



## AN ABSTRACT OF THE THESIS OF

Saeed S. Banawas for the degree of Master of Science in Veterinary Science presented on November 11, 2010.

Title: Characterization of Germinant Receptors in *Clostridium perfringens* Non-Food-Borne Isolates

Abstract Approved:

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*C. perfringens* is a Gram-positive, spore-forming, 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 . These two major diseases are caused only by *C. perfringens* Type A isolates that produce the *C. perfringens* enterotoxin (CPE). Interestingly, *C. perfringens* isolates involved in FP carry CPE-encoding gene (*cpe*) on the chromosome while isolates causing NFB GI illnesses (i.e., sporadic diarrhea and antibiotic-associated diarrhea) carry a plasmid

borne copy of the *cpe*. *C. perfringens* is able to form highly resistance spores that can survive in the environment for years. These spores are the infectious cell morphotype, and in presence of favorable condition, they can germinate and return to active growth to cause disease. Spore germination is an early and essential stage in the progression of *C. perfringens* infection in humans and animals. The germination process can be initiated by a variety of chemicals, including nutrients, cationic surfactants, and enzymes termed germinant. Germination of *Clostridium* species has been less well studied than *Bacillus* species. However, recent findings have identified the germinants of spores of *C. perfringens* FP and NFB isolates.

Our overall goal was to characterize the germinant (*ger*) receptors of spores of NFB isolates. Here through the construction of a *gerAA* knock out mutant we characterized the role of GerAA in the germination of spores of *C. perfringens* NFB isolate F4969. Result from these study indicate that in contrast to the minor role of GerAA in germination of spores of *C. perfringens* FP isolates, GerAA has a major role in spore germination of *C. perfringens* NFB isolates. Indeed, F4969 and SB103 spores germinated less than wild-type spores with nutrient broth, the mixture L-Asn and KCl (AK) and the non-nutrient germinant, dodecylamine. In addition, *gerAA* mutant spores had lower rates of DPA release than wild-type spores in the presence of AK and dodecylamine. These defects became evident in the slower outgrowth exhibited by SB103 spores, but not on overall spore viability. Collectively, these results indicate, in contrast to the role of GerAA in FP spores, that GerAA is a major germinant receptor protein in NFB spores.

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Characterization of Germinant Receptors in *Clostridium perfringens* Non-Food-Borne  
Isolates

by

Saeed S. Banawas

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Saeed S. Banawas, Author

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## CONTRIBUTION OF AUTHOR

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. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the manuscript in chapter 2.



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# **Characterization of Germinant Receptors in *Clostridium perfringens* Non-Food-Borne Isolates**

## **Chapter 1**

### **General Introduction and Literature review**

*Clostridium perfringens* is a Gram-positive, anaerobic, endospore-forming, and rod-shaped bacterium. This bacterium produces a variety of toxins (37). *C. perfringens* is found naturally in soil, water, wastewater, and the intestinal tract of most humans and animals (4). *C. perfringens* is thought to be the most widely occurring pathogenic bacteria of the genus *Clostridium*, which also includes pathogenic *Clostridium botulinum*, *Clostridium difficile* and *Clostridium tetani* and the industrially relevant *Clostridium acetobutylicum* (19). In 1892, *C. perfringens* was first described by Welch and Nuttall and was commonly known as *Clostridium welchii* (19). *C. perfringens* can grow at temperatures between 20°C to 50°C, and exhibits hemolysis when grown on blood agar plate (19). It causes a wide array of diseases in both humans and animals including gas gangrene, cellulitis, septicemia, necrotic enteritis and food poisoning (26).

Based on the ability of producing major four toxins, *C. perfringens* are classified into 5 types A-E (Table 1). In many countries including the United States, *C. perfringens* food poisoning is ranked as one of the most common cause of food borne infections (27). Most of the food poisoning strains belong to type A and only a few to type C. The virulence of *C. perfringens* comes from many factors. First, the ability to produce 17 toxins (39). Second, it has a shorter doubling time (as short as 15 min) (94)

compared to other *Clostridium* species. Third, the ability to form highly resistant spores (29, 35).

Table 1: Typing of *C. perfringens* based on the production of four major toxins.

<i>C. perfringens</i> type	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Source (37)

### 1.1. Major toxins of *C. perfringens*

#### Alpha toxin:

Alpha ( $\alpha$ ) toxin is produced by all strains of *C. perfringens*. The  $\alpha$  toxin gene (*plc*) is located on the chromosome of *C. perfringens*. This toxin is regulated at the transcriptional level by the products of the *virR* and *virS* genes (45, 73, 82).  $\alpha$  toxin is a protein with a molecular weight of about 43-kDa dependent on zinc-containing metalloenzyme phospholipase C enzyme with lecithinase and sphingomyelinase activity.  $\alpha$  toxin has two domains, with the N-terminal domain exhibits phospholipase activity while the C-terminal domains is involved in membrane binding (75).  $\alpha$  toxin causes tissue damage by hydrolysis of lipid lecithin in the mammalian cell membranes

(75), lysing blood and endothelial cells (75). The ability of  $\alpha$  toxin to lyse blood cells is used in the reverse cAMP test for the identification of *C. perfringens* in diagnostic tools (47).  $\alpha$  toxin is the major virulence factor for gas gangrene by causing extensive tissue damage, hepatic toxicity and myocardial dysfunction (47).

### **Beta toxin:**

The beta ( $\beta$ ) toxin is mostly found on a large plasmid in *C. perfringens* type B and D isolates (Table 1) (19).  $\beta$  toxin is the major virulence factor in necrotic enteritis and enterotoxaemia in many domesticated livestock including lamb, sheep, and fowl, and it is also the causative agent of human necrotic enteritis (26, 85).  $\beta$  toxin is a pore forming toxin that forms cation dependent channel in susceptible membranes (44). Pig-bel or human necrotic enteritis is caused by the consumption of under-cooked meat product contaminated with *C. perfringens* type C spores by immunocompromised individuals (32).  $\beta$  toxin is sufficient to induce typical necrotizing intestinal lesions in the rabbit ileal loop model (78). Treatment of disease consists of administration of  $\beta$ -toxoid (84). Ongoing works are on the way to develop safer  $\beta$ -toxin vaccine for human and veterinary use (26).

### **Epsilon Toxin:**

Epsilon ( $\epsilon$ ) toxin is thought to be the third most potent clostridia toxin after botulinum and tetanus neurotoxins (26). Type B and C are only the main strains to produce  $\epsilon$  toxin. Moreover,  $\epsilon$  toxin is ranked by the CDC as a category B bioterrorist agent.  $\epsilon$ -toxin gene (*etx*) is encoded by a large plasmid in type B and type C isolates (5), and has a limited host range including lambs, sheep, cattle and goats (25). In North America, type B affects the newborn lambs and causes dysentery, while type D causes enterotoxaemia predominantly in sheep (26). The  $\epsilon$  toxin is produced as an inactive protoxin of approximately 32.7-kDa (24). Strong evidence suggests that  $\epsilon$ -toxin is a pore forming toxin that can increase vascular permeability in the brain, kidney, and intestine (68-70). Because its rapid onset of disease, that leads to mortality, vaccines has been the usual method of choice for prevention (74).

**Iota toxin:**

Iota ( $\iota$ ) toxin, is a binary toxin found in *C. perfringens* type E and *C. spiroforme* (26).  $\iota$  toxin causes diarrhea in animals especially in domesticated livestock (26). It is composed of two noncovalently lined components: Ia, an ADP-ribosyltransferase with a molecular weight of  $\sim$  47.5-kDa, and Ib, with a molecular weight of  $\sim$  71.4-kDa is involved in the binding and internalization, respectively, of the toxin (68). Ia, which is the light chain, causes ADP-ribosylation of globular skeletal muscle and muscle actin at Arg-177, inducing cell death and cytoskeletal disarray. Ib, which is the heavy chain, is required to internalize Ia in the cytosol (3).



**Other toxins:**

The above toxins are mainly used for toxinotyping of *C. perfringens* isolates. However, there are other toxins that have major roles in the pathogenesis of certain diseases and are listed below.

**CPE:** The *Clostridium perfringens* enterotoxin (CPE) is the most important virulence factor for Food Poisoning (FP) and Non-Food-Borne Gastrointestinal Diseases (NFBGID) in human (76). Less than 5% of the *C. perfringens* population, mostly type A isolates, can produce CPE (36). Importantly, the CPE encoded by the *cpe* gene can be located on the chromosome or on a large plasmid (8); most of *C. perfringens* type A FP isolates carry a chromosomal copy of the *cpe*, while all NFBGID isolates carry a plasmid copy of the *cpe* (8, 9, 30, 86). The only virulence factor that is associated uniquely with sporulation in the genus *Clostridium* is CPE (36). CPE is a 35-kDa protein, and it is heat-labile and pH labile (36). It is released into the intestinal lumen when sporulating cells lyse to release their spores. CPE binds to its receptor protein belonging to the claudin family of proteins. Then, CPE toxin binds to other co-receptors to form a 90-kDa small complex. However, it is a precursor for formation of a SDS-resistant large-complex of ~155-kDa. This large complex simulates a pore causing membrane permeability alteration in sensitive mammalian cell. These CPE-induced membrane alterations cause direct histopathological damage to the small

intestine, especially the tips of the villi (36, 39). The symptoms include acute abdominal pain and diarrhea; nausea, fever and vomiting (36, 39).

**Beta-2 Toxin.** A  $\beta$ -2 toxin has been found mostly in *C. perfringens* type C isolates (16) , as well as in type A isolates (20). Some studies showed that a  $\beta$  2 toxin is associated with necrotizing enterocolitis in a number of domestic animal and livestock (20). Further studies have explained that  $\beta$ -2 toxin link with the CPE is implicated in 75% of *C. perfringens* type A isolates causing sporadic-diarrhea (SD) and antibiotic-associated diarrhea (AAD) (15). The  $\beta$ -2 toxin is a 28 kDa protein and encoded by the *cpb2* located on the plasmid with the *cpe* gene in the most of the isolates that cause AAD and SD (15). Moreover, the  $\beta$ -2 toxin showed no homology to  $\beta$  toxin of type B isolates (18) and little is known about its mechanism of action. In fact, *cpe* and *cpb2* are on the same plasmid help to clarify why NFB diseases in humans caused by *C. perfringens* type A isolates carrying plasmid *cpe* lasts 1-2 weeks (7). In contrast, food-borne diseases caused by *C. perfringens* type A isolates carrying chromosomal *cpe* lasts for 24-48h (7, 38), suggesting that  $\beta$  -2 toxin has an important role in GI disease.

### **1.2. *C. perfringens* Food Poisoning (FP) and Non-Food-Borne Gastrointestinal Diseases (NFBGID).**

*C. perfringens* type A food poisoning (FP) is ranked as the third most commonly reported human food-borne illness. *C. perfringens* type A FP is affecting more than 250,000 individuals annually and causing millions of dollars in economic

loss each year in the USA (46). Most of *C. perfringens* type A that carries a chromosomal copy of the *cpe* are capable of causing the GI diseases of this food-borne illness (36). Also, most of *C. perfringens* type A carry a plasmid copy of the *cpe* are the major factor for having non-foodborne human GI diseases (86). In fact, NFB isolates are responsible for ~20% of causes of AAD and SD (86). *C. perfringens* spores of FP isolates are more resistant and metabolically dormant to various environmental stress factors than *C. perfringens* spores of NFBGID isolates. *C. perfringens* FP isolates have more resistance than those of *C. perfringens* NFBGID isolates to i) osmotic, nitrite, and pH induced stress, iii) prolonged frozen storage, and iv) pressure assisted thermal processing (PATP) (29, 30, 33, 46, 47, 63). These resistance properties of spores of FP isolates are allowing them to survive in processed and poultry meats, and other products that are most commonly implicated in *C. perfringens* type A FP outbreaks (36).

### **1.3. Sporulation**

*Bacillus* and *Clostridium* species can initiate the process of sporulation in response to environmental signals (14, 65). In *B. subtilis*, these environmental signals are sensed by specific histidine kinases that transduce the signal through a phosphoryley, ultimately phosphosylating the master regulator of sporulation, Spo0A (13). The first event of morphological change during sporulation is an asymmetrical division into a small prespore and the mother cells. The subsequent morphological and

biochemical events are tightly regulated by four RNA polymerase sporulation-specific sigma factors,  $\sigma^F$ ,  $\sigma^E$ ,  $\sigma^G$ ,  $\sigma^K$  (54, 71).  $\sigma^F$  is the earliest  $\sigma$  factor and becomes active in the prespore, while  $\sigma^E$  becomes active in the mother cell and these events take place in early stage of sporulation, resulting in the engulfment of the prespore by the mother cell. Next,  $\sigma^G$  and  $\sigma^K$  will become active in the forespore and mother cell compartment, respectively (21, 71). While the major steps in the sporulation process are well conserved in *C. perfringens*, this latter species has the unique ability to produce a major virulence factor during sporulation, the CPE (22, 55).

#### **1.4. Bacterial spore germination**

Bacterial spores are metabolically dormant and resistant to many environmental stresses such as radiation, heat, and toxic chemicals (53, 63). However, to return to life, dormant spores must undergo germination and then outgrowth (41, 42). The germination process is initiated upon binding of the germinants to their cognate germinant receptors (GR) localized in the spore's inner membrane. Upon germinant/GR binding a series of biophysical and biochemical events take place: i) the release of monovalent cations (i.e.,  $\text{Na}^+$  and  $\text{K}^+$ ) and the spore core's large depot of dipicolinic acid (DPA) present as a 1:1 chelate with divalent cations, primarily with  $\text{Ca}^{2+}$  (54). These small molecules, at least in *Bacillus* species, activate downstream effectors such as the cortex-lytic enzymes (CLEs) (43, 48, 49), and initiate the degradation of the spore peptidoglycan (PG) cortex allowing an increase in water

uptake to levels similar to that of growing cells (80) and consequently lead to resumption of metabolism.

#### 1.4.1 Germinant Receptor (GR)

The GR proteins are highly conserved proteins of the GerA family of proteins present in the majority of endospore forming species of the orders of *Bacillales* and *Clostridiales* (59). In *Bacillus* and *Clostridium* species, these proteins are localized in the spore's inner membrane at levels of 10s of GR per spore (23, 50). The genetic architecture of the GR proteins is highly heterogenous among endospore forming species of the order of *Bacillales* and *Clostridiales* (59). Indeed, the classical tricistronic *gerA*-type operon found in *B. subtilis* and in pathogenic *Bacillus* species, although represents ~ 50% of all GR proteins sequenced, there is a wide variability on their organization suggesting different mechanisms of germinant/GR interactions (59). The classical GerA-type of GR is composed by three subunits, A, B, and C (41, 42, 80). However, there is no intact tricistronic *gerA*-type operon in the *C. perfringens* sequenced genome (43), suggesting that the GR proteins in *C. perfringens* can work as a independent GRs (61, 63). In *C. perfringens*, the main GR proteins involved in spore germination are the GerKA-KC, while GerAA and GerKB GR proteins play at most an auxiliary role (61, 63). Previous studies have shown that GerKA-KC are essential for *C. perfringens* spore germination with nutrients, L-Asparagine, KCl, the co-germinants sodium and inorganic phosphate (NaPi) and the non-nutrient germinants

Ca-DPA and dodecylamine (61, 63, 64). Most striking is that, in contrast to *B. subtilis*, the *C. perfringens* GerKA-KC and GerKB GR proteins are required for full spore viability, as *gerKA-KC* and *gerKB* spores give lower titers than wild-type spores even in presence of lysozyme, enzyme that is capable of recovering spores that have undergone partial cortex hydrolysis (61, 63). The GerKA-KC GR protein is also essential for the normal release of DPA from the spore core and therefore, transducing the germination signal to the downstream effectors (63).

#### **1.4.2 Signal transduction.**

Upon germinant/GR binding, the GR proteins transduce the germination signal to downstream effectors through an unknown mechanism. However, several biophysical events take place: i) a large efflux of more than 75% of the spore's depot of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{H}^+$  through an energy independent process (87). ii) this large efflux is followed by the release of the spore's large depot of dipicolinic acid (DPA), chelated at a 1:1 ratio with divalent cations, mainly  $\text{Ca}^{2+}$  (80). These latter events are believed to be dependent on a DPA gated channel that is presumably composed in part by SpoVA proteins (59). While in *B. subtilis*, the release of small molecules and Ca-DPA is essential for the activation of the downstream effectors (48, 49, 83, 88), in *C. perfringens* these biophysical events have no role (57). Interestingly, a lipoprotein present in the spore inner membrane and termed GerD is present only in endospore former species of the order of *Bacillales*, is essential for normal germination of *B.*

*subtilis* spores (66, 67). GerD is present at levels of 1000s of molecules per spore, and might act as a signal amplifier between the GR and the SpoVA proteins (66, 67). However, such a protein is absent in endospore forming species of the order of *Clostridiales*, indicating that the signal transduction mechanism is significantly different between *Bacillales* and *Clostridiales* and is a matter of further research.

### 1.4.3 Cortex hydrolysis.

The hydrolysis of the spore's PG cortex is the hallmark of the germination process as it allows full core hydration and resumption of metabolism (80). In *B. subtilis*, three cortex lytic enzymes (CLEs) are involved in the hydrolysis of the spore's peptidoglycan cortex (31, 80). Although, *cwlJ* and *sleB* spores can go through germination normally, double mutant spores are not capable of degrading the cortex (10, 80). SleL has a less essential role in germination of *B. anthracis* (17). These enzymes only recognize the spore's PG cortex which has a spore specific modification, the replacement of muramic acid residues with muramic- $\delta$ -lactam, which prevents that the spore's PG is degraded by hydrolytic enzymes from the vegetative cells, and also acts as a recognition substrate for CLEs (72). CwlJ and SleB are uniquely synthesized during sporulation in the mother cell and the forespore compartment, respectively. Both enzymes remain inactive in the dormant spore and upon release of DPA from the spore core they become activated. In *C. perfringens*, although no homologues of CwlJ and SleB are present, two CLEs have been well

characterized, SleC and SleM (29, 35, 40, 56, 58, 62, 81). In contrast to the redundancy of CwlJ and SleB in *B. subtilis*, in *C. perfringens*, SleC is the sole major CLEs essential for cortex hydrolysis and DPA release (62). In addition, the mechanism of activation of the CLEs significantly differs between *B. subtilis* and *C. perfringens* (59). *B. subtilis* CLEs are activated by the release of Ca-DPA, which acts on CwlJ, while the partial cortex deformation produced by the partial hydration during DPA release activates SleB. In *C. perfringens*, SleC is prototypically activated by CspB, a member of the subtilisin family of proteases (58). However, the mechanism of CspB activation remains unclear, and seems to be independent to release of DPA and low core hydration.

### **1.5 Applications of bacterial spore germination**

A detail understanding of the germination mechanism of bacterial spores will allow the identification of new drug targets, decontamination, therapeutic development, and preventive measurement. For instance, identification of compounds that trigger spore germination would be easy to kill or alternatively to stop spore germination preventing the return of dormant spores into active cells.



### **Objective of this study**

In *C. perfringens*, spore germination is an important and early event in the development of any of the *C. perfringens*-associated diseases. Because it is an anaerobic bacteria and the fact that *C. perfringens* spores are ubiquitous in the environment, understanding of *C. perfringens* spores germination is a key for the development of new strategies to stop *C. perfringens* disease. In this study we will initiate the dissection of the role of the GR of spores of *C. perfringens* non-food-borne gastrointestinal illnesses.

The specific aims in this research are:

- 1- Construction of a germination receptor (*gerAA*) knockout mutant in *C. perfringens* NFB strain F4969.
- 2- Characterization of *gerAA* mutant spores with nutrient and non-nutrient germinants.

## Chapter 2

### **Role of GerAA in germination of spores of *Clostridium perfringens* non-food-borne isolate F4969.**

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## 2.1 Abstract

*C. perfringens* is an anaerobic pathogenic bacterium capable of causing a wide spectrum of diseases in both animals and humans. Germination of *C. perfringens* spores is considered the earliest and most essential step for initiation of the disease. Previous studies have dissected the role of germinant receptors in *C. perfringens* spores of food poisoning isolates. In this study, we have established the role of the germinant receptor protein GerAA in germination of spores of non-food-borne gastrointestinal disease isolates. Our results show that GerAA is essential for normal germination with nutrient broth, the mixture of L-Asn and KCl (AK) and the non-nutrient germinant, dodecylamine. SB103 spores also released DPA at a slower rate than that of wild-type spores. Although the ability of *gerAA* mutant spores to outgrowth was affected, they gave rise to similar titers as wild-type spores. In summary, the GerAA germinant receptor protein has different roles in the germination of spores of food poisoning versus none-food-borne gastrointestinal disease isolates.

## 2.2 Introduction

*C. perfringens* is a Gram-positive, spore-forming, anaerobic pathogenic bacterium capable of causing a wide variety of diseases in both humans and animals (36). However, the two most common illnesses in humans are *C. perfringens* type A Food Poisoning (FP) and Non-Food-Borne (NFB) gastrointestinal (GI) illnesses (7, 36). These two major diseases are caused only by *C. perfringens* Type A isolates that carry the *C. perfringens* enterotoxin (CPE), present in only 5% of Type A isolates (76, 93). Interestingly, isolates involved in FP carry *cpe* in the chromosome while isolates that cause NFB GI illnesses (i.e., sporadic diarrhea and antibiotic associated diarrhea) carry a plasmid borne copy of the *cpe* (9). In both FP and NFB isolates, the spores are considered as the infectious morphotype. Numerous studies have highlighted the ability of FP spores to be better fitted to survive the harsh conditions present in FP environments than those of NFB spores. For example, FP spores are more resistant than NFB spores to: i) heat treatments (77); ii) low temperatures (4°C and -20°C) (34); iii) nitrite induced stress (33). However, in order to cause diseases spores of both FP and NFB isolates must germinate to return to vegetative cell.

The process of bacterial spore germination is triggered when compounds, called germinants, bind to their cognate germinant receptor located in the spore's inner membrane. Upon germinant-ligand binding of the cognate germinant receptor, starts the release of monovalent cations (i.e., Na<sup>+</sup> and K<sup>+</sup>) and the spore core's large depot of dipicolinic acid (DPA) present as a 1:1 chelate with divalent cations, primarily Ca<sup>2+</sup>

(59, 80). The release of these small molecules, at least in *Bacillus* species, activate downstream effectors such as the cortex-lytic enzymes (CLEs) (48, 57, 59), and initiate the degradation of the spore PG cortex allowing an increase in water uptake to levels similar to that of growing cells (80).

Recent studies (1, 51, 56-64) have dissected the mechanism of germination of *C. perfringens* spores. However, those studies used a FP isolate SM101, and also have highlighted significant differences with spore germination of NFB isolates. Most importantly, FP spores are able to germinate with L-Asparagine, KCl, the mixture of L-Asparagine and KCl (AK), the co-germinants Na<sup>+</sup> and inorganic phosphate, and the non-nutrient germinant dipicolinic acid chelated at a 1:1 ratio with Ca<sup>2+</sup>, through the main germinant receptor GerKA-KC (51, 61, 63, 64). In contrast, NFB spores are able to germinate with L-alanine, L-valine, and with the mixture AK but not with L-Asparagine and KCl separately (51, 61, 63, 64). However, there is a lack of knowledge of the role of the germinant receptors in NFB spore germination. Consequently, to begin dissecting the role of the germinant receptors in germination of NFB spores, we have constructed a *gerAA* knock out mutant in the NFB strain F4969 and characterized the germination phenotype of *gerAA* mutant spores. Our results indicate that, *gerAA* mutant (SB103) spores germinate slower and release slower levels of DPA than F4969 wild-type spores during AK-triggered germination. The cationic surfactant also released lower amounts of DPA in SB103 than in wild-type spores. Finally, *gerAA* mutant spores had a slower outgrowth than wild-type spores but were able to give rise to similar amounts of colonies as wild-type spores.

## 2.3 Material and Methods

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

**Construction of *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* (63) was introduced into *C. perfringens* strain F4969 by electroporation (11) and Cm<sup>r</sup> colonies were screened for the insertion of the Targetron by PCR using detected primer CPP211 and CPP206 (Table 2.2). The PCR condition was placed in a thermal cycler (Techne) for the first stage of 1.5 min at 94 °C (denaturation). Then, the second stage was subjected to 32 cycles, each consisting 1 min at 94 °C, 1 min at 47 °C (annealing), and 2 min at 72 °C (extension). The final stage was additional extension for 5 min at 72 °C. To cure the Cm<sup>r</sup> coding vector, one Cm<sup>r</sup> Targetron-carrying clone was subcultured for 48h in FTG medium without Cm, and single colonies were patched onto BHI agar, with or without Cm, giving strain SB103.

**Construction of a *gerAA* complemented strain.** To construct a *gerAA* mutant strain complemented with wild-type *gerAA* as follows. A 2.2-kb DNA fragment carrying 446-bp upstream and 213-bp downstream of *gerAA* was PCR amplified with Phusion<sup>TM</sup> High-Fidelity DNA Polymerase using primer CPP857/ CPP858 (forward and reverse primers had KpnI and SalI sites at their 5' end, respectively). This PCR used 10 ng of template DNA (F4969), 25 pM each primer, 200 μM deoxynucleotide

triphosphates (Fermentas), 2.5 mM MgCl<sub>2</sub> and 1 U of *Taq*DNA polymerase (Fermentas) in a total volume of 50 µl. The PCR reaction mixture was placed in a thermal cycler (Techne) for the first stage of 1.5 min at 94 °C (denaturation). Then, the second stage was subjected to 32 cycles, each consisting 1 min at 94 °C, 1 min at 50 °C (annealing), and 2 min at 72 °C (extension). The final stage was additional extension for 5 min at 72 °C. This PCR was extracted and cloned into pCR-XL-TOPO giving plasmid pSB16. Next, plasmid pSB16 was extracted and then digested with KpnI and Sall and cloned between KpnI and Sall sites of plasmid pJIR751 giving plasmid pSB17. pSB17 was introduced into the *C. perfringens gerAA* strain SB103 by electroporation, and Em<sup>r</sup> transformants were selected. The presence of both plasmid pSB17 and the original *gerAA* deletion in the latter strain were confirmed by PCR detected primer CPP211 and CPP206 (Table 2.2).

**DNA Isolation.** All DNA was isolated from *C. perfringens* isolates used in this study. Aliquots 1ml of freshly tryptone-glucose-yeast extract TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, and 0.1% cysteine) culture and spun down at 13,200 rpm for 1 min. Supernatant solution was discarded and the cell pellet was washed 3 times with 1X TES (1.25 mM Tris-HCl pH 7.0, 0.625 mM EDTA, 0.125 mM NaCl). Then, the pellet was re-suspended in 1 ml lysis solution (50 µl 1 M Tris pH.8.0, 25 µl 0.5 M EDTA pH.8.0, 500 µl 20% glucose, 0.02 g lysozyme, 5 µl 20 mg/ml proteinase K, and 420 µl distilled water) and incubated for 1h at 37°C. After that, the sample was centrifuged and the supernatant removed. The remaining pellet was re-suspended in 0.5 ml 100 mM TE (100 mM Tris-HCl pH 7.0, 10 mM EDTA, pH 8.0) and 125 µl of

10% sarkosyl solution. The sample was mixed by inversion, and then incubated at 50 °C for 15 min. After that, phenol: chlorophorm extracting was performed on the sample, using an equal amount of phenol: chlorophorm to sample. The supernatant was transferred to a new centrifuge tube and 5µl of RNase A(10 mg/ml) added. Sample was incubated at room temperature for 20-60 min. Following this, another phenol: chlorophorm extraction was performed. After the extraction, 40 µl of 5 M NaCl and 1 ml of cold absolute ethanol were added to the sample and mixed them by several inversions. After mixing, the sample was centrifuged at 13.200 rpm for 10 min to resolve the DNA pellet. Sample supernatant was discarded and placed upright in a 37°C incubator to thoroughly dry and remove any remaining ethanol from DNA pellets. Finally, the DNA pellet was re-suspended in 50 µl of TE( 10mM Tris-HCl pH.7.0, 1mM EDTA) and isolated DNA sample is stored at -20°C.

**Spore preparation.** Starter cultures of *C. perfringens* isolates were prepared by overnight growth at 37°C in fluid thioglycollate (FTG) broth (Difco) as described (28). To start sporulation culture of *C.perfringens*, 0.4 ml of FTG starter culture was inoculated into 10 ml of Duncan-Strong (DS) sporulation medium (12) . Then, DS was incubated for 24 h at 37°C to form spores and confirmed by phase-contrast microscopy. Spore preparations were prepared by scaling-up the last procedure. After that, Spores were purified by repeated washing with sterile distilled water until the spores were more than 99% free of sporulation cells, germinated cells, and cell debris. Clean spores were suspended in distilled water at an optical density at 600 nm (OD<sub>600</sub>) of ~ 6 and stored at -20°C.



**Spores germination assay.** Spore suspension was heat activated at 75°C for 15 min. Then, spores cooled down at room temperature and incubate at 40 °C for 10 min. Spore germination were routinely measured by monitoring the OD<sub>600</sub> of spore culture (Smartspec™ 3000 Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA) which fall ~50 % upon complete spore germination and level of germination were confirmed by phase-contrast microscopy. The rate of germination was expressed as the maximum rate of loss of OD<sub>600</sub> of the spore suspension, relative to the initial value. The extent of spore germination was calculated by measuring the decrease in OD<sub>600</sub> and expressed as percentage of initial. All values reported are average of three experiments performed on at least three independent spore preparations.

All germination solution were prepared at 100 mM germinants in 25 mM Tris.HCl buffer ( pH 7.0). Germination was also carried in TGY. Germination was also assayed with Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM).

**DPA release.** Chemical DPA release was measured by incubating spores (OD<sub>600</sub> of 6) that had been previously heat activated at 75°C for 15 min. Then, spores cooled down at room temperature and incubate at 40 °C with pre-heating 100 mM AK 40 °C to allow adequate measurement of DPA release. Germinated cultures were centrifuged (13,200 rpm, 5 min), and 0.2 ml of the supernatant fluid was mix with 0.2 ml of distilled water (dH<sub>2</sub>O) and 0.1 ml of assay reagent ( 25 mg of Cysteine, 310 mg of FeSO<sub>4</sub> +H<sub>2</sub>O, and 80 mg of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). The mixture was centrifuged for 4 min and

measured at OD<sub>440</sub>. Initial DPA was determined by measuring the amount of DPA on the supernatant of an aliquot 1 ml of sample boiled (100°C for 1 h) as described above.

Another way to measure the DPA was assayed by measuring the DPA release during nutrient-triggered spore germination. The assay was measured by heat activated a spore suspension ( OD of 6) and incubating at 40 °C with 100 mM AK pH 7.0, to allow adequate measurement of DPA release.

Dodecylamine germination was assessed by measured DPA release by incubating untreated spores (OD<sub>600</sub> of 1.5) with 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4) at 60°C. Aliquots (1 ml) of germinating cultures were centrifuged for 3 min in a microcentrifuge and DPA in the supernatant fluid was measured at an absorbance of 270 nm as previously described(6). Initial DPA levels in dormant spores were measured by boiling 1 ml aliquots for 60 min, centrifuging in a microcentrifuge for 3 min, and measuring the OD<sub>270</sub> of the supernatant fluid (6).

**Colony formation assay.** To evaluate the colony-forming ability of spores of strains F4969 and SB103, spores at an OD<sub>600</sub> of 1 ( around 10<sup>8</sup> spores/ml) were heat activated at 75 °C for 15 min, and aliquots of various dilution were plated on BHI agar, incubated at 37 °C anaerobically for 24 h, and colonies were counted.

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

## 2.4 Results

**Germination of *C. perfringens* spores of FP and NFB isolates.** Previous studies have shown that spores of *C. perfringens* FP isolates germinate well with L-Asn and KCl, while spores of *C. perfringens* NFB isolates germinate only in the presence of the mixture of L-Asn and KCl (AK) (52). To validate previous results, we repeated similar experiments with spores of a representative FP isolate, strain SM101, and spores of a representative NFB isolate, strain F4969. As expected, spores of SM101 and F4969 germinated well in presence of the mixture AK (Fig. 2.1A). However, in presence of KCl and L-Asn, only spores of SM101 spores germinated, while F4969 spores germinated poorly (Fig. 2.1BC). These results confirmed previous findings that suggest that the AK-triggered germination pathway significantly differs between FP and NFB isolates.

**Identification of putative germinant receptor (GR) homologues in *C. perfringens* non-food-borne isolate F4969.** To investigate if differences in the AK-triggered germination pathway between strains SM101 and F4969 are due to differences at the GR-level, we subjected the draft assembly *C. perfringens* F4969 genome to BLASTP analyses to identify GR protein homologues using *C. perfringens* SM101 GR as baits. Four ORFs (AC5\_0662, AC5\_0663, AC5\_0664, and AC5\_1261) encoding proteins with high similarity (>95%) to GR proteins of *C. perfringens* SM101 were identified (Fig. 2.2A). Similarly as in *C. perfringens* SM101, the genome of *C. perfringens* F4969 encoded a *gerK* locus that contained a bicistronic operon composed by *gerKA*

and *gerKC*, which is flanked by a monocistronic *gerKB* encoded in opposite orientation and upstream of the *gerK* operon (Fig. 2.2A). Interestingly, the monocistronic *gerAA* gene was found in opposite orientation relative to *C. perfringens* SM101 (Fig. 2.2A). BLASTP analyses also revealed that the predicted amino acid sequence between GR protein of SM101 and F4969 was higher than 95% (Fig. 2.2B), suggesting that while both isolates have the same number of highly similar GR proteins, their role in AK-triggered germination might be different.

**GerAA is required for germination of spores of NFB isolates in nutrient rich media.** In spores of *C. perfringens* FP isolates, GerAA has no role in germination with nutrient rich media (63). Therefore, to initiate dissecting the role of the GR in spore germination of *C. perfringens* NFB isolates, we constructed a *gerAA* mutation in *C. perfringens* F4969 spores. Strikingly, while wild-type spores germinated well in presence of nutrient rich media (TGY), *gerAA* mutant spores germinated to a significantly ( $p$ -value < 0.001) lesser extent (Fig 2.3A). However, this was not unique to TGY vegetative media, as similar results were also observed in two different amino acid rich media used for tissue culture (Fig. 2.3BC). These results suggest that GerAA is required for nutrient germination of NFB spores.

**GerAA is required for AK-triggered germination.** A previous study showed that GerAA had only an auxiliary role during germination of FP spores and only at low concentrations of AK (63). Therefore, we evaluated whether the *gerAA* gene product has a role in germination of spores NFB isolates. As expected, wild-type spores fully

germinated with AK (Fig. 2.4A), however, *gerAA* mutant spores germinated poorly. Results were confirmed by phase contrast microscopy, indicating that  $\geq 90\%$  of wild-type spores became phase dark (indicative of full germination), while only  $\geq 88\%$  of *gerAA* mutant spores remained phase bright (indicative of dormant spore).

After the germinant binds to its cognate receptor, the next measurable event in spore germination is the release of the spore core's large deposit of Ca-DPA (80). Therefore, release of DPA during germination of wild-type and SB103 spores with AK was assayed. As expected, wild-type spores released the majority of their DPA within the first 10 min of incubation (Fig. 2.4B). However, it was most surprising that SB103 spores only released  $\sim 20\%$  of their DPA during the first 10 min of germination, and up to 40% after 60 min of germination (Fig. 2.4B). Similar results were observed when DPA release was measured through a chemical assay (Fig. 2.4C). Collectively, these results indicate that GerAA is required for DPA release and germination in presence of AK.

#### **Effect of *gerAA* mutation on dodecylamine germination of spores of NFB isolates.**

The cationic surfactant, dodecylamine, can germinate spores of many *Bacillus* and *Clostridium* species. In *B. subtilis* spores, dodecylamine triggers the release of DPA from the spore core by opening a DPA channel in the spore's inner membrane (79). In *C. perfringens* FP spores, GerAA has shown to have no role in dodecylamine germination (63). Therefore, we performed similar assays with NFB wild-type and *gerAA* mutant spores. When wild-type spores were incubated with dodecylamine, the

majority of the spore core's DPA content was released within the first 10 min of incubation (Fig. 2.5). In contrast, although SB103 spores released less than half of total DPA within the first 10 min of incubation the majority of DPA was released after 60 min of incubation (Fig. 2.5). These results suggest that GerAA is required for normal DPA release during germination of *C. perfringens* NFB spores with dodecylamine.

**Effect of *gerAA* mutation on spore outgrowth and colony forming ability of *C. perfringens* spores.** Previous results in *C. perfringens* FP spores have shown that GerAA has no role in spore outgrowth and colony forming ability (63). Since a *gerAA* mutation significantly affects the germination phenotype of *C. perfringens* NFB spores, we evaluated the effect of such a mutation on spore outgrowth and colony forming ability. Surprisingly, SB103 spores had significantly ( $p$ -value  $< 0.01$ ) slower outgrowth than wild-type spores (Fig. 2.6A). However, when the colony forming ability of SB103 spores was measured, similar titers to that of wild-type spores were found (Fig. 2.6B). These results indicate that GerAA, although affects the ability of *C. perfringens* NFB spores to germinate and outgrow normally, it has no role in their ability to form colonies.

**Effect of complementation of a *gerAA* mutant with wild-type *gerAA*.** To provide conclusive evidence that the germination phenotype of *gerAA* mutant spores was due to a *gerAA* deletion, we introduced a wild-type *gerAA* into *gerAA* mutant strain SB103 and assayed their germination phenotype. Strikingly, when spores of SB103 (pSB17) (*gerAA* mutant complemented with wild-type *gerAA*) were assayed with the various

germination assays described above, there was no significant complementation of the germination phenotype (data not shown). The inability to complement certain germination-related genes is not novel as has been previously reported (57, 61, 63). Therefore, to be certain that the germination phenotype of *gerAA* mutant spores was not due to a secondary mutation elsewhere in the genome, we constructed a second mutant and assayed its spore germination phenotype. Results indicate that both *gerAA* mutants have essentially identical germination phenotype, indicating that the suggested roles of GerAA are indeed due to a *gerAA* mutation and not a secondary mutation.

## 2.5 Discussion

Due to the anaerobic nature of *C. perfringens*, spores are considered the infectious morphotype for the wide range of *C. perfringens*-associated diseases. Therefore, germination of *C. perfringens* spores could be considered the earliest and most essential step for the progression of any *C. perfringens*-associated disease. Recent studies (51, 63) have shown that significant differences exist at the germinants/GR specificity between spores of two different source of isolation FP and NFB GI illnesses. Most importantly is the ability of FP isolate to germinate with FP-related germinants such as KCl and NaPi, and the L-Asn (63, 64). While NFB isolates are only able to germinate in presence of the mixture AK (63, 64). In this context, results offered by this work contribute to our understanding of the differential AK germination pathway between spores of FP and NFB isolates.

A major conclusion of this work is that GerAA is required for AK germination pathway of spores of NFB isolates. Previous studies indicate that, at least in *C. perfringens* FP isolates, GerKA-KC are the main GR proteins (61, 63), with GerAA and GerKB proteins playing at most an auxiliary role in germination of *C. perfringens* spores. Now we find that GerAA is essential for normal germination of *C. perfringens* NFB isolates. This conclusion got support from the fact that poor germination of SB103 spores was observed with nutrient rich media and AK mixture. The fact that *gerAA* mutant spores released lower amounts of DPA than wild-type spores when incubated with AK mixture clearly suggest that GerAA are involved in transduction of the germination signal to downstream effectors. These results indicate that the role of the GR, or at least GerAA, in FP and NFB isolates might be substantially different. It was most surprising that deletion of *gerAA* significantly affected the ability of NFB spores to outgrow but not the colony forming ability. This suggest that the defects in germination observed above lead to an overall slower germination process that by no means affects the spore's viability as is the case with the FP GerKA-KC and GerKB GR proteins (61, 63). However, the role of GerKA-KC and GerKB in germination of NFB spores cannot be discarded and is matter of further studies.

A second major conclusion offered by this work is the fact that GerAA is required for optimal DPA release with the cationic surfactant dodecylamine. This cationic surfactant triggers germination by opening a gated channel called "DPA channel" localized in the spore's inner membrane and composed in part by the SpoVA proteins (57, 88-92). In *B. subtilis* species, spores lacking all GR release wild-type

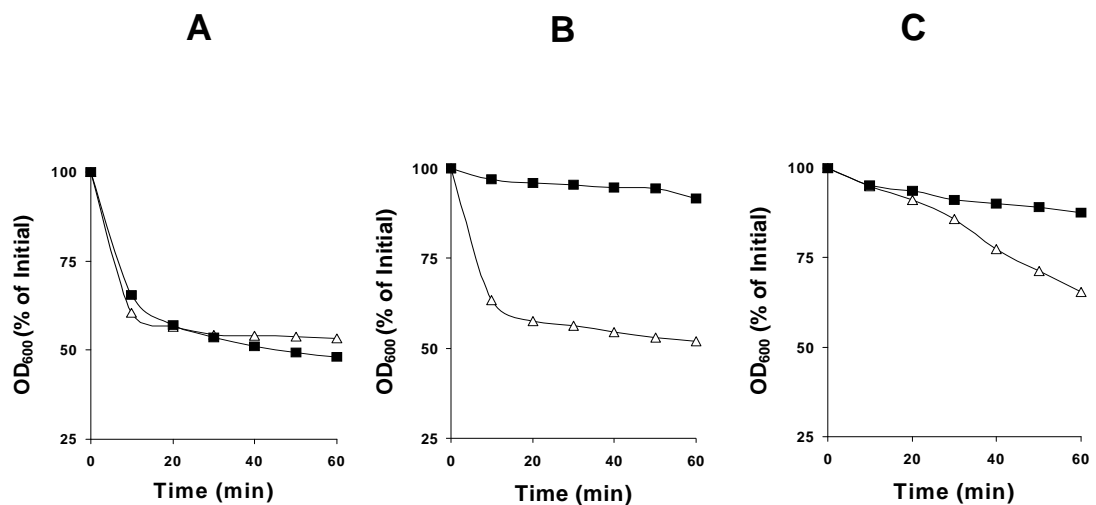


level of DPA (79). In contrast, the FP GR, GerKA-KC (63), and now the NFB GR, GerAA, are required for normal release of DPA from the spore core in presence of dodecylamine. This difference in functionality between *B. subtilis* and *C. perfringens* GR might reflect important differences in the mechanism of signal transduction of the germination signal from the GR to downstream effectors such as the SpoVA proteins. Indeed, a major difference between members of *Bacillus* and *Clostridium* gene is the presence of a lipoprotein, GerD, in the former group (59). The GerD protein is localized in the spore's inner membrane at levels 100-fold higher than the GR, but at 10-fold lower levels than the SpoVA proteins (66, 67). Although the precise role of the GerD protein has not been proven, it is suggested that its role in germination is to amplify the germination signal from the GR to the SpoVA proteins (59). Therefore, given the fact that *C. perfringens* lacks this lipoprotein, the results presented in this and previously (63) studies suggest, but by no means prove, that the FP GerKA-KC GR proteins and now the NFB GerAA GR protein might be directly interacting with the DPA channel. Further studies to identify the role of GerKA-KC and GerKB receptor proteins in dodecylamine germination of NFB spores are being conducted in our lab.

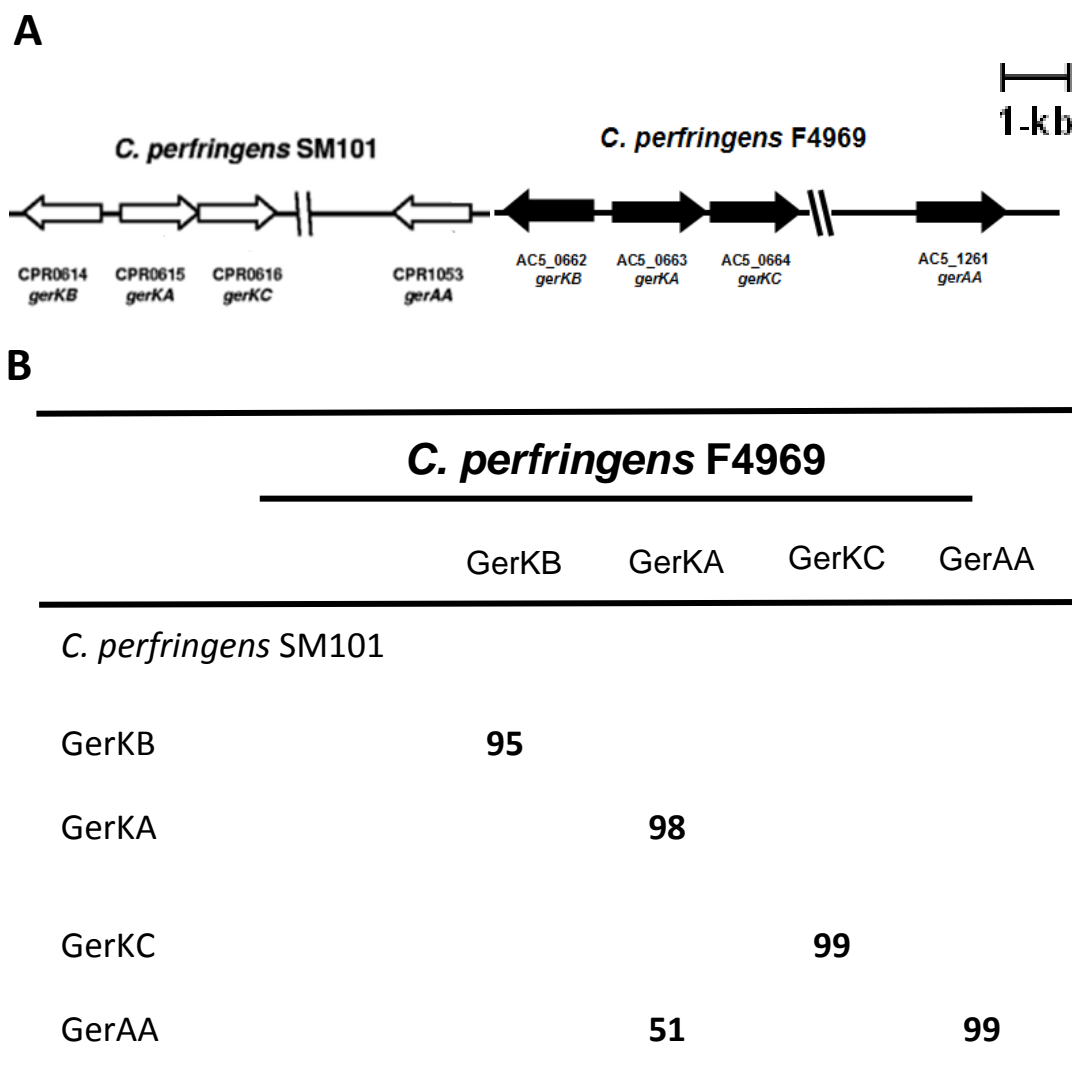
Finally, this work allows the development of a preliminary model of the AK germination pathway between spores of FP and NFB isolates (Fig. 7) as follows: i) In spores of *C. perfringens* FP isolates, the AK mixture acts mainly through the GerKA-KC receptor proteins, while GerAA and GerKB have an auxiliary role mainly at low concentrations (~ 10 mM) of AK (61, 63). Upon binding of AK to the GR, GerKA-KC

is again the major receptor protein involved in triggering the release of the spore core's large depot of DPA. However, in spores of *C. perfringens* NFB isolates, the AK mixture seems to act through the GerAA receptor, although the role of the other receptor proteins cannot be excluded. GerAA is also essential for the initiation of the release of DPA from the spore core; ii) In spores of *C. perfringens* FP isolates, the main GR proteins, GerKA-KC, are required for normal release of DPA from the spore core with dodecylamine. In spores of NFB isolates, GerAA is required for dodecylamine triggered DPA release. However, the roles of the NFB GR proteins GerKA-KC and GerKB needs to be evaluated to fully understand this differential AK-germination pathway.

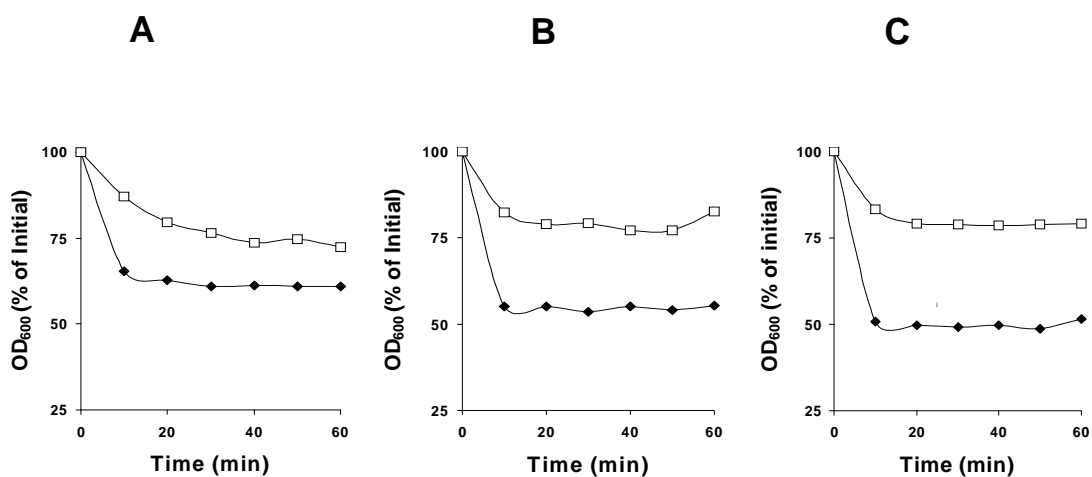
## Figures



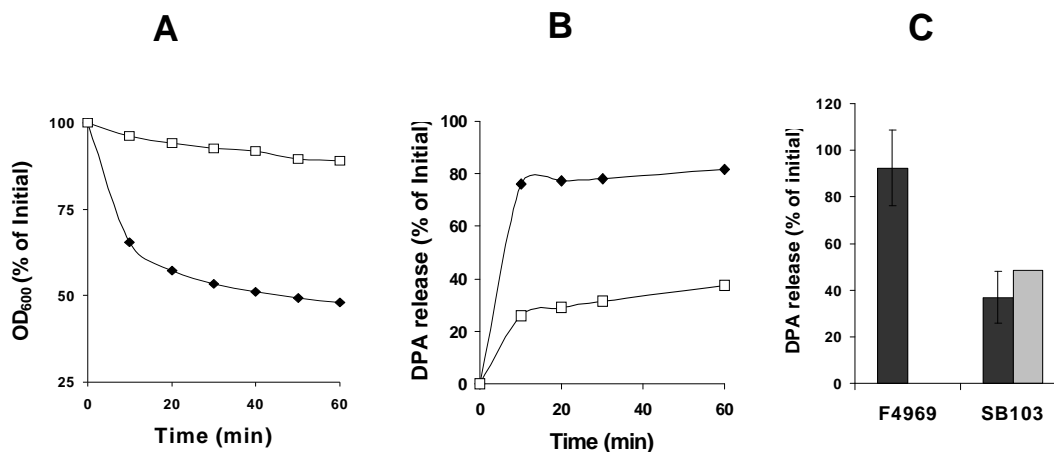
**Fig.2.1 A,B,C. Germination of *C. perfringens* wild-type F4969 and *C. perfringens* wild-type SM101 with various germinants.** Heat activated spores of strains F4969 (■) and SM101 (Δ) were germinated with: A) 100 mM AK (100 mM L-Asn and 100 mM KCl) in 25 mM Tris-HCl (pH 7.0); B) with 100 mM KCl in 25 mM Tris-HCl (pH 7.0); C) with 100 mM L-Asn in 25 mM Tris-HCl (pH 7.0). Decrease in OD<sub>600</sub> was measured as described in Materials and Methods.



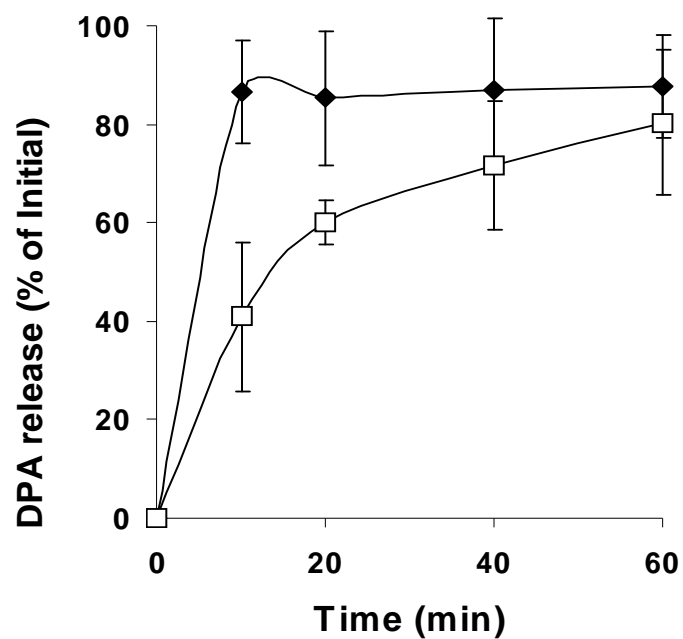
**Fig. 2.2. Analysis of genes encoding germinant receptors in *C. perfringens*.** (A) Comparison of genes encoding germinant receptor proteins in SM101 and F4969. Data were obtained from the Entrez Genome website (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view1>). (B) Percent amino acid sequence similarity between nutrient germinant receptor protein homologues from and *C. perfringens* SM101 and F4969.



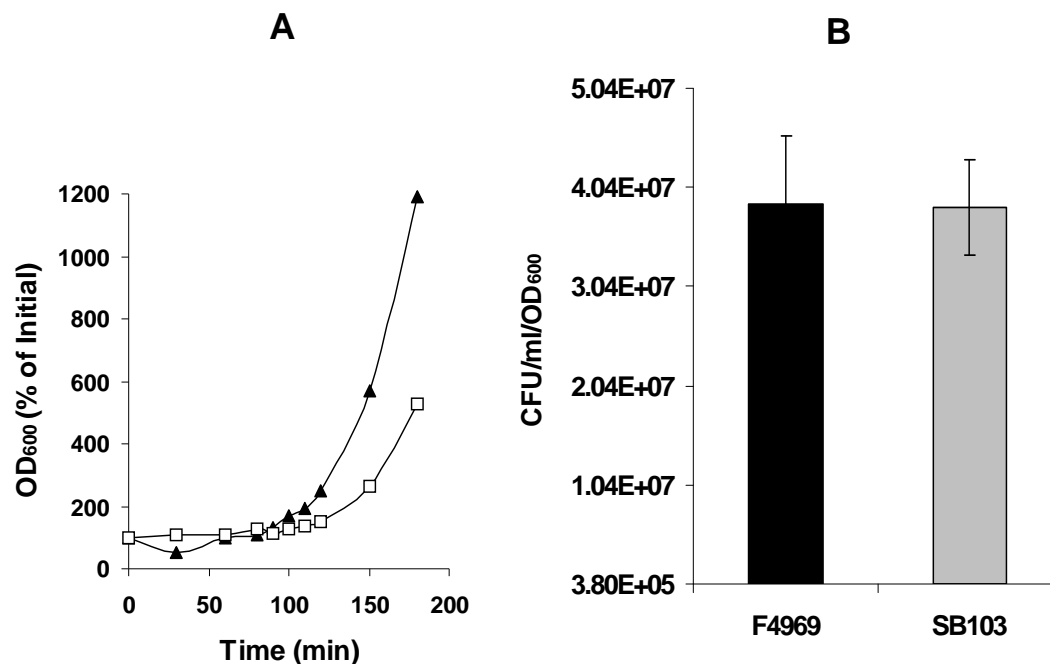
**Fig.2.3 ABC. Germination of *C. perfringens* wild-type F4969 and SB103 spores in nutrient rich media.** Heat activated spores of strains F4969 (◆) and SB103 (*gerAA* mutant) (◻) were incubated at 37°C with: A) tryptone-glucose-yeast extract (TGY); B) Dulbecco's Modified Eagle Medium (DMEM); C) Eagle's minimal essential medium(EMEM), and germination was measured as described in Materials and Methods.



**Fig. 2.4. A-C Germination of *C. perfringens* wild-type and *gerAA* mutant spores with AK.** A) Germination of heat activated spores of strains F4969 (wild-type) (◆) and SB103 (*gerAA* mutant) (□) were germinated with 100 mM AK in 25 mM Tris-HCl (pH 7.0) and OD<sub>600</sub> was measured as described in Materials and Methods. B,C) DPA release during AK-triggered germination of F4969 wild-type and SB103 spores. Heat activated spores of F4969 (wild-type) (◆) and SB103 (□) were incubated with 100 mM AK in 25 mM Tris-HCl (pH 7.0) and DPA released was measured: B) direct measurement at absorbance at 270 nm; C) chemical assay (black bar) after 10 min and (gray bar) after 60 min incubating with 100 mM AK, as described in Materials and Methods.

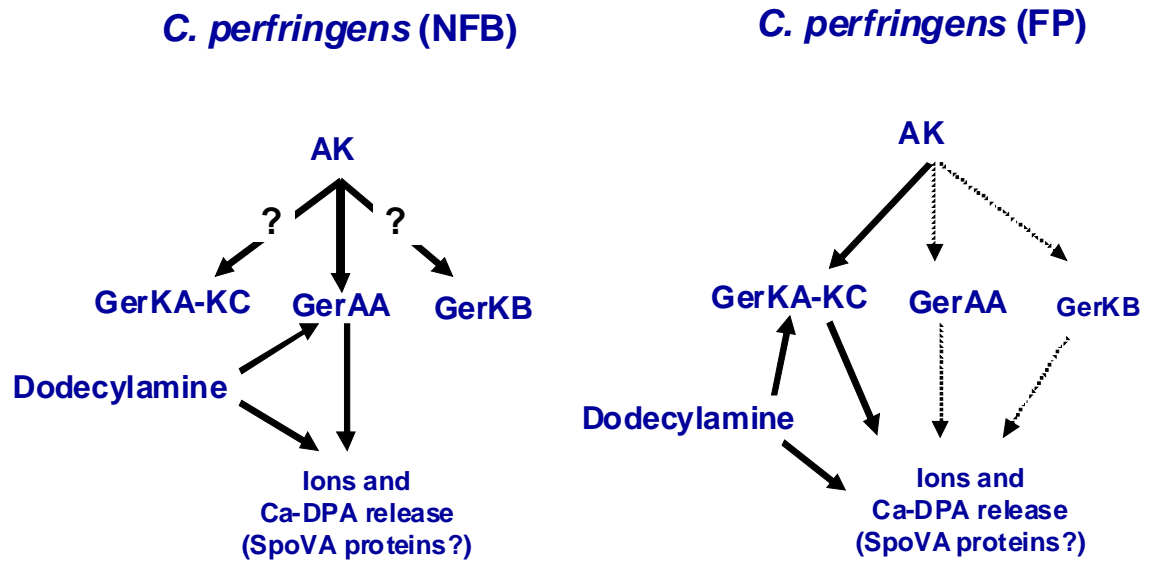


**Fig.2.5. Dodecylamine germination of spores of *C. perfringens* strains.** Spores of strains F4969 (wild-type) (♦), and SB103 (□), were incubated at 60 °C with 1 mM dodecylamine (pH 7.4), and DPA release was measured in Materials and Methods.



**Fig 2.6. Outgrowth of spores of *C. perfringens* strains.** A) *C. perfringens* spore outgrowth. Heat activated spores of strains F4969 (wild-type) (▲), and SB103 (*gerAA* mutant) (□) were incubated anaerobically in TGY broth at an initial OD<sub>600</sub> of 1, and the OD<sub>600</sub> of the cultures was measured. Error bars denote standard deviations. B) *C. perfringens* spore colony forming ability. Heat activated spores of F4969 (wild-type) (black bar) and SB103 (*gerAA* mutant) (gray bar) at an OD<sub>600</sub> ~ 1.0 were plated, incubated overnight at 37°C under anaerobic conditions and titers were calculated as described in Materials and Methods.





**Fig. 2.7. Model of AK germination pathway in *C. perfringens* FP and NFB spores.**

## Tables

**Table 2-1.** Bacterial strains and plasmid used for this study

Strain or plasmid	Relevant characteristic	Source or reference
<i>C. perfringens</i> strains		
SM101	Electroporatable derivative of food poisoning type A isolate NCTC8798; carries a chromosomal <i>cpe</i> gene	(95)
F4969	NON-Food-borne GI disease isolate; carries <i>cpe</i> gene on plasmid	(8)
Plasmid		
pCR-XL-TOPO	<i>E. coli</i> vector; encodes resistance to kanamycin (Kmr; 50µg/ml)	Invitrogen
pJIR750ai	<i>C. perfringens/E. coli</i> shuttle vector containing an <i>L1.LtrB</i> intron retargeted to the <i>plc</i> gene	(63)
pJIR751	<i>C. perfringens/E. coli</i> shuttle vector; Em <sup>r</sup> .	(2)
pDP13	pJIR750ai with IBS, EBS1d, and EBS2 retargeted to insert in <i>gerAA</i>	(63)
pSB16	~2.2-kb <i>kpn1-sal1</i> fragment carry <i>gerAA</i> gene cloned into pCR-XL-TOPO	This study
pSB17	pSB16 into pJIR 751	This study

**Table 2-2.** Primers used in this study

Primer name	Primer sequence <sup>a</sup>	Gene	Position <sup>b</sup>	Use
CPP206	5' CAAGTATTAATCCTCCAATAACAG 3'	<i>gerAA</i>	+1102 to +1126	Detected primer
CPP211	5' CTTTAATGGGAATTATAGCA 3'	<i>gerAA</i>	-264 to -244	Detected primer
CPP857	5' <u>GGTACC</u> GCTACC CTT GCT ATG GTT GAT GT-3'	<i>gerAA</i>	+469 to +447	CP
CPP858	5' <u>GTCGACT</u> TG AGC TGC TTC CAT GAG AGC-3'	<i>gerAA</i>	-1625 to -1604	CP

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- CP, construction of complementing plasmid

## Chapter 3

### Conclusion

*Clostridium perfringens* is a pathogenic anaerobic bacterium that is able to produce more than 17 toxins, allowing *C. perfringens* to cause a wide variety of diseases in humans and animals. Beside toxin production, *C. perfringens* is able to form highly resistance spores that can survive in the environments for years. These spores are the infectious cell morphotype, and in presence of favorable condition, these spores germinate and return to active growth to cause disease. Spore germination is an early and essential stage in the progression of *C. perfringens* infection in human and animal. It can be initiated by a variety of chemicals, including nutrients, cationic surfactants, and enzymes termed germinant. Germination of *Clostridium* species has been less well studied than *Bacillus* species. However, recent findings have identified the germinants of spores of *C. perfringens* food poisoning (FP) and non-food borne (NFB) isolates.

In my study we construct and characterize *gerAA* gene knockout mutant from *C. perfringens* F4969. Results showed that a role of SB103 (*gerAA* mutant) on nutrient and non-nutrient germinants germination. We found that GerAA has a major role on AK, and Lysine trigger germination as well as dodecylamine germination. Moreover, GerAA is required for NFB F4969 spore outgrowth, but not for colony forming efficiency.

In conclusion, the result showed a major difference in germination between spores of wild-type F4969 and SB103 (*gerAA* knockout mutant). These results indicate that there are significant differences in the GerA-type receptors between FP and NFB isolates.

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