to cause the disease, initiated with spore germination, outgrowth, and transform into the vegetative cells [50–52].

To initiate spore germination process, spores have to get in contact with germinants such as, amino acids, sugars, purine nucleosides, inorganic salts, or combinations of them [65, 83]. Followed by binding to the germinant receptor (GR)

located in the inner membrane of the spores, followed by the release of monovalent cations and large depot of DPA-chelated with divalent cations [65, 83, 88]. After releasing cations and other molecules, water is uptaken inside the spore's core leading to increase in the spore's core water activity. In *Bacillus* species, these cations activate the enzymatic action such as cortex-lytic enzyme (CLEs) on the spore's cortex peptidoglycan (PG) [65, 83, 88]. The activation of CLEs leads to degradation of the spore's cortex peptidoglycan, which enables the rehydration of spore's core followed by enzymatic and metabolic resumption [3, 5, 65, 86].

Studies have demonstrated a correlation between divalent cations and the effectiveness of bacterial spore germination [18, 24, 32]. In *Bacillus* species, ions can inhibit spore germination, but divalent ions specially DPA-chelated ions such as Mg^{2+} and Ca^{2+} can increase spore germination [20]. Previous study has shown that exogenous Ca-DPA can stimulate significantly spore germination in *C. perfringens* [66]. However, it is unclear whether Ca^{2+} or DPA alone can induce spore germination. There was in need to understand whether DPA or it's associated divalent cations are responsible for induction of germination and track internal and external signal pathways. Consequently, in this study, we found that the divalent cations at pH 6.0 are able to stimulate germination alone without the need for DPA, and the endogenous signal most importantly can stimulate spore's germination rather than endogenous.

2.3 Material and Methods:

Strains used in this study. Three *C. perfringens* food-borne isolates [SM101, NCTC10239, NCTC 8239] [10, 101] were used and maintain them in cooked meat media (Difco, BD Diagnostic Systems, Sparks, MD, USA) and stored at -20°C.

Spore preparation and purification. Sporulating cultures of *C. perfringens* were prepared as described previously by (Paredes-Sabja) [63]. Briefly, *C. perfringens* isolates were prepared by inoculating 0.1- 0.2 ml from the cooked meat culture into 10 ml fresh fluid thioglycollate media (FTG) vegetative medium, incubated at 37°C for 24 hours [37]. Next, we inculcated 0.4 ml from the previous FTG culture to a new 10 ml FTG and incubated at 37°C for 9 to 12 hours [37]. For sporulation cultures, we inoculate 0.4 ml from the previous fresh active FTG culture into 10 ml fresh Duncan-Strong (DS) sporulation medium (1.5% protease peptone, 0.4% yeast extract, 0.1% sodium thioglycolate, 0.5% sodium phosphate dibasic (Na_2HPO4) (andydrous), 0.4% soluble starch) and incubate at 37°C for 16-18 hours [16]. Then the percentage of spore formation was confirmed by phase-contrast microscopy (Leica MDLS, Leica microsystems).

However, if the spore percentage is less than 80%, we have to restart the spore preparation processes again from first FTG. Large scale spore preparation was performed by scaling up the culture volume and following the same small scale procedure.

Spore purification was done by repeated washing with autoclaved cold sterile distilled water, centrifuging at least 10 times and sonicating several times for 10 second until the spore suspensions were 99% free of sporulating cells and cell debris. After that, we suspended the spores into autoclaved distilled water and adjusted an optical density at 600 nm (OD_{600}) of ~ 6 by using SmartspecTM 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), and stored at -20 °C until used [63].

Spore germination. The free purified spores' suspensions were heat-activated for 10 min at 80°C and then cooled down at water bath room temperature 21°C for 5 minutes. The spore germination was measured by mixing heat-activated 33 µl of spores (OD_{600} of 1) suspension with 167 µl of the pre-warmed germinant solutions, 50mM Ca-DPA pH: 8.0, and 50 mM of each of $CaCl_2$, $C_6H_{10}CaO_6$, Ca $(NO_3)_2$, $MgCl_2$, $MnCl_2$, $MgSO_4$, and $ZnCl_2$ adjusted to pH 6.0 with 25mM Tris HCL buffer, and 100mM of KCL pH: 6.0 and 25 mM Tris-HCl buffer pH: 6.0 in 96-well microtiter plate and incubated at 37 °C for 60 min. Spore germination was routinely monitored by measuring OD_{600} changes using a SynergyTM MX multi-mode microplate reader (BioTek® Instruments, Inc., Winooski, VT, USA). The ~60% decrease in OD_{600} indicates complete spore germination as found in our previous studies [66]. The level of spore germination was also confirmed by phase-contrast microscopy (Leica MDLS, Leica microsystems) after 60 min post-

inoculation, as fully germinated spores' changes from phase-bright to phase-dark. The extent of germination was calculated by measuring the percentage decrease in OD_{600} and express as percentage of initial. The rate of germination was determined by measuring the OD_{600} of germinating spores every 2.5 minutes and the maximum rate was expressed as a percentage of maximal loss of OD_{600} per minute, relative to the initial value of spore suspension. All values at least consist of two experiments with at least two independent spore preparations.

To examine the effects of pH on the germination rate, germinants were prepared in a 25mM Tris-HCl buffer (pH 6.0) at 37°C. Similar with previous experiments that we did, all values at least two experiments with at least two independent spore preparations.

All solutions were prepared at 50 mM in 25 mM Tris-HCl buffer (pH 6.0). For ionic chelators (EDTA, and EGTA) were prepared firstly at 50 mM with 25 mM Tris.HCl buffer (pH 9.0) to make it dissolve and then adjust to the pH to 6.0.

Confirmation of spore germination using colony formation assay. *C. perfringens* SM101 spore germinated with various germinants ($CaCl_2$, Ca-DPA, and DPA) were heat-treated and plated onto BHI agar to confirm the spore's germination ability. Each germinated sample contained 50µl of spore concentration (33µl/200µl) with 950µl of distasted water and heated it at 100 °C for 20 minutes.

Each sample aliquots of serial dilutions were plated onto BHI agar and were incubated at 37 °C anaerobically for 24 h and colonies were counted.

In heat-treated sample, if the spores are fully germinated, they will become heat sensitive, so we can expect no colonies on the plate, and in contrast if the spores are not germinated, we can expect colonies in the plate because spores are heat resistant and get activated by heat.

2.3.5 Statistical analyses. The student's t-test was used for specific comparisons

2.4. Results:

Germination of spores of *C. perfringens* SM101 with Ca-DPA, DPA and Ca^{2+}.

Previous studies have shown that in *Bacillus* and *Clostridium* species exogenous Ca-DPA significantly induce spores germination [36, 66, 83]. However, it is unclear whether Ca-DPA or single Ca^{2+} or DPA, can induce bacterial spore germination. Recent studies on *C. difficile* demonstrated that exogenous Ca^{2+} with co-germinant taurocholate can efficiently induce spore germination [36]. We assumed this could also be true for *C. perfringens* spores. To confirm our hypothesis, we first tested germination of spores of *C. perfringens* FP wild type strain SM101 in the presence of 50mM Ca-DPA (pH 8.0), 50 mM DPA (pH 6.0), and 50 mM $CaCl_2$ (pH 6.0). Similar germination was also carried out with 100 mM KCl (pH 6.0) as a positive control and with 25mM Tris HCL buffer (pH 6.0) as a negative control.

As expected, *C. perfringens* SM101 spores germinated well with KCL, i.e., a significant OD_{600} decrease (~60%) was observed when SM101 spores was incubated with 100 mM KCl (pH 6.0) at 37 °C for 1.5 h. However, under similar germination assay, SM101 spores did not germinate with 25mM Tris-HCL (pH 6.0), as ~10-15% OD_{600} decrease was observed (Fig. 2.1). Interestingly, when SM101 spores incubated with Ca-DPA or $CaCl_2$, ~ 50% or 55% OD_{600} decrease, respectively, was observed. These results indicated that either Ca-DPA or $CaCl_2$ can induce significant level of spore germination in *C. perfringens* SM101,

although not at the level with KCl. In contrast, DPA alone did not induce germination of spores of SM101, as no significant difference in OD_{600} decrease was observed between SM101 incubated with DPA versus 25mM Tris-HCL (pH 6.0). These results are confirmed by using phase-contrast microscopy, ~ 80-95% of SM101 spores became phase-dark after 90 minutes of incubation with KCl, Ca-DPA or $CaCl_2$, while ~ 90% of SM101 spores remained phase bright in the presence of 25mM Tris-HCL (pH 6.0) or DPA (data not shown).

For further confirmation of germination, we evaluated the colony formation by heat-treated germinated samples. As vegetative cells are heat sensitive, fully-germinated spores should be killed at 100 °C and thus should not produce any colonies after plating onto brain heart infusion (BHI) plate and incubated at 37 °C overnight. As expected, Ca-DPA- or $CaCl_2$-germinated samples treated at 100 °C for 20 min did not form any colony on the agar plate (data not shown), indicating that the spores in the samples were 100% germinated and thus killed at 100 °C. In contrast, DPA-germinated samples heated at 100 °C for 20 min generated colonies on BHI agar at a level similar to that should be generate from non-germinated original spores in the sample, indicating that the spores in the sample remained un-germinated. Collectively, these findings indicated that $CaCl_2$ alone is sufficient to induce spore germination in C. perfringens SM101.

Germination of *C. perfringens* SM101 spore with different pH and concentration of $CaCl_2$.

In order to identify optimum pH and concentration of $CaCl_2$ that can induce maximum rate of germination of *C. perfringens* spores. The $CaCl_2$ germination was tested at pH ranging from 4.0 to 9.0 with 25mM Tris HCL buffer [Fig.2.2A-B]. According to our results, pH 6.0 at 50mM $CaCl_2$ was the optimum condition for $CaCl_2$ germination [Fig.2.2A]. The extent of germination was significantly ($P < 0.05$) higher at pH 6.0 and no significant ($P > 0.05$) germination was observed at below pH 6.0 [Fig.2.2B]. These results are confirmed by using phase-contrast microscopy, as ~ 90% of SM101 spores became phase-dark after 60 minutes of incubation with $CaCl_2$ at pH 6.0 (data not shown).

C. perfringens SM101 spores were able to germinate significantly after 60 minutes with $CaCl_2$ concentrations at 50 mM and 100 mM [Fig. 2.2C]. The extent of germination was significantly ($P < 0.05$) higher with 50 mM $CaCl_2$ and no significant germination was observed at below or above 50 mM $CaCl_2$ [Fig.2.1D]. These results are confirmed by using phase-contrast microscopy; ~ 90% of SM101 spores became phase-dark after 60 minutes of incubation with 50 mM $CaCl_2$ (pH 6.0) (data not shown). Collectively, these results show that 50mM $CaCl_2$ at pH 6.0 is the optimum condition to induce best germination of *C. perfringens* SM101 spores.

Germination of *C. perfringens* SM101 spores with different calcium salts.

We initially found that 50 mM $CaCl_2$ (pH 6.0) is able to induce highest level of *C. perfringens* SM101 spore germination. To determine if other exogenous Ca^{+2}, not chloride can induce *C. perfringens* SM101 spore germination, spores were incubated with 50mM (pH 6.0) of each of $CaCl_2$, $C_6H_{10}CaO_6$, and Ca $(NO_3)_2$, and DPA; 50mM Ca-DPA (pH 8.0) , 100mM KCl (pH 6.0), and 25mM Tris-HCl (pH 6.0).

Our result indicated that SM101 spores were germinated, as expected, in the presence of two positive controls, KCL, and Ca-DPA, (~60% and 40% decrease in OD_{600} expressed as a percentage of initial, respectively [Fig.2.3A]. Under similar experimental conditions, all tested Ca^{2+} ions [$CaCl_2$, Ca $(NO_3)_2$, and $C_6H_{10}CaO_6$] induced germination of SM101 spores although at variable levels; 55%, 50% and 35% decrease in OD_{600} expressed as a percentage of initial, respectively. While, as expected, SM101 spores did not germinate with negative control buffer or DPA alone (8% and 10%, respectively, decrease in OD_{600} expressed as a percentage of initial) [Fig.2.3A]. These results are confirmed by using phase-contrast microscopy; ~ 90% of SM101 spores became phase-dark after 60 minutes of incubation with 50 mM of $CaCl_2$, Ca $(NO_3)_2$, and $C_6H_{10}CaO_6$ at pH 6.0 (data not shown).

To determine if Ca^{2+} can induce spore germination in other FP isolates, we tested two other FP strains [NCTC10239, and NCTC 8239] using a similar experimental condition [Fig2.3B]. Our results showed that spores of both strains germinated with different Ca^{2+} ions similarly as with SM101 spores, as 35-40% decrease of OD_{600} expressed as a percentage of initial. These results are confirmed by using phase-contrast microscopy ~ 85-90% of both FP strains' spores became phase-dark after 60 minutes of incubation with 50 mM of each [$CaCl_2$, Ca $(NO_3)_2$, and $C_6H_{10}CaO_6$] at pH 6.0 (data not shown). Collectively, these results suggest that different Ca^{2+} ions are able to induce spore germination in most *C. perfringens* FP strains.

Germination of *C. perfringens* SM101 spores with other spore core-specific divalent cations.

Previous studies demonstrated that, in *C. botulinum,* magnesium sulfate increases the rate of spore germination and plays a minor role in inducing *C. difficile* spore germination [36, 72]. Other studies suggested that minerals have a role in accelerating germination process in *Bacillus megaterium* spore germination by 93% very rapidly in the presence of $MnCl_2$ [41]. In addition, studies demonstrated that in *C. difficile* Mn^{+2} has no role in spore germination, while other studies with *C. perfringens* NCTC 8238 spores found minor germination with Mn^{+2} (16% loss in OD_{600}) without adjusting pH [2, 36].

To test whether other spore core-specific divalent cations can induce *C. perfringens* spore germination, we incubated *C. perfringens* SM101 spores with 50mM (pH6.0) of each of $MgCl_2$, $MgSO_4$, $MnCl_2$, $ZnCl_2$, $CaCl_2$; and with 100mM KCL (pH 6.0) (as a positive control), with 25mM Tris-HCL (pH 6.0) and 50mM DPA (pH 6.0) (as a negative controls).

Our results show that, i) *C. perfringens* SM101 spores germinated in the presence of $MgCl_2$, $MnCl_2$ and $MgSO_4$; 40%, 50% and 35% loss of OD_{600} expressed as a percentage of initial, respectively [Fig.2.4]. These results are confirmed by using phase-contrast microscopy ~ 80-85% of SM101 spores became phase-dark after 60 minutes of incubation (data not shown). However, *C. perfringens* SM101 spores did not germinate in the presence of $ZnCl_2$ and Tris-HCL buffer; 13% and 10% loss of OD_{600} expressed as a percentage of initial, respectively [Fig.2.4]. These results are confirmed by using phase-contrast microscopy ~ 90% of SM101 spores remained phase-bright after 60 minutes of incubation with $ZnCl_2$ and Tris-HCL buffer (data not shown). Collectively, these results indicated that spore core-specific divalent cations can trigger spore germination in *C. perfringens*.

Germination of *C. perfringens* spores with different pH and concentration of $MgCl_2$ and $MnCl_2$.

In order to identify optimum pH and concentrations that can induce germination of *C. perfringens* spores in the presence of $MgCl_2$ and $MnCl_2$, the

germinant solution was tested at pH ranging from 4.0 to 9.0 [Fig.2.5A-B]. Our results indicated that the optimum conditions for Mg^{+2} and Mn^{+2} were at pH 6.0 (50mM) [Fig.2.2A]. The extent of germination was significantly ($P < 0.05$) higher at pH 6.0 [Fig.2.5B]. These results are confirmed by using phase-contrast microscopy ~ 85-90% of SM101 spores became phase-dark after 60 minutes of incubation with 50 mM of $MgCl_2$, or $MnCl_2$ at pH 6.0 (data not shown). Overall, $MgCl_2$ and $MnCl_2$ can efficiently induce SM101 spore germination similarly as with $CaCl_2$. These results confirm that divalent cations are sufficient to induce *C. perfringens* spore germination at pH 6.0.

In order to identify optimum concentration of $MgCl_2$ and $MnCl_2$ that can induce germination of *C. perfringens* spores, the germinant solution was tested at various concentrations ranging from 5mM to 250mM [Fig.2.5C-D]. *C. perfringens* SM101 spores were able to germinate significantly after 60 minutes of incubation with 50 mM of each of $MgCl_2$ or $MnCl_2$ [Fig. 2.5C]. The extent of germination was significantly ($P < 0.05$) higher at 50 mM and no significant germination was observed with higher or less than 50 mM [Fig.2.5D]. These results are confirmed by using phase-contrast microscopy ~ 85-90% of SM101 spores became phase-dark after 60 minutes of incubation with 50 mM of $MgCl_2$ or $MnCl_2$ at pH 6.0 (data not shown). These results show that the optimum concentration to induce SM101 spore germination of both Mg^{+2} and Mn^{+2} were at 50mM, pH 6.0.

To investigate whether Mg^{2+} and Mn^{2+} can also induce germination of spores of other FP strains, we incubated spores of FP strains NCTC10239 or NCTC 8239 with 50 mM of $MgCl_2$. $MgSO_4$ or $MnCl_2$ for 60 mins at 37 C and OD_{600} decrease measured [Fig2.6]. Our result found that both strains germinated well with Mg^{2+} and Mn^{2+} similarly to each other with slight differences; 30% and 35% decrease of OD_{600}. These results are confirmed by phase-contrast microscopy, ~ 75-80% spores became phase-dark after 60 minutes of incubation with 50 mM of $MgCl_2$, $MgSo_4$ and $MnCl_2$ at pH 6.0 (data not shown). There was a significant difference in spore germination between SM101 versus NCTC10239 and NCTC8239. SM101 spores germinated better with [$MgCl_2$, $MgSo_4$ and $MnCl_2$] compared to that of NCTC10239 and NCTC8239; 20% more germination with SM101 spores than that of NCTC10239 and NCTC8239 spores. Interestingly, SM101 and NCTC10239 spores germinated very well with $MnCl_2$ comparing to $MgCl_2$ and $MgSo_4$.

Exogenous Ca^{2+} is essential for *C. perfringens* SM101 spore germination.

As our results showed that exogenous Ca^{2+} alone is able to induce spore germination in all FP isolates [Fig.2.3A-B], we aimed to understand the role of endogenous Ca^{2+} in the germination process. To examine if the endogenous or exogenous Ca^{2+} is needed to induce spore germination, spores of wild-type SM101

were incubated with 100 mM KCl (pH 6.0) and mixtures of [100mM KCL and 50mM EGTA], [100mM KCL and 50mM $CaCl_2$-EGTA], and [50mM $CaCl_2$-EGTA], where EGTA is calcium specific chelator [Fig.2.7].

Our results showed that, in the presence of mixtures [KCl and EGTA], SM101 spores germinated well, meaning that the release of Ca^{2+} from the spore-core was not required for inducing spore germination. However, in the presence of a mixture of [$CaCl_2$-EGTA and KCL], a reduced level spore germination was observed, meaning that the extra calcium in the present of EGTA decreasing fall of OD_{600} after 20 minutes of incubation. Finally, in the presence of a mixture of [Ca^{+2}-EGTA], SM101 spore germination is blocked, meaning that the extra calcium is needed to initiate spore germination. Collectively, our results indicated that the exogenous, but not the endogenous, Ca^{2+} is essential to initiate spore germination.

Exogenous Mg^{2+} and both exogenous and endogenous Mn^{2+} are essential for *C. perfringens* SM101 spore germination.

To examine if the endogenous or exogenous Mg^{2+} is needed to induce spore germination, spores of wild-type SM101 were incubated with 100 mM KCL, or mixtures of [100mM KCl and 50mM EDTA], [100mM KCL and 50mM $MgCl_2$-EDTA], [50mM $MgCl_2$-EDTA], where EDTA is a general metal ion chelator [Fig.2.8A].

Our results showed that, SM101 spore germinated well in the presence of mixtures [KCl and EDTA] (40% decrease of OD_{600}), meaning the release of Mg^{+2} from the core spores is not required for inducing SM101 spore germination. However, in the presence of a mixtures of [KCL and $MgCl_2$-EDTA], SM101 spores just stopped germinating after 30 minutes by (22% decrease of OD_{600}), and with mixtures of [$MgCl_2$-EDTA-50mM] spores germination is completely blocked, meaning that the extra magnesium is needed to initiate spore germination. Collectively, our results indicated that the exogenous, but not the endogenous, Mg^{2+} is essential to initiate spore germination.

To examine if the endogenous or exogenous Mn^{2+} is needed to induce spore germination, spores of wild-type SM101 were incubated with 100mM KCL, and a mixtures of [100mM KCl and 50mM EDTA], [100mM KCL and 50mM $MnCl_2$-EDTA], and [50mM $MnCl_2$-EDTA] [Fig.2.8B]. Our results indicated that in the presence a mixture of [KCl and EDTA], spores germinated well reducing by ~40% decrease of OD_{600}, meaning that the release of Mn^{2+} from the spore-core is not required for start inducing SM101 spore germination. Surprisingly, in the presence of [KCL and $MnCl_2$-EDTA] or [$MnCl_2$ and EDTA] SM101 spores completely stopped germinating, which is different than Mg^{2+} and Ca^{2+} pathway. These results suggested that both exogenous and endogenous Mn^{2+} is essential to initiate spore germination.

2.5 Discussion

The previous studies indicated that the ability of FP isolates to be adapted to the environment much higher than other isolates of *C. perfringens* [54, 62]. In addition, the great ability of FP isolates to grow in meat products may be due to the presence of appropriate ionic media, for example potassium is found as a higher percentage then the sodium, magnesium, manganese and zinc, respectively with different proportions depending on the type of meat [34, 54]. Therefore, the significance of studying and understanding FP isolates' germination pathways should limit their consequences. Recent studies have shown that bacterial spores from *Bacillus* and Clostridium species are capable of germinating in an ionic medium [17, 28, 36, 41]. In *C. difficile,* calcium ions have been known to enhance spore's germination more than any other divalent or monovalent cations in the presence of taurocholate, which is needed as a co-germinant for *C. difficile* spores [28]. While in *C. perfringens* potassium ion, sodium ion, and calcium chelated DPA are able to trigger spore's germination either as germinants or co-germinants [51, 54]. Generally, there is a broad range of ionic types that are able to enhance spore germination possibly because ions have some catalytic or physical function [17].

However, we did not clarify if the calcium ion or DPA alone is able to trigger *C. perfringens* spore germination. There might be some role of divalent cations, that present in the meat products or in the spore core, in spore germination.

The results of this study suggested that the spore core-specific, but not non-spore specific, divalent cations contributed to stimulating spore germination. The possible explanation for that is the divalent cations could be essential to activate cortex-lytic enzymes, which is important to initiate spore germination [41]. Another explanation could be that specific spore core-specific divalent cation such as Ca^{2+} and Mg^{2+} are needed to release DPA from the spore core, which is also important in the germination process [2]. Nevertheless, the effect of ions on spore germinating tend to differ greatly between spore-forming bacteria. For instance, Mn^{2+} shows real enhancement in *B. megatherium* spores' germination [41] and in *C. perfringens*, while in *C. difficile* there is no effect of Mn^{2+} [36]. We do not have a comprehensive explanation of about the reason behind these differences between spore-forming bacteria.

In several studies spore germination stages of *Bacillus* species have been studied well [83, 88, 92]. However, we still do not have detailed knowledge about molecular signals that can initiate bacterial spore germination process. So, the other objective of this study is to understand the endogenous and exogenous molecule signal specifically divalent cations. Our results indicated that the internal calcium

and magnesium signal is not important to complete germination, but the external signals of them are needed to induce spore germination. Interestingly, both internal and external signals of manganese play important role in inducing spores' growth. The possible reason for the different sign of manganese during the stage of induction of spore germination is due to the importance of manganese in cortex-lytic enzymes necessary for completing the spore germination process. Further studies are needed at molecular and protein levels to help to understand this difference in these ionic pathways during the germination process.

Figures

Fig.2.1 Germination of spores of *C. perfringens* SM101with DPA, Ca-DPA, $CaCl_2$.

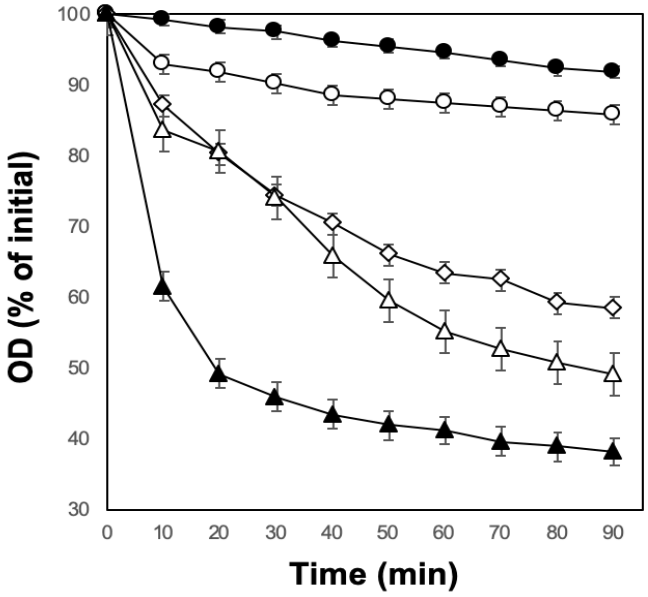

Fig.2.1 Germination of spores of *C. perfringens* SM101 with DPA, Ca-DPA, and $CaCl_2$. Heat-activated spores of strain SM101 were germinated for 90 min at 37°C with 25mM Tris-HCl buffer (pH 6.0) (filled circle), 50mM DPA (pH 6.0) (open circle), 50mM Ca-DPA (pH 8.0) (open diamonds), 100mM KCl (pH 6.0) (filled triangles), 50mM $CaCl_2$ (pH 6.0) (open triangles). Error bars represent standard deviations from the mean of at least duplicate experiments with three independent spore preparations.

Fig 2.2 A-D Effect of pH and concentration on $CaCl_2$ induced germination of *C. perfringens* spores.

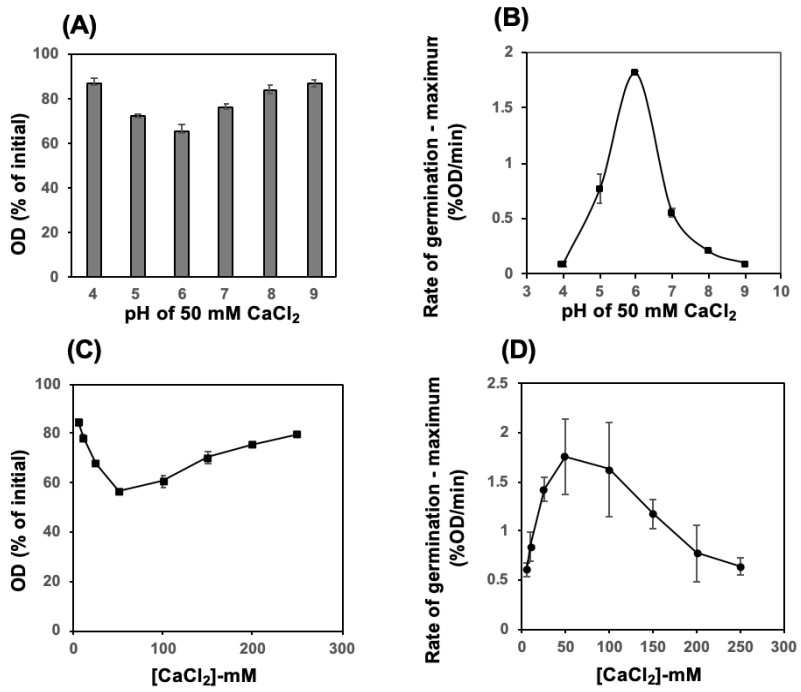

Fig 2.2 A-D Effect of pH and concentration on $CaCl_2$ germination of *C. perfringens* SM101. Heat-activated spores of SM101 were incubated with 50 mM $CaCl_2$ in 25 mM Tris-HCl at various adjusted pHs. The extents of germination (A) after 60 min of incubation at 37 °C and maximum rates of germination (B) were calculated as described in Material and methods. Error bars represent standard deviations from the mean of triplicate experiments with three independent spore preparations, (C, D) calcium chloride (pH 6.0) at various concentrations and extent of germination after 60 min (C) and maximum rates of germination (D) were calculated as

described in materials and methods. Error bars represent standard deviations from the mean of triplicate experiments with three independent spore preparations.

Fig.2.3 A-B Germination of spores of *C. perfringens* FP isolates with different calcium salts.

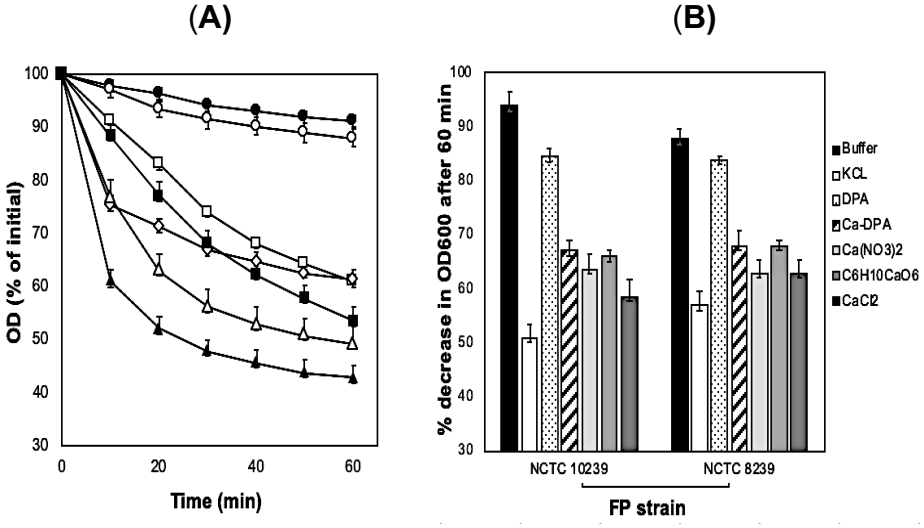

Fig.2.3 A-B Germination of spores of *C. perfringens* SM101 (A) and other FP strains (NCTC10239 and NCTC8239) (B) with DPA and various Ca^{2+}. Heat-activated spores of strains SM101, NCTC10239 and NCTC8239 were germinated at 37°C with 100 mM KCl (pH6.0) (filled triangles, white bars), 50 mM $CaCl_2$ (pH 6.0) (open triangles, dark gray bar), 50 mM $Ca(NO_3)_2$ (pH 6.0) (filled squares, light gray bar), 50 mM $C_6H_{10}CaO_6$ (pH 6.0) (open squares, medium gray bar), 50 mM Ca-DPA (pH 8.0) (open diamonds, strips bar), 50 mM DPA (pH 6.0) (open circle, dot bar), and 25mM Tris-HCl (pH 6.0) (filled circle, black bar). At various times, the OD_{600} was measured as described in Methods. Error bars represent standard deviations from the mean of at least duplicate experiments with three independent spore preparations.

Fig. 2.4 Germination of *C. perfringens* SM101 spores with other divalent cations.

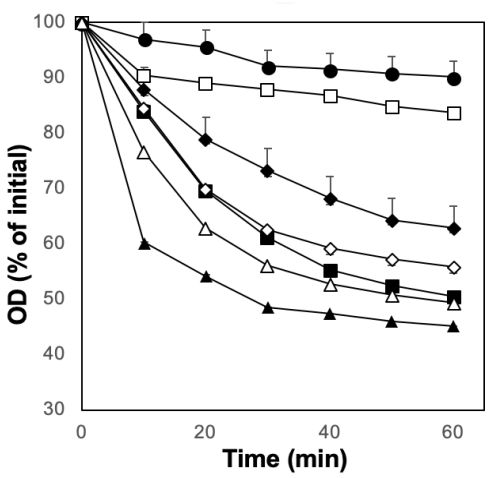

Fig. 2.4 Germination of *C. perfringens* SM101 spores with other divalent cations. Heat-activated spores of SM101 were incubated in 25mM Tris-HCl buffer (pH 6.0) with 100mM KCl (pH 6.0) (filled triangle), 50 mM $CaCl_2$ (pH 6.0) (open triangle), 50 mM $MnCl_2$ (pH 6.0) (filled square), 50 mM $MgCl_2$ (pH 6.0) (open diamond), 50 mM $MgSO_4$ (pH 6.0) (filled diamond), 25mM Tris-HCL buffer (pH 6.0) (filled circle), 50 mM $ZnCl_2$ (pH 6.0) (open square). Error bars represent standard deviations from the mean of at least duplicate experiments with three independent spore preparations.

Fig 2.5 Effect of pH and concentration on $MgCl_2$- and $MnCl_2$-germination of *C. perfringens* spores.

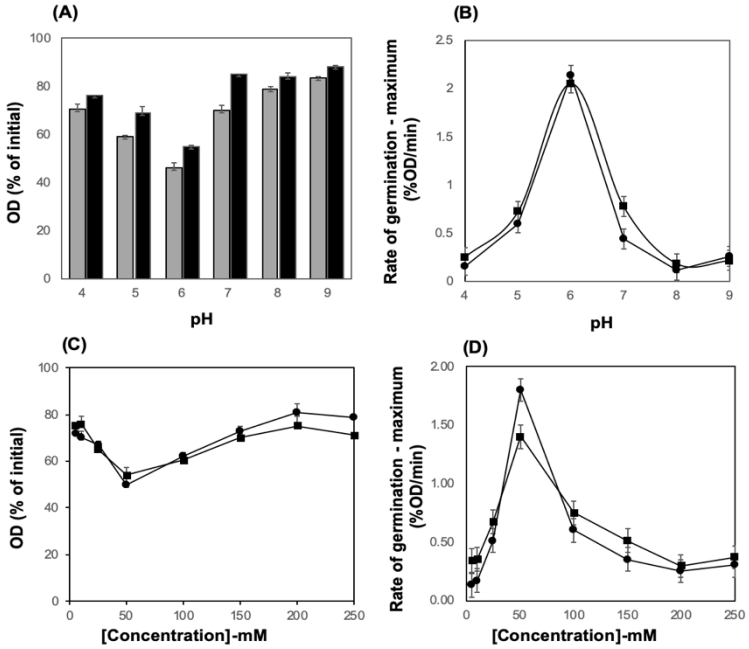

Fig 2.5 A-B Effect of pH and concentration on $MgCl_2$- and $MnCl_2$-germination of *C. perfringens* SM101 spores. (A-B) Heat-activated spores of SM101 incubated with 50mM $MnCl_2$ (grey bar and filled square) and 50mM $MgCl_2$ (black bar and filled circle) at various adjusted pHs. The extents of germination (A) after 60 min of incubation at 37°C and maximum rates of germination (B) were calculated as described in Material and methods. Error bars represent standard deviations from the mean of triplicate experiments with three independent spore preparations. (C-D) Heat-activated spores of SM101 incubated at pH 6.0 with various concentrations of $MnCl_2$ (filled square) and $MgCl_2$ (filled circle). The extents of germination (C)

after 60 min of incubation at 37°C and maximum rates of germination (D) were calculated as described in Material and methods. Error bars represent standard deviations from the mean of triplicate experiments with three independent spore preparations.

Fig. 2.6 Germination of spores of *C. perfringens* FP isolates with divalent cations.

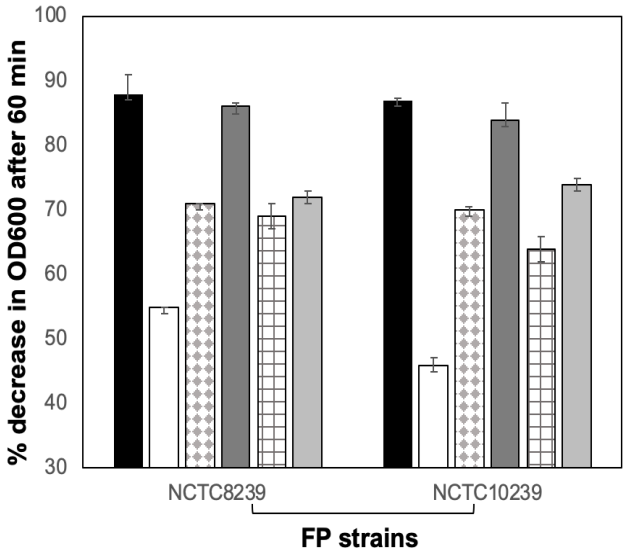

Fig. 2.6 Germination of spores of *C. perfringens* FP isolates with Mg^{2+} and Mn^{2+} cations. Heat-activated spores incubated with 50 mM prepared with Tris HCL buffer at pH 6.00 buffer (black bars), 100mM KCL pH 6.0 (white bar), 50 mM $MnCl_2$ pH 6.0 (large grid bar), 50mM $MgSo_4$ pH 6.0 (light gray bar), 50mM $MgCl_2$ pH 6.0 (diamond bar), 50mM $ZnCl_2$ pH 6.0 (dark gray bar).Error bars represent standard deviations from the mean of at least duplicate experiments with three independent spore preparations.

Fig.2.7 Germination of *C. Perfringens* SM101 spore in the presence of Ca^{2+} and specific Ca^{2+} blocker (EGTA).

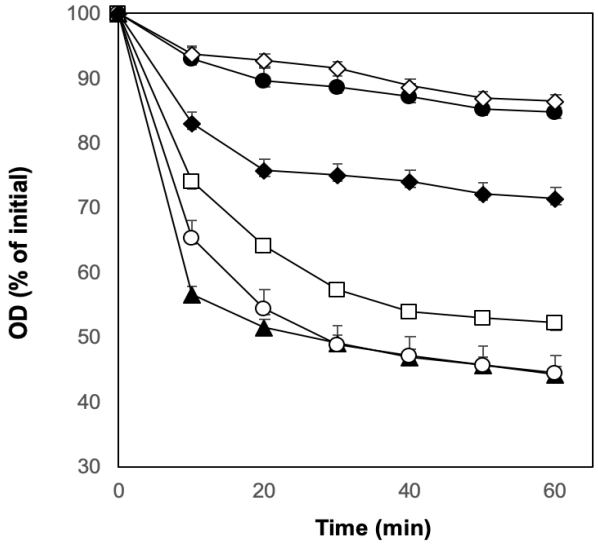

Fig.2.7 Germination of *C. perfringens* SM101 spores in the presence of Ca^{2+} and specific Ca^{2+} blocker (EGTA). Heat-activated spores were incubated for 60 min at 37°C with 50 mM Tris-HCl buffer (pH 6.0) (filled circle), 100 mM KCl (filled triangle), 50mM EGTA-100mMKCl (open circle), 50 mM $CaCl_2$ (open squares), 50 mM EGTA-$CaCl_2$ and 100 mM KCl (filled diamond), 50 mM $CaCl_2$-EGTA (open diamond) and OD_{600} was measured. Error bars represent standard deviations from the mean of at least duplicate experiments with two independent spore preparations.

Fig.2.8 Germination of *C. perfringens* SM101 spores in the presence of the $MgCl_2$, $MnCl_2$, and general metal ion blocker (EDTA).

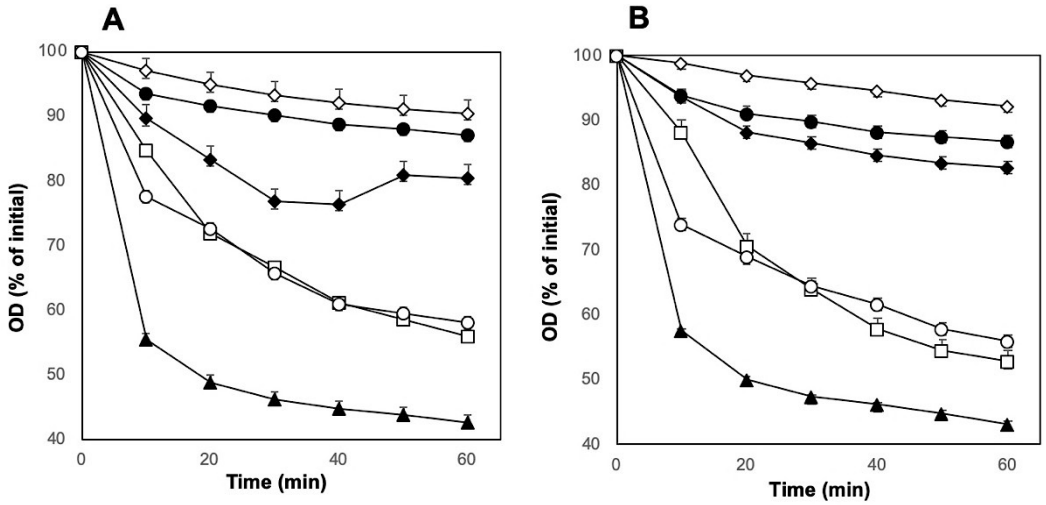

Fig.2.8 Germination of *C. perfringens* SM101 spores in the presence of $MgCl_2$, $MnCl_2$, and general metal ion blocker (EDTA). Heat-activated spores incubated with 50 mM prepared with Tris HCL buffer at pH 6.00 for 60 min at 37°C. (A) KCL (filled triangle), 50mM EDTA- 100mM KCL (open circle), 50mM $MgCl_2$ (open squares), 50mM EDTA-$MgCl_2$ and 100mM KCL (filled diamond), 50mM $MgCl_2$-EDTA (open diamond), and 25mM Tris HCL buffer (filled circle), (B) 100mM KCL (filled triangle), 50mM EDTA- 100mM KCL (open circle), 50 mM $MnCl_2$ (open squares), 50mM EDTA-$MnCl_2$ and 100mM KCL (filled diamond), 50mM $MnCl_2$-EDTA (open diamond), and 25mM Tris HCL buffer (filled circle). Error bars represent standard deviations from the mean of at least duplicate experiments with two independent spore preparations.

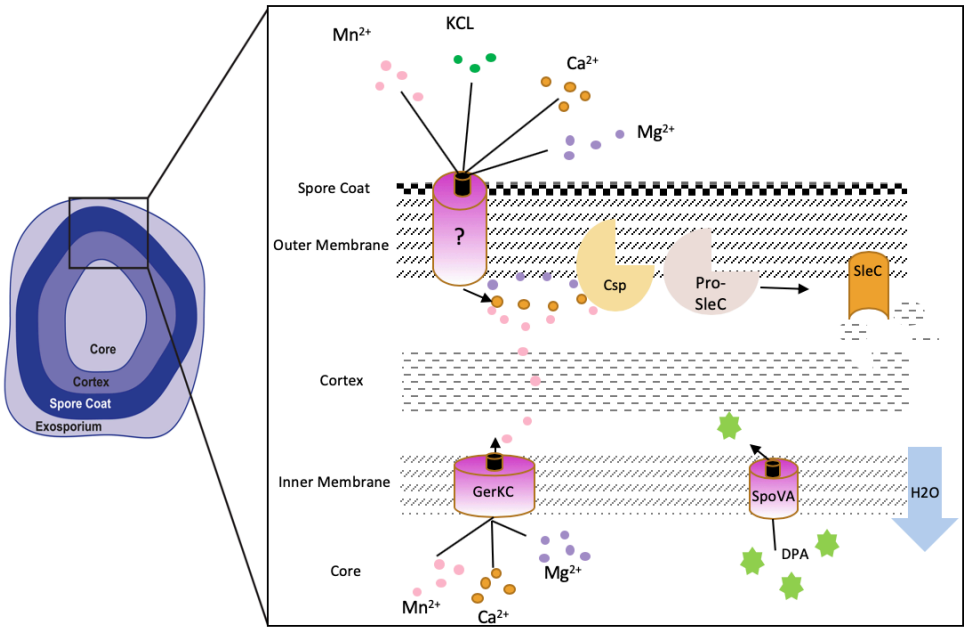

Fig.2.9 Possible model of divalent cations induced germination pathway in *C. perfringens* FP isolates**.**

Chapter 3

Conclusion

Clostridium perfringens is an anaerobic bacterium that is able to produce metabolically dormant spores, which can tolerate environmental stresses and survive for many years. When the environment is favorable, *C. perfringens* spores germinate and cause the disease. The germination process is initiated when bacterial spores sense a variety of chemicals, including salts, amino acids, cations and enzymes. Previous study has shown that dipicolinic acid (DPA) chelated with calcium (Ca-DPA) can stimulate significantly spore germination in *C. perfringens*. However, it is unclear whether Ca^{2+} or DPA alone can induce spore germination. Therefore, in this study we aimed to evaluate the possible role of Ca^{2+} and other divalent cations present in spore core (Mn^{2+} and Mg^{2+}) in germination of *C. perfringens* spores.

Our study demonstrates that (i) Ca-DPA, but DPA alone, induced germination of spores of *C. perfringens*, suggesting that Ca^{2+} might have signaling role in spore germination; ii) all tested calcium salts (calcium-chloride, -carbonate, or -nitrate) induced spore germination, indicating that Ca^{2+} ion is critical for spore germination; (iii) other spore-specific divalent cations (Mn^{2+}, and Mg^{2+}), but not Zn^{2+}, could induce spore germination, suggesting that all spore core-specific

divalent cations are involved in *C. perfringens* spore germination. (iv) endogenous Ca^{2+} and Mg^{2+} are not necessary for *C. perfringens* spore germination; surprisingly, exogenous and partly endogenous Mn^{+2} is needed to induce spore germination.

Bibliography:

1. Amaha M, Ordal ZJ (1957) effect of divalent cations in the sporulation medium on the thermal death rate of *Bacillus* coagranulas var. thermoacidurans. J Bacteriol 74:596–604
2. Ando Y (1974) Ionic germination of spores of *Clostridium perfringens* type A. Jpn J Microbiol 18:433–439. doi: 10.1111/j.1348-0421.1974.tb00831.x
3. Banawas S, Korza G, Paredes-Sabja D, Li Y, Hao B, Setlow P, Sarker MR (2015) Location and stoichiometry of the protease CspB and the cortex-lytic enzyme SleC in *Clostridium perfringens* spores. Food Microbiol 50:83–87. doi: 10.1016/j.fm.2015.04.001
4. Banawas S, Paredes-Sabja D, Korza G, Li Y, Hao B, Setlow P, Sarker MR (2013) The *Clostridium perfringens* Germinant Receptor Protein GerKC Is Located in the Spore Inner Membrane and Is Crucial for Spore Germination. J Bacteriol 195:5084–5091. doi: 10.1128/JB.00901-13
5. Banawas S, Sarker MR (2018) l-lysine (pH 6.0) induces germination of spores of *Clostridium perfringens* type F isolates carrying chromosomal or plasmid-borne enterotoxin gene. Microb Pathog 123:227–232. doi: 10.1016/j.micpath.2018.07.022

6. Beaman TC, Gerhardt P (1986) Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl Environ Microbiol 52:1242–1246
7. Bender GR, Marquis RE (1985) Spore heat resistance and specific mineralization. Appl Environ Microbiol 50:1414–1421
8. Bhunia AK (2018) *Clostridium botulinum, Clostridium perfringens, Clostridium difficile.* In: Bhunia AK (ed) Foodborne Microbial Pathogens: Mechanisms and Pathogenesis. Springer, New York, NY, pp 209–228
9. Chen Y, Miyata S, Makino S, Moriyama R (1997) Molecular characterization of a germination-specific muramidase from Clostridium perfringens S40 spores and nucleotide sequence of the corresponding gene. J Bacteriol 179:3181–3187
10. Collie RE, Kokai-Kun JF, McClane BA (1998) Phenotypic Characterization of Enterotoxigenic *Clostridium perfringens* Isolates from Non-foodborne Human Gastrointestinal Diseases. Anaerobe 4:69–79. doi: 10.1006/anae.1998.0152
11. Collie RE, McClane BA (1998) Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. J Clin Microbiol 36:30–36
12. Córdoba MG, Aranda E, Medina LM, Jordano R, Córdoba JJ (2001) Differentiation of *Clostridium perfringens* and *Clostridium botulinum* from non-toxigenic clostridia, isolated from prepared and frozen foods by PCR-DAN based methods.

Food Nahr 45:125–128. doi: 10.1002/1521-3803(20010401)45:2<125::AID-FOOD125>3.0.CO;2-8

13. Cowan AE, Koppel DE, Setlow B, Setlow P (2003) A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: Implications for spore dormancy. Proc Natl Acad Sci 100:4209–4214. doi: 10.1073/pnas.0636762100
14. Curran HR, Brunstetter BC, Myers AT (1943) Spectrochemical Analysis of Vegetative Cells and Spores of Bacteria. J Bacteriol 45:485–494
15. Driks A, Setlow P, Setlow P (2000) Morphogenesis and Properties of the Bacterial Spore. Prokaryotic Dev 191–218. doi: 10.1128/9781555818166.ch9
16. Duncan CL, Strong DH (1968) Improved medium for sporulation of *Clostridium perfringens*. Appl Microbiol 16:82–89
17. Fleming HP, Ordal ZJ (1964) RESPONSES OF *BACILLUS SUBTILIS* SPORES TO IONIC ENVIRONMENTS DURING SPORULATION AND GERMINATION1. J Bacteriol 88:1529–1537. doi: 10.1128/JB.88.6.1529-1537.1964
18. Foerster HF, Foster JW (1966) Response of *Bacillus* Spores to Combinations of Germinative Compounds. J Bacteriol 91:1168–1177
19. Fujita K, Katahira J, Horiguchi Y, Sonoda N, Furuse M, Tsukita S (2000) *Clostridium perfringens* enterotoxin binds to the second extracellular loop of

claudin-3, a tight junction integral membrane protein. FEBS Lett 476:258–261. doi: 10.1016/s0014-5793(00)01744-0

20. Gould, G. W., and A. Hurst. (1969) The bacterial spore.
21. Granger AC, Gaidamakova EK, Matrosova VY, Daly MJ, Setlow P (2011) Effects of Mn and Fe Levels on *Bacillus subtilis* Spore Resistance and Effects of Mn^{2+}, Other Divalent Cations, Orthophosphate, and Dipicolinic Acid on Protein Resistance to Ionizing Radiation. Appl Environ Microbiol 77:32–40. doi: 10.1128/AEM.01965-10
22. Grant KA, Kenyon S, Nwafor I, Plowman J, Ohai C, Halford-Maw R, Peck MW, McLauchlin J (2008) The identification and characterization of *Clostridium perfringens* by real-time PCR, location of enterotoxin gene, and heat resistance. Foodborne Pathog Dis 5:629–639. doi: 10.1089/fpd.2007.0066
23. Grass JE, Gould LH, Mahon BE (2013) Epidemiology of foodborne disease outbreaks caused by *Clostridium perfringens*, United States, 1998-2010. Foodborne Pathog Dis 10:131–136. doi: 10.1089/fpd.2012.1316
24. Gupta KG, Sidhu R, Yadav NK (1971) effect of monovalent and divalent ions upon the germination of *Bacillus* spores in the presence of nisin. J Food Sci 36:896–898. doi: 10.1111/j.1365-2621.1971.tb15555.x
25. Guzewich JJ, Bryan FL, Todd ECD (1997) Surveillance of Foodborne Disease I. Purposes and Types of Surveillance Systems and Networks. J Food Prot 60:555–566. doi: 10.4315/0362-028X-60.5.555

26. Hatheway CL (1990) Toxigenic clostridia. Clin Microbiol Rev 3:66–98
27. Hoffmann S, Batz MB, Morris JG (2012) Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J Food Prot 75:1292–1302. doi: 10.4315/0362-028X.JFP-11-417
28. Hudson KD, Corfe BM, Kemp EH, Feavers IM, Coote PJ, Moir A (2001) Localization of GerAA and GerAC Germination Proteins in the *Bacillus subtilis* Spore. J Bacteriol 183:4317–4322. doi: 10.1128/JB.183.14.4317-4322.2001
29. Igura N, Kamimura Y, Islam MS, Shimoda M, Hayakawa I (2003) Effects of minerals on resistance of *Bacillus subtilis* spores to heat and hydrostatic pressure. Appl Environ Microbiol 69:6307–6310. doi: 10.1128/aem.69.10.6307-6310.2003
30. Ireland JAW, Hanna PC (2002) Amino Acid- and Purine Ribonucleoside-Induced Germination of *Bacillus anthracis* ⌬Sterne Endospores: gerS Mediates Responses to Aromatic Ring Structures. J BACTERIOL 184:8
31. Jay JM, Loessner MJ, Golden DA. 2 (2005) Modern Food Microbiology
32. Jaye M, Ordal ZJ (1965) Germination of Spores of *Bacillus megaterium* with Divalent Metal-Dipicolinate Chelates. J Bacteriol 89:1617–1618
33. Kiu R, Caim S, Painset A, Pickard D, Swift C, Dougan G, Mather AE, Amar C, Hall LJ (2019) Phylogenomic analysis of gastroenteritis-associated *Clostridium perfringens* in England and Wales over a 7-year period indicates distribution of clonal toxigenic strains in multiple outbreaks and extensive involvement of

enterotoxin-encoding (CPE) plasmids. Microb Genomics 5:e000297. doi: 10.1099/mgen.0.000297

34. Kiu R, Hall LJ (2018) An update on the human and animal enteric pathogen *Clostridium perfringens*. Emerg Microbes Infect7.doi:10.1038/s41426-018-0144-8
35. Kochan TJ, Foley MH, Shoshiev MS, Somers MJ, Carlson PE, Hanna PC (2018) Updates to *Clostridium difficile* Spore Germination. J Bacteriol 200. doi: 10.1128/JB.00218-18
36. Kochan TJ, Somers MJ, Kaiser AM, Shoshiev MS, Hagan AK, Hastie JL, Giordano NP, Smith AD, Schubert AM, Carlson PE, Hanna PC (2017) Intestinal calcium and bile salts facilitate germination of *Clostridium difficile* spores. PLoS Pathog 13:e1006443. doi: 10.1371/journal.ppat.1006443
37. Kokai-Kun JF, Songer JG, Czeczulin JR, Chen F, McClane BA (1994) Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. J Clin Microbiol 32:2533–2539
38. Krawczyk AO, Berendsen EM, Jong A de, Boekhorst J, Wells-Bennik MHJ, Kuipers OP, Eijlander RT (2016) A transposon present in specific strains of *Bacillus subtilis* negatively affects nutrient- and dodecylamine-induced spore germination. Environ Microbiol 18:4830–4846. doi: 10.1111/1462-2920.13386

39. Laaberki M-H, Dworkin J (2008) Role of Spore Coat Proteins in the Resistance of *Bacillus subtilis* Spores to Caenorhabditis elegans Predation. J Bacteriol 190:6197–6203. doi: 10.1128/JB.00623-08

40. Labbe, R. (1989) *Clostridium perfringens*. In: Food-borne Bacterial Pathogens Marcel Decker, New York

41. Levinson HS, Sevag MG (1953) stimulation of germination and respiration of the spores of *Bacillus megatherium* by manganese and monovalent anions. J Gen Physiol 36:617–629. doi: 10.1085/jgp.36.5.617

42. Li J, McClane BA (2008) A novel small acid soluble protein variant is important for spore resistance of most *Clostridium perfringens* food poisoning isolates. PLoS Pathog 4:e1000056. doi: 10.1371/journal.ppat.1000056

43. Li J, Paredes-Sabja D, Sarker MR, McClane BA (2016) *Clostridium perfringens* Sporulation and Sporulation-Associated Toxin Production. Microbiol Spectr 4. doi: 10.1128/microbiolspec.TBS-0022-2015

44. Luisa BG (2012) Muscle as Food. Elsevier

45. McClane, B. A. (2001) *Clostridium perfringens*, p. 351-372. In M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food Microbiology: fundamentals and frontiers, 2 ed. ASM Press, Washington, D.C.

46. McClane BA (1994) *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. Toxicology 87:43–67. doi: 10.1016/0300-483x(94)90154-6

47. McClane BA (1996) An overview of *Clostridium perfringens* enterotoxin. Toxicon 34:1335–1343. doi: 10.1016/S0041-0101(96)00101-8

48. McClane BA, Chakrabarti G (2004) New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin. Anaerobe 10:107–114. doi: 10.1016/j.anaerobe.2003.11.004

49. McClane BA, Robertson SL, Li J. (2013) *Clostridium perfringens*, p 465–489 In Doyle MP, Buchanan RL. (ed), Food microbiology: fundamentals and frontiers, 4th ed ASM Press, Washington, DC

50. McClane, Bruce A.. (2007) *Clostridium perfringens*, p 423-444. In Doyle, Michael P. and Beuchat, Larry R.(ed), Food Microbiology: Fundamentals and Frontiers, Third Edition. doi:10.1128/9781555815912.ch19.

51. Moir A, Kemp EH, Robinson C, Corfe BM (1994) The genetic analysis of bacterial spore germination. J Appl Bacteriol 77:9S-16S

52. Moir A, Smith DA (1990) The genetics of bacterial spore germination. Annu Rev Microbiol 44:531–553. doi: 10.1146/annurev.mi.44.100190.002531

53. Nagler K, Moeller R (2015) Systematic investigation of germination responses of *Bacillus subtilis* spores in different high-salinity environments. FEMS Microbiol Ecol 91. doi: 10.1093/femsec/fiv023

54. Novak JS, Juneja VK, McClane BA (2003) An ultrastructural comparison of spores from various strains of *Clostridium perfringens* and correlations with heat

resistance parameters. Int J Food Microbiol 86:239–247. doi: 10.1016/s0168-1605(02)00550-0

55. Novak JS, Tunick MH, Juneja VK (2001) Heat Treatment Adaptations in *Clostridium perfringens* Vegetative Cells. J Food Prot 64:1527–1534. doi: 10.4315/0362-028X-64.10.1527
56. Olguín-Araneda V, Banawas S, Sarker MR, Paredes-Sabja D (2015) Recent advances in germination of *Clostridium* spores. Res Microbiol 166:236–243. doi: 10.1016/j.resmic.2014.07.017
57. Olsen SJ, MacKinnon LC, Goulding JS, Bean NH, Slutsker L (2000) Surveillance for foodborne-disease outbreaks--United States, 1993-1997. MMWR CDC Surveill Summ Morb Mortal Wkly Rep CDC Surveill Summ 49:1–62
58. Orsburn B, Melville SB, Popham DL (2008) Factors contributing to heat resistance of *Clostridium perfringens* endospores. Appl Environ Microbiol 74:3328–3335. doi: 10.1128/AEM.02629-07
59. Paidhungat M, Setlow B, Daniels WB, Hoover D, Papafragkou E, Setlow P (2002) Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Appl Environ Microbiol 68:3172–3175. doi: 10.1128/aem.68.6.3172-3175.2002
60. Paidhungat M, Setlow B, Driks A, Setlow P (2000) Characterization of Spores of *Bacillus subtilis* Which Lack Dipicolinic Acid. J Bacteriol 182:5505–5512. doi: 10.1128/JB.182.19.5505-5512.2000

61. Paidhungat M, Setlow P (2001) Localization of a Germinant Receptor Protein (GerBA) to the Inner Membrane of *Bacillus subtilis* Spores. J Bacteriol 183:3982–3990. doi: 10.1128/JB.183.13.3982-3990.2001

62. Paredes-Sabja D, Gonzalez M, Sarker MR, Torres JA (2007) Combined effects of hydrostatic pressure, temperature, and pH on the inactivation of spores of *Clostridium perfringens* type A and Clostridium sporogenes in buffer solutions. J Food Sci 72:M202-206. doi: 10.1111/j.1750-3841.2007.00423.x

63. Paredes-Sabja D, Raju D, Torres JA, Sarker MR (2008) Role of small, acid-soluble spore proteins in the resistance of *Clostridium perfringens* spores to chemicals. Int J Food Microbiol 122:333–335. doi: 10.1016/j.ijfoodmicro.2007.12.006

64. Paredes-Sabja D, Setlow P, Sarker MR (2009) SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. J Bacteriol 191:2711–2720. doi: 10.1128/JB.01832-08

65. Paredes-Sabja D, Setlow P, Sarker MR (2011) Germination of spores of Bacillales and Clostridiales species: mechanisms and proteins involved. Trends Microbiol 19:85–94. doi: 10.1016/j.tim.2010.10.004

66. Paredes-Sabja D, Torres JA, Setlow P, Sarker MR (2008) *Clostridium perfringens* Spore Germination: Characterization of Germinants and Their Receptors. J Bacteriol 190:1190–1201. doi: 10.1128/JB.01748-07

67. Paredes-Sabja D, Udompijitkul P, Sarker MR (2009) Inorganic Phosphate and Sodium Ions Are Cogerminants for Spores of *Clostridium perfringens* Type A Food

Poisoning-Related Isolates. Appl Environ Microbiol 75:6299–6305. doi: 10.1128/AEM.00822-09

68. Perez-Valdespino A, Li Y, Setlow B, Ghosh S, Pan D, Korza G, Feeherry FE, Doona CJ, Li Y-Q, Hao B, Setlow P (2014) Function of the SpoVAEa and SpoVAF proteins of *Bacillus subtilis* spores. J Bacteriol 196:2077–2088. doi: 10.1128/JB.01546-14
69. Petit L, Gibert M, Gourch A, Bens M, Vandewalle A, Popoff MR (2003) *Clostridium perfringens* epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells. Cell Microbiol 5:155–164. doi: 10.1046/j.1462-5822.2003.00262.x
70. Piggot PJ, Hilbert DW (2004) Sporulation of *Bacillus subtilis*. Curr Opin Microbiol 7:579–586. doi: 10.1016/j.mib.2004.10.001
71. Popham DL, Helin J, Costello CE, Setlow P (1996) Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc Natl Acad Sci U S A 93:15405–15410. doi: 10.1073/pnas.93.26.15405
72. R.a AK, Gh B, Morteza S, Gh.h R, Gh.a G (2002) The effect of magnesium sulfate on spore germination, outgrowth and toxin production of *Clostridium botulinum* type A. 5:225–233

73. Raju D, Waters M, Setlow P, Sarker MR (2006) Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiol 6:50. doi: 10.1186/1471-2180-6-50

74. Rode LJ, Foster JW (1962) Ionic and non-ionic compounds in the germination of spores of *Bacillus megaterium* texas. Arch Für Mikrobiol 43:201–212. doi: 10.1007/BF00406436

75. Rooney AP, Swezey JL, Friedman R, Hecht DW, Maddox CW (2006) Analysis of Core Housekeeping and Virulence Genes Reveals Cryptic Lineages of *Clostridium perfringens* That Are Associated With Distinct Disease Presentations. Genetics 172:2081–2092. doi: 10.1534/genetics.105.054601

76. Sarker MR, Carman RJ, McClane BA (1999) Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. Mol Microbiol 33:946–958. doi: 10.1046/j.1365-2958.1999.01534.x

77. Sarker MR, Shivers RP, Sparks SG, Juneja VK, McClane BA (2000) Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. Appl Environ Microbiol 66:3234–3240. doi: 10.1128/aem.66.8.3234-3240.2000

78. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM Foodborne Illness Acquired in the United States—Major Pathogens - Volume 17, Number 1—January 2011 - Emerging Infectious Diseases journal - CDC. doi: 10.3201/eid1701.p11101
79. Sella SRBR, Vandenberghe LPS, Soccol CR (2014) Life cycle and spore resistance of spore-forming *Bacillus atrophaeus*. Microbiol Res 169:931–939. doi: 10.1016/j.micres.2014.05.001
80. Setlow p (2007) I will survive: DNA protection in bacterial spores. - Abstract - Europe PMC. https://europepmc.org/article/med/17336071. Accessed 31 Mar 2020
81. Setlow B, Melly E, Setlow P (2001) Properties of Spores of *Bacillus subtilis* Blocked at an Intermediate Stage in Spore Germination. J Bacteriol 183:4894–4899. doi: 10.1128/JB.183.16.4894-4899.2001
82. Setlow B, Wahome PG, Setlow P (2008) Release of Small Molecules during Germination of Spores of *Bacillus* Species. J Bacteriol 190:4759–4763. doi: 10.1128/JB.00399-08
83. Setlow P (2003) Spore germination. Curr Opin Microbiol 6:550–556. doi: 10.1016/j.mib.2003.10.001
84. Setlow P (2006) Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals - Setlow - 2006 - Journal of Applied Microbiology - Wiley Online Library. https://sfamjournals.onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.2005.02736.x. Accessed 31 Mar 2020

85. Setlow P (2013) Summer meeting 2013 – when the sleepers wake: the germination of spores of *Bacillus* species. J Appl Microbiol 115:1251–1268. doi: 10.1111/jam.12343

86. Setlow P (2014) Germination of Spores of *Bacillus* Species: What We Know and Do Not Know. J Bacteriol 196:1297–1305. doi: 10.1128/JB.01455-13

87. Setlow P (2014) Spore Resistance Properties. Microbiol Spectr 2. doi: 10.1128/microbiolspec.TBS-0003-2012

88. Setlow P, Wang S, Li Y-Q (2017) Germination of Spores of the Orders Bacillales and Clostridiales. Annu Rev Microbiol 71:459–477. doi: 10.1146/annurev-micro-090816-093558

89. Singh U, Mitic LL, Wieckowski EU, Anderson JM, McClane BA (2001) Comparative biochemical and immunocytochemical studies reveal differences in the effects of *Clostridium perfringens* enterotoxin on polarized CaCo-2 cells versus Vero cells. J Biol Chem 276:33402–33412. doi: 10.1074/jbc.M104200200

90. Smedley JG, Fisher DJ, Sayeed S, Chakrabarti G, McClane BA (2004) The enteric toxins of *Clostridium perfringens*. Rev Physiol Biochem Pharmacol 152:183–204. doi: 10.1007/s10254-004-0036-2

91. Sorg JA, Sonenshein AL (2008) Bile salts and glycine as cogerminants for *Clostridium difficile* spores. J Bacteriol 190:2505–2512. doi: 10.1128/JB.01765-07

92. Swerdlow BM, Setlow B, Setlow P (1981) Levels of H+ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. J Bacteriol 148:20–29

93. Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, Adams V, Moore RJ, Rood JI, McClane BA (2014) Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Future Microbiol 9:361–377. doi: 10.2217/fmb.13.168

94. Van der Sluis, W. (2000) Clostridial enteritis is an often underestimated problem. World Poult 16:42-43

95. Velásquez J, Schuurman-Wolters G, Birkner JP, Abee T, Poolman B (2014) *Bacillus subtilis* spore protein SpoVAC functions as a mechanosensitive channel. Mol Microbiol 92:813–823. doi: 10.1111/mmi.12591

96. Vepachedu VR, Setlow P (2004) Analysis of the germination of spores of *Bacillus subtilis* with temperature sensitive *spo* mutations in the *spoVA* operon. FEMS Microbiol Lett 239:71–77. doi: 10.1016/j.femsle.2004.08.022

97. Vepachedu VR, Setlow P (2005) Localization of SpoVAD to the Inner Membrane of Spores of *Bacillus subtilis*. J Bacteriol 187:5677–5682. doi: 10.1128/JB.187.16.5677-5682.2005

98. Vepachedu VR, Setlow P (2007) Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. J Bacteriol 189:1565–1572. doi: 10.1128/JB.01613-06

99. Willardsen RR, Busta FF, Allen CE (1979) Growth of *Clostridium perfringens* in Three Different Beef Media and Fluid Thioglycollate Medium at Static and Constantly Rising Temperatures. J Food Prot 42:144–148. doi: 10.4315/0362-028X-42.2.144

100. Zhang Y, Mathys A (2019) Superdormant Spores as a Hurdle for Gentle Germination-Inactivation Based Spore Control Strategies. Front Microbiol 9. doi: 10.3389/fmicb.2018.03163

101. Zhao Y, Melville SB (1998) Identification and Characterization of Sporulation-Dependent Promoters Upstream of the Enterotoxin Gene (*cpe*) of *Clostridium perfringens*. J Bacteriol 180:136–142. doi: 10.1128/JB.180.1.136-142.1998

102. McDonnell, J. L. (1986). Toxins of *Clostridium perfringens* type A, B, C, D, and E, p. 477-517. In F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, Oxford.

103. Moir, A., Corfe, B. M., & Behravan, J. (2002). Spore germination. Cellular and Molecular Life Sciences (CMLS), 59(3), 403–409. doi: 10.1007/s00018-002-8432-8

104. Influence of sporulation medium and divalent ions on the heat resistance of Alicyclobacillus acidoterrestris spores - Yamazaki - 1997 - Letters in Applied Microbiology - Wiley Online Library. https://sfamjournals.onlinelibrary.wiley.com/doi/abs/10.1046/j.1472-765X.1997.00194.x. Accessed 13 Apr 2020