AN ABSTRACT OF THE THESIS OF

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Title: Characterization of Germination of *Clostridium perfringens* Spores from Various Sources

Abstract approved:

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*Clostridium perfringens* is a pathogenic anaerobic bacterium that is able to produce more than 17 toxins, allowing *C. perfringins* 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.

The first focus of this project was to compare the nutrient and non-nutrient induced germination of spores of FP versus NFB isolates. The result showed that spores of FP isolates can germinate with KCl, L-asparagine and a mixture of KCl and L-asparagine (AK), while spores of NFB isolates germinate well only with the AK mixture. While dodecylamine is a universal germinant for spores of both FP and NFB isolates, a 1:1 chelate of $Ca^{2+}$ and dipicolinic acid (Ca-DPA) can induce germination of spores of most FP isolates but none of NFB isolates. These results suggest a possible difference between spore germination mechanism in FP versus NFB isolates and this might be, at least in part, due to the differences in germination machinery.

The second focus of this study was to investigate the germination requirements of spores of *C. perfringens* animal isolates (AI). Result from this study found that although AI spores germinated poorly in nutrient broth, they germinated well in tissue culture media, specially RPMI 1640. Most notably, was the ability of L-threonine to trigger germination of most AI spores, followed by L-lysine and to a lesser extent L-alanine. There was no correlation in the germinant requirements and the source of isolation of AI. Interestingly, this study also reports novel germinants for FP and NFB spores which include L-glutamine and L-aspartic acid and to a lesser extent L-lysine and L-arginine.

Collectively, the present study contributes to the understanding of the germinant specificity of spores of *C. perfringens* AI and the differential germinant requirements between FP and NFB spores.

# Characterization of Germination of *Clostridium perfringens* Spores from Various Sources

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## Chapter 1

## General Introduction and Literature Review

*Clostridium perfringens* is a Gram-positive, anaerobic, spore-forming, and rod-shaped bacterium. *C. perfringens* belongs to *Clostridium* genus which includes more than 100 species, some of them are pathogens, such as *C. botulinum, C. tetani, C. difficile,* and some have industrial benefits, such as *C. acetobutylicum, C. thermocellum*(18). *C. perfringens* is ubiquitous in the environment and part of normal intestinal flora of humans and animals (32). It is responsible for a wide variety of diseases in both humans and animals including gas gangrene, antibiotic-associated diarrhea, septicaemia, necrotic enteritis and food poisoning (22). *C. perfringens* was first recognized in the 1940's and 1950's as a causative agent of food poisoning and now ranks as the third most commonly reported food-borne pathogen (33).

The pathogenesis of *C. perfringens* attributes to several factors. First, it is able to produce more than 15 different toxins, combination of specific toxins allows *C. perfringens* to cause different diseases. However, individual strains produce only a subset of these toxins (32). Second, it has a rapid doubling time, in less than 10 min it can multiply and proliferate to a large number of organisms and cause contamination (25). Another key factor in *C. perfringens* pathogenesis is, its ability to produce highly resistant spores that allow to survive under harsh conditions for years (25). Spore

properties like heat resistance and specificity of germinants are also important virulence factors involve in the pathogenesis of *C. perfringens*.

## 1.1 Major Toxins of *C. perfringens*

*C. perfringens* is classified into five groups, A through E, based on the production of four major toxins (α, β, ε, and ι). These toxins have different roles in the pathogenesis of *C. perfringens* through their biological activity inside the host. List of major lethal toxins and their characteristics:

**Alpha toxin:**

Alpha toxin is produced by all type of *C. perfringens* with greater amounts by type A isolates. It is responsible for all the symptoms associated with gas gangrene. It is encoded by *plc* gene which is located on the chromosome. This toxin has lethal activites, such as phospholipase C, sphinomyelinase and host cell signaling activities4 (68). It consists of two domains, the N-terminal domain exhibits phospholipase activity while the C-terminal domain is involved in membrane binding (66). Degradation of phosphatidylcholine and sphingomyelin followed by membrane disruption cause tissue damage and lyse of blood cells and epithelial cells (66). The ability of alpha toxin to lyse blood cells used as diagnostic tools to identify *C. perfringens* by using the reverse CAMP test (40).

**Beta toxin:**

Beta toxin is an extracellular toxin produced by *C. perfringens* type B and C isolates. It is the major virulence factor of necrotic enteritis in human known as pigbel and many domesticated livestock including sheeps, lambs, and especially pigs with a high mortality rate (76). The gene encoding beta toxin *cpb* is carried in a large plasmid. It is a pore forming toxin inactivated rapidly by trypsin in the gastrointestinal tract (37). Immunized guinea pigs by beta toxoid do not develop the disease (76). The regulation of this toxin remains unknown.

**Epsilon toxin:**

This toxin is produced by *C. perfringens* type B and type D animal isolates and (*etx)* gene is carried in a large plasmid (76). It is the most potent toxin classified as Category B Bioterrorist agent after tetanus and botulinum toxins (64). It is synthesized as an inactive toxin which can be activated in the intestinal tract by trypsin. It causes enterotoxemia and necrotic enteritis in cattle which result in significant economic losses (82). Recent evidence suggested its biological activity as pore forming toxin which increases vascular permeability of GI wall (56).

**Iota toxin:**

Iota toxin is a binary toxin produced as protoxin by *C. perfringens* type E strains (55, 76). It consists of two subunits: Ia subunit acts as ADP-ribosyltransferase modifies host cell actin and induces cell death, and Ib subunit is involved in the binding and internalization of the Ia subunit into the host cell (23). Both subunits (*iap*

and *ibp*) are encoded in a large plasmid and nontoxic only when combined. The toxin is implicated in sporadic diarrheic outbreaks among domesticated livestock (3).

## Other toxins:

In addition to the major lethal toxins used in the *C. perfringens* classification listed above, there are other toxins produced by some isolates of *C. perfringens*. Some of these toxins have a minor role in *C. perfringens* pathogenesis such as kappa toxin, theta toxin and delta toxin. However, numerous toxins play major role in *C. perfringens* pathogenesis listed below.

### *Clostridium perfringens* Enterotoxin (CPE):

CPE is the most studied virulence factor attributes to *C. perfringens* food poisoning (FP) and non-food born gastrointestinal diseases (NFBGID) in humans. Small percentage (< 5%) of the *C. perfringens* type A isolates produce CPE (33). It is synthesized as a 35 kDa protein in the mother cell during sporulation and released into the intestinal lumen when these sporulating cells lyse to release spores (33).The CPE is encoded by a *cpe* gene that can be located on the chromosome as well as on a large plasmid (10), with chromosomal copy of the *cpe* gene is carried by the majority of *C. perfringens* type A FP isolates, while a plasmid copy of the *cpe* is carried by all NFBGID isolates (11, 26)

CPE is a heat labile protein can be activated by heating for 5 min at 60°C (34). Also, trypsin treatment increases CPE activity by three fold (40). CPE has a cytotoxic

activity and acts as pore forming protein which binds via its C-terminal domain to surface receptor in the host epithelial cells then followed by binding to different proteins to form a large complex ~155 kDa (76). This complex induces lesions altering membrane permeability and ion transport system which lead to apoptosis of the host cell (6, 74).

**Beta-2 Toxin (CPB2):**

A beta-2 toxin has been recently discovered and found mainly in *C. perfringens* type C isolate and also in type A isolates (19). Beta-2 toxin is associated with necrotizing enterocolitis in domestic animals and livestock (84). Lately, a study showed that beta-2 toxin along with the CPE is involved in 75% of *C. perfringens* type A isolates causing antibiotic associated diarrhea (AAD) and sporadic-diarrhea (SD) (15). The beta-2 toxin is a 28 kDa toxin encoded by the *cpb2* gene which is located on the same plasmid as the *cpe* gene in the majority of the isolates causing AAD and SD (7, 15). Trypsin treatment cleaves beta-2 toxin into inactive species (31) This toxin has no homology to beta toxin of type B isolates (16). Even though a little is known about beta-2 toxin mechanism of action, a recent work suggested its function as a pore-forming toxin (31).

**Necrotic Enteritis Toxin B-like (NetB):**

NetB is a newly discovered toxin responsible for necrotic enteritis disease. It has been identified as the major virulence factor in necrotic enteritis of poultry (24), a

disease that costs the international poultry industry huge losses, estimated more than US$ 2 billion per year (81). NetB is a 33 kDa protein chromosomally encoded and present in ~ 15 % of *C. perfringens* type A isolates recovered from chickens (29). It has limited amino acid sequence similarity to other pore forming toxins such as *C. perfringens* beta-toxin (38% identity) and displays cytotoxic activity against chicken leghorn male hepatoma cell line (24). The major feature of NE is acute death, with high mortality rates and the incidence of *C. perfringens* in the intestinal tract and in processed poultry meat being high (82). Clinical signs are including depression, dehydration, somnolence, ruffled feathers, diarrhea and decreased feed consumption (82).

## 1.2 Spore resistance

In addition to toxin production, *C. perfringens* is able to produce spores. These spores are dormant and resist to moist heat, cold, UV radiation, desiccation, and many other environmental factors (20). *C. perfringens* strains exhibit considerable variation in their heat resistance (67) and spores of FP isolates possess significantly greater heat resistance than spores of *C. perfringens* from other sources (62, 67).

A number of factors are responsible for spore resistance including, spore coats, the cortex peptidoglycan (PG) structure, the relatively impermeable spore inner membrane, low water content in the spore core, the high levels of pyridine-2,6-dicarboxylic acid (DPA) in the spore core, and saturation of the spore chromosome with a group of a/b small, acid-soluble proteins (SASP) (43).

## 1.3 Bacterial Spore Germination

Spores of *Bacillus* and *Clostridium* species are formed during sporulation process which is triggered by integrating a wide range of environmental and physiological signals that arise from nutrient depletion, cell density, Krebs cycle (44, 78). In a recent study, it was found that inorganic phosphate (Pi) is required for initiation of *C. perfringens* sporulation (54, 57). Bacterial spores are metabolically dormant and highly resistant to various environmental stresses such as heat, cold, UV radiation and desiccation (43, 59). However, dormant spores monitor the environment, and under favorable conditions, spores germinate, outgrow, resume vegetative growth and then release toxins and cause disease (33). Thus, spore germination is interesting subject and it has been attracting researcher because through this process spores can cause a variety of life-threatening diseases (71). Over the past 20 years, a great amount of knowledge has been gained about *B. subtilis* spore germination, however recent finding elucidate significant differences between *C. perfringens* and *B. subtilis* spore germination (46-54).

Dormant spores initiate germination by sensing the presence of nutrients, termed germinants, in the environment. Germinants known to date include amino acids, sugars or purine nucleosides, yet combination of specific nutrients also triggers germination, for example a mixture for asparagine, glucose, fructose and KCl (AGFK) triggers germination of *B. subtilis* spores as well as *C. perfringens* spores (56, 74). However, the precise mechanism that triggers germination is unclear. Once spores

initiate germination, it goes through subsequent events, each event has different biophysical and biochemical effects:

- First, monovalent ions ($H^+$, $K^+$ and $Na^+$) are released from the spore core through an energy independent mechanism (79). The release of $H^+$ raises the core pH from ~ 6.5 to 7.7, a change that is essential for spore metabolism when hydration levels of the spore core are high enough for enzymatic activity (71).
- Second, release of the spore core's large depot (~ 10% of spore dry wt) of pyridine-2, 6-dicarboxylic acid (dipicolonic acid [DPA]) associated with its divalent cations, predominantly $Ca^{2+}$ (Ca-DPA). This event follows ion release (79).
- Third, increase core hydration by replacement of Ca-DPA with water, causes some decrease in spore wet-heat resistance but not sufficient for protein mobility (12, 70).
- Fourth, hydrolysis of the spore's peptidoglycan (PG) cortex is a later event in germination, and in *B. subtilis* is triggered at least in part by Ca-DPA release (2, 41).
- Fifth, degradation of the PG cortex eliminates the physical constraint allowing the core to expand and take up water to levels found in vegetative cells and resumption of metabolism (58).

The entire events comprise the germination process, take place without detectable energy metabolism (71). Spores also can be germinated with "non-nutrient" agents including lysozyme, salts, high pressure, $Ca^{2+}$-DPA and cationic surfactants

such as dodecylamine (17, 69). These non-nutrient agents might well act through several of the components of the nutrient germination pathway (4). It is also likely that Ca-DPA released from one spores might stimulate germination of neighboring spores (69).

## Objective of this study

*C. perfringens* spores are ubiquitously found in the environment, and therefore are easily aquired by the host. Once inside the host, these spores must germinate in order to cause disease. The wide variety of diseases caused by *C. perfringens* in different hosts suggests that the germinant specificity of *C. perfringens* spores has adapted to better fit the host. Results from this study will contribute to our understanding of the germinant specificity of spores of *C. perfringens* isolates.

.The specific objectives of this research are:

- To identify and characterize the nutrient and non-nutrient induced germination of spores of FP versus NFB isolates
- To identify and characterize the nutrient and non-nutrient induced germination of spore of various *C. perfringens* animal isolate

# Chapter 2

# Further Comparison of Germination of Spores of *Clostridium perfringens* Food Poisoning Versus Non-Food Borne Isolates.

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Submitted to Food Microbiology

## 2.1 Abstract

*Clostridium perfringens* type A isolates carrying a chromosomal enterotoxin gene (*cpe*) are primarily associated with food poisoning (FP), while *C. perfringens* type A isolates carrying a plasmid-borne *cpe* with non-food-borne (NFB) gastrointestinal (GI) diseases. Spores of both types of isolates are thought to be the infectious cell morphotype, and in presence of favorable conditions, these spores germinate and return to active growth to cause GI diseases. In this study, we compared the nutrient and non-nutrient induced germination of spores of FP versus NFB isolates. We show that spores of FP isolates can germinate with KCl, L-asparagine and a mixture of KCl and L-asparagine (AK), while spores of NFB isolates germinate well only with the AK mixture. While dodecylamine is a universal germinant for spores of both FP and NFB isolates, a 1:1 chelate of $Ca^{2+}$ and dipicolinic acid (Ca-DPA) can induce germination of spores of most FP isolates but none of NFB isolates. Our PCR and nucleotide sequence analyses of the *csp-sleC* locus of FP and NFB isolates also showed significant differences; while NFB isolates carry a tricistronic *cspABC* operon, FP isolates encode a monocistronic *cspB* and a significantly divergent *sleC* variant. Collectively, these results suggest a possible difference between spore germination mechanism in FP versus NFB isolates and this might be, at least in part, due to the differences in germination machinery.

## 2.2 Introduction

*Clostridium perfringens* is a gram-positive, spore-forming anaerobic bacterium capable of causing a wide variety of histotoxic and gastrointestinal (GI) diseases in humans and animals (33, 34). The most common *C. perfringens* associated illnesses in humans are *C. perfringens* type A food poisoning (FP) and non-food borne (NFB) GI diseases (i.e., sporadic diarrhea and antibiotic associated diarrhea) (33). Reports have shown that these *C. perfringens* GI-illnesses are associated with mostly *C. perfringens* type A isolates (among type A-E) that produce the *C. perfringens* enterotoxin (CPE) (33). Several studies have shown that all NFB disease isolates carry a copy of the *cpe* gene on a plasmid, while the majority of *C. perfringens* type A FP isolates carry a chromosomal copy of the *cpe* gene (10, 11, 26).

The molecular basis involved in the differences in pathogenesis between FP and NFB isolates is not clearly understood. However, it is well established that spores are the causative morphotype of *C. perfringens* associated FP and NFB diseases in humans (33), and striking differences in spore resistance between these two groups of isolates seem to contribute to their differential pathogenic traits. For example, spores of FP isolates are more suited for the FP environment than spores of NFB isolates due to higher resistance of spores of FP isolates to heat (67), cold (4ºC) and freezing (-20ºC) temperatures (28), and chemicals used in food industry settings (27). The main factors involved in spore resistance to heat, radiation and a variety of chemicals are the followings. i) Saturation of spore DNA with α/β-type small acid soluble proteins (SASPs) (61, 62). ii) High levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid

[DPA]) in the spore core (46). iii) A highly dehydrated spore core that contributes to protein immobility, protecting essential enzymes and protein from environmental stress (8). However, spores of FP and NFB isolates have similar levels of SASPs (60) and DPA (38).

To cause disease, bacterial spores break dormancy through germination when they sense compounds termed germinants, including nutrients, a 1:1 chelate of $Ca^{2+}$ and pyridine-2,6-dicarboxylic acid (dipicolinic acid) [DPA] (Ca-DPA) and cationic surfactants (i.e., dodecylamine) (71). Spore germination in *Bacillus* species is normally triggered by the binding of nutrient germinants to receptors located in the spore's inner membrane, leading to the release of monovalent cations (i.e., $Na^{+}$ and $K^{+}$) and the spore core's large depot of DPA present as a 1:1 chelate with divalent cations, primarily $Ca^{2+}$ (71). These small molecules are replaced by water leading to spore core hydration. The release of the latter molecules triggers the action of cortex-lytic enzymes (CLEs) on the spore's large peptidoglycan (PG) cortex (71), which in turn allows the core to swell significantly through additional water uptake to a value comparable to that of growing cells (71). This elevated core hydration allows restoration of metabolism leading to spore outgrowth (12, 71). In contrast to *Bacillus* species, *C. perfringens* has no tricistronic *gerA*-type receptor, but only a monocistronic *gerAA* and a *gerK* locus that contains a bicistronic *gerKA-KC* operon, and monocistronic *gerKB* upstream of and in the opposite orientation to that of *gerKA-KC* (36, 73). GerKA and/or GerKC are required for L-asparagine germination and have partial roles in germination with KCl and a mixture of L-asparagine and KCl

(AK) (53). However, GerAA and/or GerKB play an auxiliary role in *C. perfringens* spore germination (51, 53). Interestingly, a recent study (53) showed that spores of FP isolates are able to germinate with either L-asparagine or KCl, while spores of NFB isolates need a mixture of AK for their germination. These findings suggested that spore germination response in FP versus NFB isolates might differ significantly and deserves further study. Therefore, in this study, we have further compared germination of spores of FP versus NFB isolates. Our current results, although shows differences in nutrient germination similar to those previously reported (53), reports novel differences in non-nutrient germination. Such as, while spores of most FP isolates did germinate with exogenous Ca-DPA, spores of all tested NFB isolates did not. However, spores of both FP and NFB isolates germinated well with the cationic surfactant, dodecylamine. Our analyses of the *csp-sleC* locus of FP and NFB isolates also showed significant differences.

## 2.3 Materials and Methods

**Bacterial strains and plasmids.** The *C. perfringens* strains used in this study included the following. FP type A isolates carrying chromosomal *cpe* gene: SM101 (an electroporatable derivative of FP type A isolate NCTC8798), NCTC8798, NCTC10239, 6263, E13 and FD1041 (10, 14, 80, 85); NFB type A isolates carrying a plasmid *cpe* gene: F4969, NB16, B40, B41, B11, F5603, F5537 (10).

**Spore preparation and germination assay.** Spores of various *C. perfringens* strains were prepared as previously described (46, 53), and suspended in distilled water at a final optical density at 600 nm ($OD_{600}$) of ~ 6 and stored at -20ºC. All spore preparations used in this work were > 99% pure as determined by phase contrast microscopy. Spore suspensions were heat activated at 80°C for 10 min, cooled in water at ambient temperature for 5 min, and incubated at 40°C for 10 min as described (53). Spore germination with nutrients or Ca-, Mn- or Mg-DPA (50 mM $CaCl_2$, $MnCl_2$ or $MgCl_2$ and 50 mM DPA adjusted to pH 8.0 with Tris-base) was routinely measured by monitoring the $OD_{600}$ of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~ 60% upon complete germination of wild-type spores; levels of germination were also confirmed by phase-contrast microscopy. The extent of spore germination was calculated by measuring the decrease in $OD_{600}$ and was expressed as a percentage of the initial $OD_{600}$. All values reported are averages of two experiments performed on at least two independent spore

preparations, and individual values varied by less than 15% from average values shown.

**DPA release.** DPA release from spores during dodecylamine germination was measured by incubating spore suspensions ($OD_{600}$ of 1.5) at 60ºC with 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4). Aliquots (1 ml) of germinating cultures were centrifuged for 5 min at 13,200 rpm in a microcentrifuge and DPA in the supernatant fluid was measured by monitoring the $OD_{270}$ as described (6, 53). The total DPA content of spores was measured by boiling an aliquot (1 ml) for 60 min, centrifugation at 13,200 rpm in a microcentrifuge for 5 min, and measuring the $OD_{270}$ of the supernatant fluid as described (6, 53). In *C. perfringens* spores ~ 90% of the material absorbing at 270 nm is DPA (52).

***csp-sleC* locus PCR analysis.** For DNA isolation, a starter culture (6 ml) of each *C. perfringens* isolate was prepared by overnight growth at 37ºC in fluid thioglycolate broth (FTG; Difco), as described previously (13). An aliquot (0.2 ml) of each FTG culture was inoculated into 10 ml TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine) (13), which was then incubated overnight. Total *C. perfringens* DNA was isolated from overnight TGY cultures by a previously described protocol (13). The isolated DNA was then subjected to screening by *csp*-specific PCR. Primers CPP292/CPP294 (Table 2.1) were used to amplify PCR products carrying the region between *sleC* and *cspB* (Fig. 2.4A) and primers CPP293/CPP295 (Table 2.1) were

used to amplify PCR products carrying the region between *cspB* and CPR2568/AC52976 (Fig. 2.4A). These PCRs used 10 ng of template DNA, 25 pM each primer, 200 μM deoxynucleotide triphosphates (Fermentas), 2.5 mM $MgCl_2$, and 1 U of *Taq*DNA polymerase (Fermentas) in a total volume of 50 μl. The reaction mixture was placed in a thermal cycler (Techne) for an initial period of 1.5 min at 95ºC (denaturation) and was then subjected to 32 cycles, each consisting of 1 min at 94ºC, 1 min at 50ºC (annealing), and 1 min at 72ºC (extension), followed by an additional period of extension for 10 min at 72ºC. After PCR, presence of amplified products was analyzed by subjecting an aliquot of each PCR sample to electrophoresis at 100 V in 1.5% agarose gels, followed by ethidium bromide staining and visualization under UV illumination.

**Sequencing of *C. perfringens* genes.** Sequences of the *sleC* ORFs from strains E13 (GenBank accession no. FJ828661), NCTC10239 (GenBank accession no. FJ828662) and FD1041 (GenBank accession no. FJ828663) and, C-terminus of *gerKA* and *gerKC* from FD1041 were obtained from PCR products amplified using Phusion™ High-Fidelity DNA Polymerase (New England BioLabs® Inc., Ipswich, MA). Briefly, primers CPP478/CPP479 and CPP480/CPP477 (Table 2.1) were used to PCR-amplify the N-terminal and C-terminal half of *sleC*, respectively, from genomic DNA of *C. perfringens* strains E13, NCTC10239 and FD1041. Primers CPP606/CPP607 and CPP608/CPP609 (Table 2.1) were used to PCR-amplify the C-terminal half of *gerKA* and *gerKC*, respectively, from genomic DNA of FD1041. These PCR products were

cloned into Zero-Blunt®-TOPO® (Invitrogen). Both strands of the DNA inserts present in these recombinant TOPO plasmids were sequenced using M13 forward and reverse primers using an ABI 3730 capillary sequence machine (Applied Biosystems, Foster City, CA, U.S.A.) at the Oregon State University Center for Genome Research and Biocomputing core facility.

## 2.4 Results

**Nutrient germination of *C. perfringens* isolates.** Although nutrient germination (i.e., L-asparagine, KCl, and a mixture of L-asparagine and KCl (AK)) has been previously described (53), we revisited these germination phenotypes for the spore preparations used in this study for comparative purposes. As expected, spores of FP isolates germinated well with L-asparagine, KCl and the AK mixture (Fig. 2.1), with the exception of FD1041 spores that only germinated in the presence of the AK mixture (Fig. 2.1). In contrast, spores of NFB isolates germinated well with the AK mixture, but exhibited null germination with L-asparagine and KCl alone (Fig. 2.1).

**Germination of *C. perfringens* spores with Ca-DPA.** The non-nutrient germinant Ca-DPA is able to induce germination of *Bacillus subtilis* and *C. perfringens* spores (53, 71). In *B. subtilis*, exogenous Ca-DPA bypasses the germinant receptors and directly activates the CLE, CwlJ (41). In contrast, in spores of *C. perfringens* FP strain SM101, exogenous Ca-DPA acts, at least in part, through GerKA and/or GerKC (51, 53). The differential nutrient germination phenotype observed between spores of FP and NFB isolates, as shown previously (53) and in Fig. 2.1, suggested that this could also be the case for Ca-DPA-germination. Consequently, we evaluated the Ca-DPA germination of spores of a FP isolate (i.e., strain SM101) and a NFB isolate (i.e., strain F4969). As expected from results of our previous study (53), spores of SM101 germinated well with Ca-DPA, but surprisingly no germination of F4969 spores was

observed (Fig. 2.2A). Phase contrast microscopy indicated that while ~ 90% of SM101 spores had become phase dark after 60 min of incubation with Ca-DPA, indicative of complete germination, less than 1% of F4969 spores had become phase grey after 60 min of incubation with 50 mM Ca-DPA, indicative of no germination (data not shown). To rule out the possibility that high Ca-DPA concentrations might act as an inhibitor of germination of F4969 spores, we evaluated lower concentrations of Ca-DPA. While SM101 spores exhibited optimum germination with 50 mM Ca-DPA, no significant increases in the maximum rate (Fig. 2.2B) and extent of germination (Fig. 2.2C) were observed with F4969 spores germinated with different Ca-DPA concentrations (0-50mM). The negative Ca-DPA-germination phenotype of spores of F4969 is not the effect of heat activation temperature (80˚C for 10 min), as no significant germination was also observed when F4969 spores were activated by 75˚C for 10 min (data not shown), the optimum heat activation condition for germination of spores of NFB isolates (53).

To evaluate whether Ca-DPA might be a universal germinant for spores of *C. perfringens* FP isolates, germination experiments were extended to spores of 7 additional *C. perfringens* isolates including, 5 FP isolates (i.e., NCTC8798, E13, 6263, FD1041, NCTC10239) and 2 NFB isolates (i.e., NB16 and B40) (9, 10). As observed with SM101 spores, spores of all tested FP isolates germinated well with Ca-DPA (Table 2.2), with the exception of FD1041 and NCTC10239 spores. In contrast, spores of NFB isolates, NB16 and B40, did not germinate with Ca-DPA (Table 2.2), results that were confirmed by phase contrast microscopy indicating that less than 1% of

spores from these isolates had become phase grey with no phase dark spores detected after 60 min of incubation with Ca-DPA.

The unique ability of spores of FP isolates to germinate with Ca-DPA might be advantageous for germination in a FP environment since the release of Ca-DPA from one spore might lead to induce germination of neighboring spores. To test this, heat activated spores of strain SM101 were incubated in supernatant from boiled SM101 spores (at an $OD_{600}$ ~6.0), which contains the majority of the spores' Ca-DPA content. No detectable decrease in $OD_{600}$ was observed after 60 min of incubation at 40ºC of SM101 spores with the boiled supernatant, and phase contrast microscopy indicated that the majority (> 95%) of the spore population remained phase bright, similarly as spores incubated in distilled water (data not shown). Although the concentration of DPA in the spore core is estimated to be > 0.8 M, well above Ca-DPA solubility (21), the boiled supernatant contained ~ 0.25 mM DPA, which is consistent with this concentration being unable to trigger germination (Fig. 2.2B,C).

**Germination of *C. perfringens* spores with DPA chelated with other cations.** Since spores of two FP and all NFB isolates were unable to germinate with Ca-DPA, we hypothesized that DPA chelated with other cations (i.e., $Mg^{2+}$ and $Mn^{2+}$) might induce germination of spores of these isolates. Spores of FP isolates exhibited a small response with either Mg-DPA or Mn-DPA, and phase contrast microscopy indicated that ~ 10% of FP spores became phase dark after 60 min of incubation, which were similar to those observed in Tris-HCl (pH 8.0) buffer (Table 2.2), suggesting that

exogenous Mg-DPA and Mn-DPA are unable to significantly initiate germination. Spores of NFB isolates exhibited no detectable germination with either Mg-DPA or Mn-DPA as observed by decrease in $OD_{600}$ (Table 2.2) and phase contrast microscopy (data not shown). These results indicated that only a 1:1 Ca-DPA chelate is capable of inducing germination of *C. perfringens* spores and that this germination trait is unique among spores of most FP isolates.

**Dodecylamine germination of *C. perfringens* isolates.** Another non-nutrient germinant is the cationic surfactant, dodecylamine, that triggers germination by opening a DPA-channel stimulating DPA-release from the core of *B. subtilis* spores (69). Although previous study showed that dodecylamine induces DPA-release from the spore core of *C. perfringens*, that finding was limited to a single FP strain SM101 (53). Thus, to evaluate if dodecylamine-germination is unique to FP isolates, or common to most *C. perfringens* isolates, other FP and NFB isolates were evaluated. Spores from all tested FP and NFB isolates released the majority of their DPA within 60 min of incubation with dodecylamine (Fig. 2.3). These results indicate that, in contrast to Ca-DPA, dodecylamine is a universal germinant for spores of most, if not all, *C. perfringens* isolates.

**Comparative analyses of the germination machinery in *C. perfringens* FP versus NFB isolates.** In an effort to identify differences in the germination apparatus that might be associated with the differential germination phenotype between FP versus

NFB isolates, all of the components that, to our knowledge, have a role in *C. perfringens* spore germination were analyzed (46, 49, 51-53). Strains SM101 (FP isolate) and F4969 (NFB isolate) were used for bioinformatic analyses since their genome sequence is completed and under assembly, respectively (36). The main receptor involved in Ca-DPA germination of SM101 spores is the GerKA and/or GerKC receptor(s). The amino acid alignment of GerKA and GerKC of SM101 versus F4969 yielded 99% similarity and 98% identity for both proteins. At least two non-conservative (at position 319, $Pro^{F4969}$ for $Thr^{SM101}$; at position 401, $Thr^{F4969}$ for $Pro^{SM101}$), and four more conservative (at position 404, $Phe^{F4969}$ for $Leu^{SM101}$; at position 439, $Asn^{F4969}$ for $Ser^{SM101}$; at position 448, $Met^{F4969}$ for $Ile^{SM101}$; at position 453, $Asp^{F4969}$ for $Glu^{SM101}$) amino acid substitutions were observed in the C-terminus of GerKA. In the case of GerKC, all substitutions were conservatives with the exception of one at the C-terminus (at position 365, $Asp^{F4969}$ for $Asn^{SM101}$). Furthermore, since most of the amino acid substitutions were observed in the C-terminal of GerKA, the GerKA C-terminus of strain FD1041 was sequenced to evaluate the possibility of this region being responsible for the lack of Ca-DPA germination in FD1041 spores. However, nucleotide sequence of C-terminal region of FD1041 *gerKA* showed 100% identity to the nucleotide sequence of SM101's *gerKA* (data not shown), suggesting that Ca-DPA germination might act through a different activation pathway that might be absent in spores of FD1041, NCTC10239 and NFB isolates.

We noticed striking differences in the *csp* locus of strains SM101 and F4969 (Fig. 2.4A). Strain SM101 has one ORF, *cspB*, while strain F4969, similarly as strain S40 (30, 39, 72), has a tricistronic operon encoding *cspABC* (Fig. 2.4A). To evaluate whether this *csp* gene organization is conserved in other FP and NFB isolates, PCR analyses on DNA of FP and NFB isolates were conducted (Fig. 2.4B,C). Consequently, PCR products of ~ 0.5-kb and ~ 0.8-kb obtained from SM101 DNA with primer pairs CPP292/CPP294 and CPP293/CPP295, respectively, is indicative of the presence of only *cspB*. In contrast, PCR products of ~ 2.2-kb and ~ 2.5-kb from F4969 DNA with primer pairs CPP292/CPP294 and CPP293/CPP295, respectively, is indicative of the presence of *cspC* and *cspA* in addition to *cspB* (Fig. 2.4A). These PCR analyses indicated that all tested FP isolates possess only *cspB* but not *cspA* and *cspC* (Fig. 2.4B,C). However, all tested NFB isolates carry an intact *cspABC* operon, indicating that NFB isolates have a unique *csp-sleC* locus. Furthermore, amino acid sequence alignment of SleC from FP versus NFB isolates revealed significant differences. The site where Csp proteins cleave the premature junction of pro-SleC (the linkage between Val-149 and Val-150) of FP strains differs from that of other strains, in the former group Val-149 has been substituted to Ala-149 (Fig. 2.5). The high selectivity of the Csp proteins of NFB strain S40 (and perhaps F4969) for Val-149-Val-150 cleavage site in pro-SleC (39) suggest that the mechanism of activation of pro-SleC into 11mature SleC might be different in spores of FP strain SM101, especially since SM101 possess only one *csp* (*cspB*) gene and the mature SleC-domain with a number of substitutions (36, and Fig. 5).

## 2.5 Discussion

Spore germination of pathogenic bacteria is an essential and early step in the biology of their pathogenesis. In *C. perfringens*, spore germination is a crucial step for the development of *C. perfringens*-caused FP illness, and for NFB GI diseases (i.e., antibiotic associated diarrhea, necrotic enteritis, sporadic diarrhea). Consequently, an understanding of the molecular mechanism of *C. perfringens* spore germination is essential for understanding the pathogenesis of the variety of illnesses caused by *C. perfringens*. In this communication, we report a number of new observations leading to differences in the germination response between spores of FP versus NFB isolates.

A major conclusion from this work is that while spores of both FP and NFB *C. perfringens* isolates were able to germinate with dodecylamine, only spores from most FP isolates were able to germinate with Ca-DPA. In contrast, none of the NFB isolates' spores were able to germinate with Ca-DPA or the other DPA chelates tested (i.e., Mg-DPA and Mn-DPA), indicating that the Ca-DPA germination pathway is uniquely confined to the FP isolates. However, this Ca-DPA germination pathway is completely different from that of *B. subtilis* spores, where endogenous Ca-DPA acts as a signaling molecule released from the spore core and allosterically activates the CLE, CwlJ (41); similarly, exogenous Ca-DPA bypasses the germinant receptors and directly activates CwlJ, triggering cortex hydrolysis (41). In spores of FP isolate SM101, Ca-DPA-mediated germination does not activate the CLEs, instead it requires the presence of GerKA and/or GerKC for the release of the spore core's DPA content and initiation of cortex hydrolysis (53). The GerKA and GerKC of sequenced NFB

isolates showed high identity (~97%) with that of FP strain SM101 (data not shown), and yet spores of NFB isolates are unable to germinate with Ca-DPA. In addition, spores of FP and NFB isolates were able to germinate with AK, in agreement with previous results (53). However, L-asparagine or KCl alone was able to induce germination of spores of most FP but none of NFB isolates. It was most interesting that spores of NCTC10239 were able to germinate with all nutrients but not with Ca-DPA. Interestingly, the GerKA and/or GerKC proteins are, at least in part, required for both nutrient and non-nutrient germination of spores of FP isolate SM101 (53). Possible explanations for the differences in spore germination phenotype between FP versus NFB isolates follows i) The GerKA and/or GerKC proteins interact or form a receptor complex with some other unidentified proteins only present in FP isolates. ii) Indeed, some of the amino acid substitutions observed in the GerKA and/or GerKC proteins might be key for germination phenotype of FP spores. However, an obvious question is whether this phenotypic trait gives advantage to FP isolates to cause disease. From the evidence found in this study (data not shown), in a FP environment (i.e., meat products) it seems unlikely that Ca-DPA released from one spore would trigger the germination of their sisters, since Ca-DPA released from a single spores will quickly dilute in the immediate environment, and the concentration of Ca-DPA required to stimulate spore germination is, to our knowledge, inexistent in any physiological and FP environment. Therefore, it seems likely that the same receptor complex involved in KCl germination might also be involved in Ca-DPA germination of spores of FP isolates.

An interesting observation is the unique machinery of FP isolates involved in PG cortex hydrolysis during spore germination. Previous work with *C. perfringens* strain S40 indicated that pro-SleC is processed into the mature SleC by an exudation fraction from spores containing the three Csp proteins (i.e., CspA, CspB and CspC) (39, 72). This fraction would proteolytically cleave the premature junction of pro-SleC in the complex (between Val-149 and Val-150) during germination, generating an active SleC (35, 72). A major difference between FP and NFB isolates is the absence of CspA and CspC in the former group. The absence of these proteins might have implications in the processing of pro-SleC into mature SleC during spore germination in FP isolates, especially since the main CLE of FP isolates, SleC (52), has a number of important substitutions, including the premature junction (Val-149 is replaced by Ala-149), that might affect processing of pro-SleC into mature SleC. However, $CspB_{SM101}$ (CspB from FP strain SM101) although has all three residues (Asp, His, and Ser) typical of the active-site of Csp and other subtilisin proteases (72), exhibits a number of amino acid substitutions not present in $CspB_{F4969}$ (CspB from NFB strain F4969). Thus the CspB and SleC proteins of FP isolates might have co-evolved to efficiently process the pro-SleC into mature SleC triggering cortex hydrolysis. Indeed, a recent study showed that SM101 *cspB* spores were unable to degrade their PG cortex, indicative of null processing of pro-SleC into mature SleC (47). The absence of CspA and CspC in FP isolates is interesting, as one would presume that because FP isolates exhibit high resistance to food processing environments and that processing of pro-SleC into mature SleC by the CspB proteins is crucial for completion of spore

germination (47, 52), they should posses a more robust and fail-proof machinery for PG cortex hydrolysis. Although it is not clear how the unique *csp-sleC* locus in FP isolates might be advantageous, it is likely that modern food processing methods has acted as evolutionary pressure for the high resistance and their unique germination apparatus to the point of possessing a selective advantage over spores of other *C. perfringens* isolates allowing efficient germination in FP environments. Further studies addressing the individual roles of Csp proteins from FP and NFB isolates are currently underway to dissect the role, if any, of the *csp-sleC* locus in differences of spore germination between FP versus NFB isolates.

# Figures

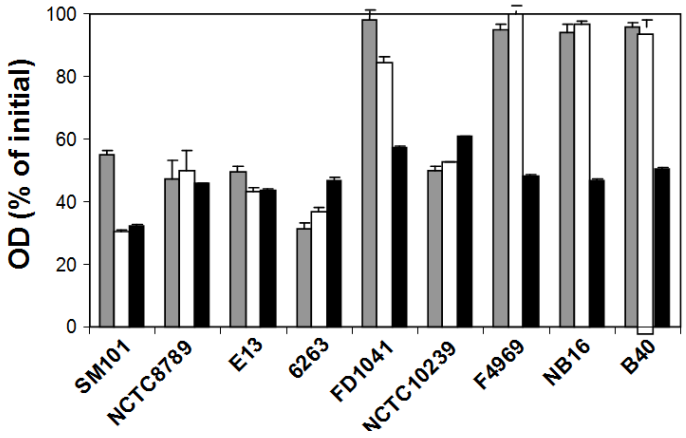


**Fig. 2.1. Nutrient germination of *C. perfringens* spores.** Heat activated spores of various *C. perfringens* FP (SM101, NCTC8798, E13, 6263, FD1041, NCTC10239) and NFB (F4969, NB16, B40) isolates were germinated with 100 mM L-asparagine (grey bars), 100 mM KCl (white bars) and a mixture of 100 mM L-asparagine and KCl (black bars), and $OD_{600}$ was measured after 60 min of incubation at 40ºC as described in Materials and Methods.

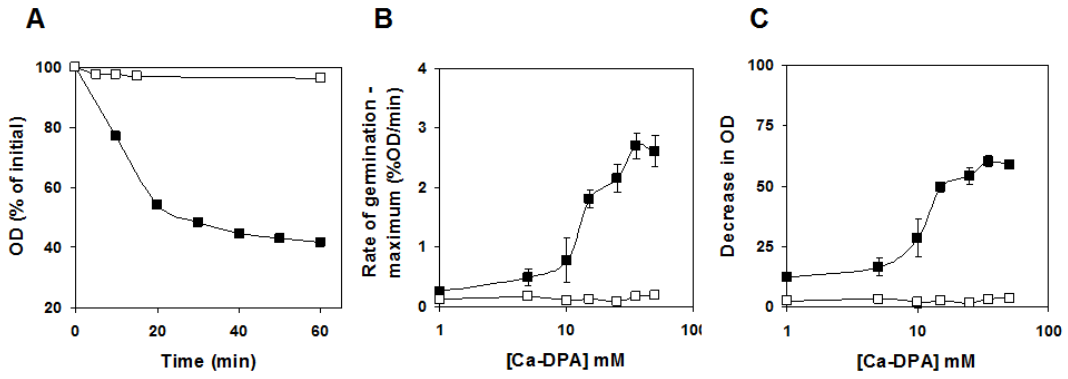


**Fig. 2.2A-C. Ca-DPA germination of *C. perfringens* spores.** A) Heat activated spores of strains SM101 (filled squares) and F4969 (open squares) were incubated with 50 mM Ca-DPA at 40ºC and $OD_{600}$ was measured as described in Materials and Methods. B,C) SM101 and F4969 spores were incubated with various concentrations of Ca-DPA and the maximum rate (B), and extent of germination after 60 min (C) were calculated as described in Materials and Methods.

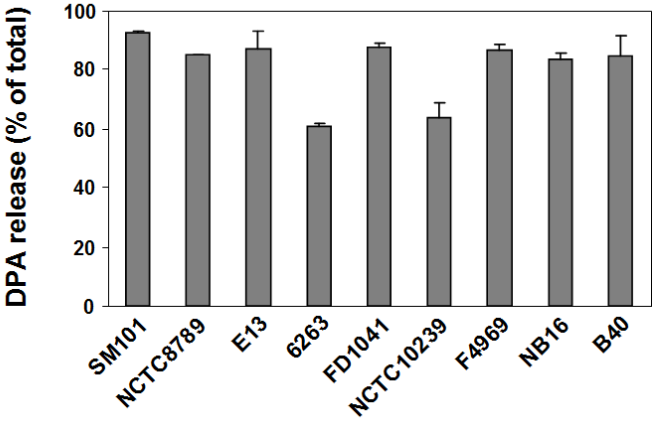


**Fig. 2.3. Dodecylamine germination of *C. perfringens* spores.** Spores of FP (SM101, NCTC8798, E13, 6263, FD1041, NCTC10239) and NFB (F4969, NB16, B40) isolates were incubated with 1 mM dodecylamine in Tris-HCl (pH 7.4) at 60ºC for 60 min, and DPA-release was measured as described in Materials and Methods.

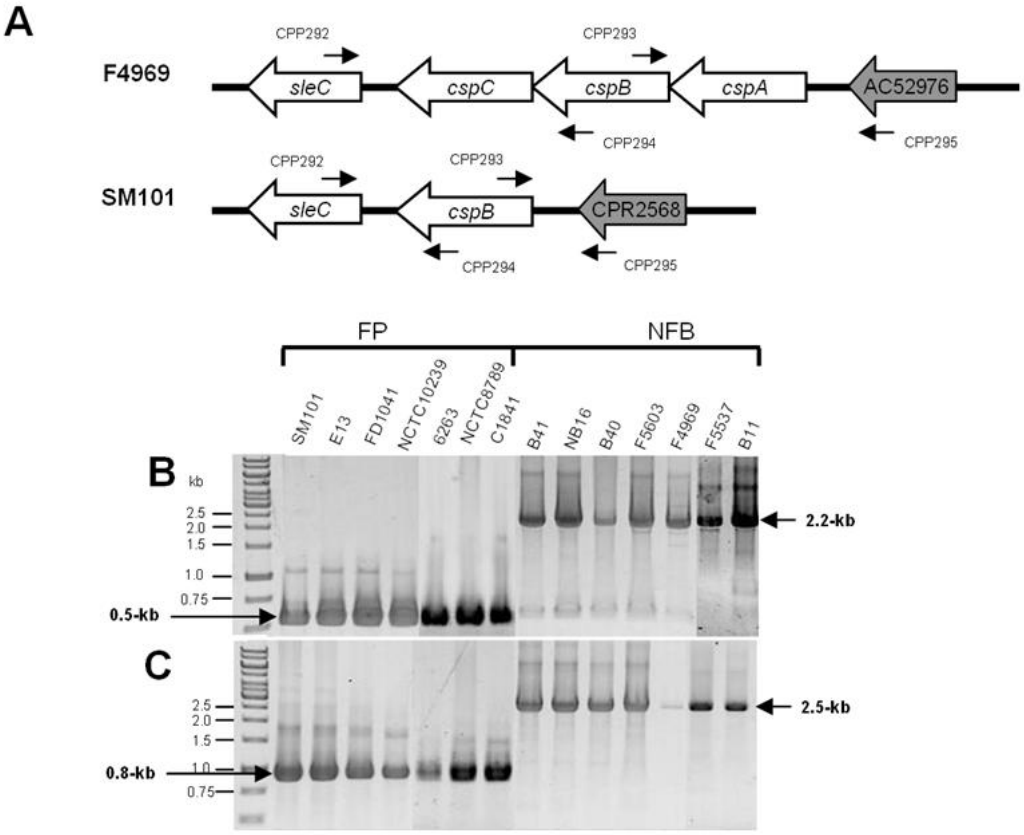


**Fig. 2.4A-C. Genetic organization of the *csp-sleC* locus of various *C. perfringens* strains.** A), Genetic organization of the *C. perfringens csp* genes from strain F4969 and SM101. AC52976 is the ORF upstream *cspA* in strain F4969, and CPR2568 is the ORF upstream *cspB* in strain SM101. B,C), PCR analysis of the *csp-sleC* locus with primers CPP292 and CPP294 (B) and with primers CPP293 and CPP295 (C) from genomic DNA of FP (SM101, E13, FD1041 NCTC10239, 6263, NCTC8798, C1841) and NFB (B41, NB16, B40, F5603, F4969, F5537 and B11) isolates.

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F4969        61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRIALQNVNLPETRGYYRQEEVID
S40          61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRIALQNVNLPETRGYYRQEEVID
st13         61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRIALQNVNLPETRGYYRQEEVID
JGS1495      61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRIALQNVNLPETRGYYRQEEVID
JGS1987      61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRIALQNVNLPETRGYYRQEEVID
ATCC13124    61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRVALQNVNLPETRGYYRQEEVID
ATCC3626     61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRVALQNVNLPETRGYYRQEEVID
JGS1721      61 IENSNQPGTIPYSFAEVIVEREGFLPVAVNGVQIYPSRIALQNVNLPETRGYYRQEEVID
SM101        61 IENSNQPNTIPYSFANVIAEKEGFLPVAVNGVQIYPARVAIQNINLPETRGYYRQEKIIN
E13          61 IENSNQPNTIPYSFANVIAEKEGFLPVAVNGVQIYPARVAIQNINLPETRGYYRQEKIIN
FD1041       61 IENSNQPNTIPYSFANVIAEKEGFLPVAVNGVQIYPARVAIQNINLPETRGYYRQEKIIN
NCTC10239    61 IENSNQPDKIPYSFANVIAEKEGFLPVAVNGVQIYPARMAIQNINLPETRGYYRQEKIIN

F4969       121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSVANYKVNYK
S40         121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSVANYKVNYK
st13        121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSVANYKVNYK
JGS1495     121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPIVPEYIVVHNGRPNDNSAANYKVNYK
JGS1987     121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSAANYKVNYK
ATCC13124   121 IQPNRLVGNFPPKIPEVEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSAANYKVNYK
ATCC3626    121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSVANYKVNYK
JGS1721     121 IQPNRLVGNFPPKIPEAEEKELPPPKGTVVLPEPVVPEYIVVHNGRPNDNSAANYKVNYK
SM101       121 IQPNRLVGNFPPKIPEPEEKELPLPKGIAVLPEPVIPEYIVVHDGRPNDNTAPNYKVNYK
E13         121 IQPNRLVGNFPPKIPEPEEKELPLPKGIAVLPEPVIPEYIVVHDGRPNDNTAPNYKVNYK
FD1041      121 IQPNRLVGNFPPKIPEPEEKELPLPKGIAVLPEPVIPEYIVVHDGRPNDNTAPNYKVNYK
NCTC10239   121 IQPNRLVGNFPPKIPEPEEKELPLPKGIAVLPEPVIPEYIVVHDGRPNDNTAPNYKVNYK

F4969       181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
S40         181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
st13        181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
JGS1495     181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
JGS1987     181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
ATCC13124   181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
ATCC3626    181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
JGS1721     181 DYIKNVACCEIFSTWSENTIRANVYAIISFTLNRIYTEWYRGKGKNFDITNSTAFDHAFS
SM101       181 DYIKNVACCEIFSTWPETTIRANVYAITSFTLNRIYTEWYRGKGKNFDITSSTAFDHAFS
E13         181 DYIKNVACCEIFSTWPETTIRANVYAITSFTLNRIYTEWYRGKGKNFDITSSTAFDHAFS
FD1041      181 DYIKNVACCEIFSTWPETTIRANVYAITSFTLNRIYTEWYRGKGKNFDITSSTAFDHAFS
NCTC10239   181 DYIKNVACCEIFSTWPETTIRANVYAITSFTLNRIYTEWYRGKGKNFDITSSTAFDHAFS
```

**Fig. 2.5. Alignment of deduced amino acid sequence of SleC (residues 61 to 240) from various *C. perfringens* strains.** Grey boxes indicate different amino acid residues. Grey arrow indicates the cleavage site during sporulation giving pro-SleC; black arrow indicates the cleavage site by Csp proteins during germination. The alignment was done with ClustalW between amino acid sequences of SleC from strains: F4969 (GenBank accession no. EDT2750), S40 (AB042154), st13 (NC_003366), JGS1495 (EDS80682), JGS1987 (EDT15414), ATCC13124 (NC_008261), ATCC3626 (EDT24307), JGS1721 (EDT72881), SM101 (NC_008262), E13 (FJ828661), FD1041 (FJ828663) and NCTC10239 (FJ828662).

# Tables

## Table 2.1. Primers used in this study

| Primer name | Primer sequence | Position[†] | Gene | Use[‡] |
|---|---|---|---|---|
| CPP292 | TACTCCATTTACTGCTACAGG | +255 to +276 | *csp-sleC*[§] | PCR |
| CPP293 | CACCCTTAGCTTTATTCTCC | -1870 to -1850 | *csp-sleC*[§] | PCR |
| CPP294 | TTAGGAATCCCTGCAACTGTA | -585 to -564 | *csp-sleC*[§] | PCR |
| CPP295 | GGATATAAACAACTTGCTACTGC | -2368 to -2345 | *csp-sleC*[§] | PCR |
| CPP477 | TTAATAAATTATCTTTGGTACATTTTCACGTCTATCC | +1280 to +1317 | *sleC*[§] | Seq |
| CPP478 | ATGACATTAGGTAGATTGAAGGTTCAATGTTT | 0 to +32 | *sleC*[§] | Seq |
| CPP479 | TCCATAACTAAAAGCATGATCAAAAGCCG | +697 to +726 | *sleC*[§] | Seq |
| CPP480 | CGGCTTTTGATCATGCTTTTAGTTATGGA | +697 to +726 | *sleC*[§] | Seq |
| CPP606 | CAAACACCAGATGATTATTATTTAAATAGGT | +765 to +796 | *gerKA* | Seq |
| CPP607 | CAAATAAAACAAACATATAAGGTCTAATCATG | +1776 to +1808 | *gerKA* | Seq |
| CPP608 | TTAGAGTTAAGCGGAGGAGCTTTG | +615 to +639 | *gerKC* | Seq |
| CPP609 | TTAAAGTGTATTTCCTGTAATTGTTGTGCC | +1095 to +1125 | *gerKC* | Seq |

[†] The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence.

[‡] PCR, primers used for PCR analysis; Seq, construction of sequencing plasmids.

[§] Relative to start codon of *sleC* from *C. perfringens* strain SM101.

**Table 2.2. Germination of *C. perfringens* spores with DPA chelated with cations**

| | Mean % decrease (± SD) in $OD_{600}$ in 60 min at 40ºC[‡] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | FP isolates | | | | | | NFB isolates | | |
| Germinant[†] | SM101 | NCTC 8798 | E13 | 6263 | FD1041 | NCTC 10239 | F4969 | NB16 | B40 |
| Tris | 2 ± 1 | 2 ± 2 | 21 ± 1 | 5 ± 2 | 8 ± 4 | 2 ± 0 | 2 ± 1 | 2 ± 2 | 0 ± 0 |
| Ca-DPA | 47 ± 3 | 45 ± 1 | 47 ± 1 | 56 ± 2 | 4 ± 1 | 10 ± 1 | 0 ± 0 | 0 ± 0 | 3 ± 1 |
| Mg-DPA | 11 ± 0 | 3 ± 0 | 16 ± 3 | 13 ± 7 | 2 ± 3 | 8 ± 4 | 2 ± 1 | 1 ± 1 | 1 ± 0 |
| Mn-DPA | 16 ± 4 | 4 ± 0 | 12 ± 4 | 11 ± 0 | 2 ± 2 | 9 ± 2 | 5 ± 4 | 2 ± 2 | 2 ± 1 |

[†] Heat activated spores were incubated with: Tris, 25 mM Tris HCl (pH 8.0); Ca-DPA, 50 mM Ca-DPA; Mg-DPA, 50 mM Mg-DPA; Mn-DPA, 50 mM Mn-DPA.

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

# Chapter 3

# Characterization of Germination of Spores of *Clostridium perfringens* Animal Isolates.

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To be Submitted to Veterinary Microbiology

## 3.1 Abstract

*Clostridium perfringens* is a pathogenic anaerobic bacterium able to produce more than 17 toxins, allowing *C. perfringens* to cause a wide variety of diseases in humans and animals. The earliest stage in *C. perfringens* infection is spore germination; therefore, the ability of spores to recognize germinants is essential for progression of infection. Previous studies have identified the germinants of spores of *C. perfringens* food poisoning (FP) and non-food borne (NFB) isolates. In this study, we have investigated the germinant requirements of spores of *C. perfringens* animal isolates (AI). Although AI spores germinated poorly in nutrient broth, they germinated well in tissue culture media, especially RPMI 1640. Most notably, was the ability of L-threonine to trigger germination of most AI spores, followed by L-lysine and to a lesser extent L-alanine. There was no correlation in the germinant requirements and the source of isolation of AI. Interestingly, this study also reports novel germinants for FP and NFB spores which include L-glutamine and L-aspartic acid and to a lesser extent L-lysine and L-arginine.

## 3.2 Introduction

*Clostridium perfringens* is a Gram-positive, anaerobic, spore-forming pathogenic bacterium ubiquitously found in the environment and as part of the intestinal flora of humans and animals (63, 75). *C. perfringens* is a prolific toxin producing species, and can produce at least 17 toxins (1, 24, 33). Specific combination of toxins allows *C. perfringens* to cause different diseases, with the most common being *C. perfringens* type A food poisoning (FP), antibiotic-associated diarrhea, gas gangrene, necrotic enteritis in poultry, enterotoxemia in food animals (33). *C. perfringens* spores are considered the infectious morphotype, and to cause disease, these spores must undergo germination and outgrowth to be converted to vegetative cells.

Bacterial spore germination is triggered when compounds, termed germinants, bind to their cognate germinant receptor (GR) located in the spore's inner membrane, triggering the release of monovalent cations (i.e., $Na^+$ and $K^+$) and the spore core's large depot of DPA present as a 1:1 chelate with divalent cations, primarily $Ca^{2+}$ (48, 71). Water replaces these small molecules, increasing the spore core water activity. In many *Bacillus* species, these small molecules activate the action of the cortex-lytic enzymes (CLEs) on the spore's large peptidoglycan (PG) cortex (48, 71). Degradation of the spore PG cortex allows a significant increase in water uptake to levels similar to that of growing cells (71).

A recent study that analyzed the core housekeeping genes (i.e., the gyrase subunit A, *gyrA* gene; the 50S ribosomal protein, *rplL* gene; and the regulatory

protein, *pfoS* gene) and the virulence genes (i.e., alpha-toxin, *plc* gene; and the kappa-toxin, *cola* gene) of 247 *C. perfringens* isolates, suggests that the different diseases are related with cryptic lineages of *C. perfringens*, suggesting that *C. perfringens* isolates have adapted to better fit their host (65). An example is that FP isolates cluster in an independent lineage from that of non-food borne isolates (NFB) and animal isolates (AI) (65). In this particular case, spores of FP isolates are not only better suited than spores of NFB isolates to survive in FP environments (42, 53, 54), but also are better fitted to germinate in FP environments (42, 53, 54). Indeed, while spores of FP isolates are able to germinate with germinants commonly found in contaminated meat products such as KCl and the co-germinants $Na^+$ and Pi, as well as with germinants such as L-asparagine and the non-nutrient germinant Ca-DPA, spores of NFB isolates germinate only with L-alanine and L-valine, and the mixture of L-asparagine and KCl (42, 53, 54). These differences in germinant specificity between FP and NFB spores are also evident in cultured intestinal epithelium cells, where NFB, but not FP spores, germinate well (45). This suggests that selective pressure in the germination apparatus, most likely in the germinant receptors (GRs), might also have acted as a driving force to favor host adaptation of *C. perfringens* isolates. This raises the hypothesis that spores from *C. perfringens* AI might have different germinant-specificity than that of spores of FP and NFB isolates. Consequently, in this study, we have characterized the germination specificities of spore of various *C. perfringens* AI. Our results indicate that there is great variability in the germination specificity of spores of AI, and that some novel germinants for spores of AI include L-threonine, L-lysine, and L-alanine.

In addition, FP and NFB spores were found to germinate with the novel germinants L-glutamine and L-aspartic, and to a lesser extent L-lysine and L-arginine.

## 3.3 Materials and Methods

**Bacterial strains.** The *C. perfringens* strains used in this study are described in Table 3.1.

**Spore preparation.** Purified spore suspensions of various *C. perfringens* isolates were prepared as previously described (53) and suspended in sterile distilled water to a final optical density at 600 nm ($OD_{600}$) of ~ 6 and stored at -20°C until use. Spore suspensions used in this work were > 99% pure as determined by phase contrast microscopy.

**Germination assay.** Spore suspensions were heat activated at 80°C for 10 min for the FP isolate SM101, and 75°C for 15 min for NFB and AI, and cooled on water at room temperature for 5 min, and incubated at 40°C for 10 min prior to mixing with the germinant solution (53). Germination was monitored by measuring the $OD_{600}$ of spore -germinant solution (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~ 60% upon complete germination of wild-type spores. Extent of germination was also confirmed by phase contrast microscopy. To measure the rate at which the spore population decreased its OD, the maximum rates of pore germination were determined by measuring the $OD_{600}$ of germinating spores every 2.5 min, the maximum slopes were calculated, and maximum rates were expressed as loss in $OD_{600}$ of the spore population relative to the initial $OD_{600}$ of the culture. All values

reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 15% from average values shown.

All germinant solutions were prepared at 100 mM in 25 mM Tris-HCl (pH 7.0), with the exception of L-tyrosine and L-tryptophan, which were used at 3 mM due to lack of solubility. Ca-DPA was prepared with 50 mM $CaCl_2$ and 50 mM DPA (adjusted to pH 8.0 with Tris-Base). Germination was also carried in Brain Heart Infusion (BHI) broth, BHI broth supplemented with 1% sodium taurocholate (ST) and 1.3% glycine, and Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM), and RPMI1640 (Lonza Walkersville).

**Colony formation assay.** Colony forming efficiency of *C. perfringens* spores was assayed by heat activating (75°C for 15 min) spores at $OD_{600}$ of 1, and aliquots of serial dilutions were plated onto BHI agar and incubated under anaerobic conditions at 37°C for 24 h, and colonies were counted. Results were expressed as colony forming unit (CFU)/ml/$OD_{600}$.

## 3.4 Results

**Germination of spores of *C. perfringens* AI with previously identified germinants (L-Asn , KCL and AK) for FP isolates.** To dissect the germination specificity of AI isolates from various sources, we first assayed germination with germinants known to trigger germination of spores of FP and NFB isolates (42, 53, 54). As expected, spores of the FP isolate SM101 germinated well in presence of L-Asn and KCl, while spores of the NFB isolate F4969 germinated well only with the mixture of L-Asn and KCl (AK) (data not shown). However, spores from most of the AI did not germinate with either L-Asn or KCl alone, with the exception of the bovine isolate 06-1084 (Fig. 3.1). When AI spores were germinated with AK mixture, spores from *C. perfringens* isolates obtained from horse (106889), poultry (JGS4122, JGS4125, JGS4126) and bovine (06-7495D and 06-10854) germinated (Fig. 3.1). Phase contrast microscopy confirmed that when spores of AI were treated with L-Asn or KCl, the majority of the spores (> 90%) of all AI remained phase bright (indicative of no germination), while ~ 70% of spores of 06-10854 changed to phase dark (indicative of complete germination) (data not shown). However, ~ 40 to 60% of the spores of those AI that germinated with AK became phase dark (data not shown).

Furthermore, we assayed the ability of spores of AI to germinate in presence of the co-germinants $Na^+$ and inorganic phosphate (Pi) (NaPi) known to trigger germination uniquely in spores of FP isolates (54). As expected, spores of our control FP isolate SM101 germinated well in the presence of NaPi at pH 6.0 but not at pH 7.0, consistent with previous results (data not shown) (54). In contrast, spores of a NFB

isolate F4969 did not germinate with NaPi at either pH tested (data not shown). Interestingly, while the majority of the spores of AI did not germinate in the presence of NaPi at either pH 6.0 or 7.0, spores of 06-10854 germinated well with NaPi at pH 6.0 but not 7.0 (Fig. 3.2). the results were confirmed by phase contrast microscopy, indicating that > 95% of spores of AI remained phase bright when treated with NaPi at either pH, while 40% of 06-10854 spores changed to phase dark after 60 min of incubation only with NaPi (6.0) (data not shown).

Another germinant that triggers germination through a *gerA*-dependent pathway is the non-nutrient germinant Ca-DPA (53). To date, results suggest that this germinant is unique to *C. perfringens* FP isolates (42, 53). We therefore assayed if Ca-DPA could trigger germination spores of AI. As expected, while spores of our control FP isolate SM101 did germinate with Ca-DPA, spores of the control NFB F4969 isolate did not germinate with Ca-DPA (data not shown). Interestingly, none of AI spores germinated well with Ca-DPA (Fig. 3.2). Phase contrast microscopy indicates that the reduction in $OD_{600}$ observed is due to ~ 5 to 20% of the spores became phase grey, indicative of stage 1 of germination (71) (data not shown). Collectively, these results suggest that spores of the majority of AI might have different germination specificity than those of FP isolates with the exception of the bovine isolate 06-10854, which although germinated with L-Asn , KCl, and NaPi, did not with exogenous Ca-DPA.

**Germination of *C. perfringens* spores with BHI broth and bile salts.** Since spores of the majority of AI did not germinate with known germinants for *C. perfringens*, we evaluated the ability of AI spores to germinate in a nutrient rich BHI broth. As expected, spores of the FP SM101 isolate germinated well in presence of BHI broth (Fig. 3.3A); however, spores of the NFB isolate F4969 germinated to a lesser extent (Fig. 3.3A). Spores of AI also germinated poorly in BHI broth to a similar extent as F4969 spores (Fig. 3.3A), suggesting that components other than those present in BHI broth might be triggering germination of AI spores. In *C. difficile*, addition of bile salt ST to BHI broth triggers significant spore germination, and glycine acts as a co-germinant with ST (77). Therefore, to test if this was also the case for spores of AI, we included ST and glycine in BHI broth. However, there was no increase in the extent of germination of spores of NFB F4969 and all surveyed AI (Fig. 3.3B). Most striking was the lesser extent of germination observed with FP SM101 spores upon addition of ST and glycine (Fig. 3.3B), suggesting an inhibitory effect on spore germination. Collectively, these results indicating that the *C. difficile* co-germinants, ST and glycine, do not trigger germination of *C. perfringens* AI spores.

To evaluate if the lack of germination in BHI broth would affect the ability of *C. perfringens* AI spores to form colonies, we evaluated the colony formation efficiency of selected AI isolates. When spores were plated into BHI agar plates, no significant difference in colony forming efficiency between spores of surveyed isolates was observed after overnight growth under anaerobic conditions (Fig. 3.4).

This suggests that although AI spores germinate slower, but eventually leads to similar titers than that of FP and NFB isolates.

**Germination of *C. perfringens* spores with tissue culture medium.** The slower germination observed with spores of AI in nutrient broth led us to hypothesis that perhaps complex mixture of amino acids and salts might be required by spores of AI to germinate. Consequently, we assayed the ability of spores of representative AI, which germinated very poorly in BHI broth, to germinate in highly nutrient-rich tissue culture media. First, we evaluated germination of AI spores in DMEM. Interestingly, incubation for 60 min with DMEM triggered significant germination of spores of JGS4122 and JGS1807, and to a lesser extent, spores of F4969 (Fig. 3.5A). Phase contrast microscopy confirmed that ~ 40 % of JGS4122 and JGS1807 became phase dark, while only ~ 10 % of F4969 spores became phase dark (data not shown). When AI spores were incubated with EMEM, only JGS1807 spores germinated to a significant extent, while spores of all other isolates germinated poorly (Fig. 3.5B). Strikingly, RPMI 1640 medium induced germination of spores of all isolates assayed to a significantly higher extent than DMEM and EMEM, with JGS1807 spores germinating to the highest extent, followed by spores of 106902 and JGS4122. Spores of SM101 and F4969 germinated to a lesser extent than all AI isolates tested (Fig. 3.5C). These results were confirmed by phase contrast microscopy, with ~ 40, 30, and 55 % of JGS1807, 106902 and JGS4122 spores became phase dark (data not shown). These results clearly indicate that the germinant specificity of *C. perfringens* AI spores

is significantly different than that of spores of FP and NFB isolates and might require mixture of various amino acids and/or salts and/or vitamins.

**Identification of new germinants for spores of *C. perfringens*.** The ability of AI spores to germinate with tissue culture media raised the hypothesis that AI spore germination is triggered by novel germinants. Therefore, in an attempt to identify novel germinants for AI spores, we assayed AI spores' germination with all amino acids (aa). When nonpolar and aliphatic aa were assayed, L-alanine triggered significant (p-value < 0.003) germination of spores of 106902, JGS4125, JGS4126 and 06-10854 (Table 3.1). However, the extent of germination of these AI spores were lower than that of SM101 spores germinated with L-alanine (Table 3.2), which was previously reported as a poor germinant for *C. perfringens* (53). Interestingly, spores of two poultry isolates (i.e., JGS4125 and JGS4126) germinated slighly with most nonpolar and aliphatic aa (Table 3.2). In contrast, no germination was observed when AI spores were germinated with aromatic aa (Table 3.3). Strikingly, when AI spores were germinated in presence of uncharged polar aa, L-threonine was able to trigger significant (p-value < 0.0002) germination in spores of most AI (Table 3.4). It is worth noting that in addition to the known germinant L-asparagine, L-glutamine was also able to trigger germination of spores of the FP SM101 isolate (Table 3.4). Assay of positively charged aa also yielded novel germinants; L-lysine was able to trigger germination of spores of the AI JGS4126 and to a lesser extent of AI JGS4125 and FP SM101 and NFB F4969 (Table 3.5). L-arginine was also able to trigger some

germination in spores of FP SM101 and AI 06-10854 (Table 3.5). Finally, the negatively charged aa L-aspartic acid and L-glutamic acid were also able to trigger germination; L-aspartic acid triggered germination of spores of FP SM101 and NFB F4969 and AI 06-10854, 106902 and JGS1807 (Table 3.6); L-glutamic acid triggered germination of the NFB F4969 and the AI 06-10854 and 106902 (Table 3.6). Collectively, these results indicate that: i) in addition to previously identified germinants, FP isolates are able to germinate well with the novel germinants L-glutamine and L-aspartic acid and to a lesser extent with L-lysine and L-arginine; ii) similarly, the NFB F4969 isolate is able to germinate with the novel germinants L-aspartic acid and L-glutamic acid, and to a lesser extent with L-lysine; iii) spores of AI are able to germinate with the novel germinants L-threonine, L-lysine, and to a lesser extent with L-alanine, L-valine, L-glycine, L-leucine, L-methionine, L-arginine.

**Characterization of novel germinants for *C. perfringens* AI spores.** To gain more insight into the specificity of the novel germinants for spores of AI described above. We examined the effects of concentrations and pH of L-threonine, L-lysine and L-glutamine on spores of strains JGS4125, JGS4126 and 06-10854, respectively. While the, minimum requirement for L-threonine were > 25 mM, > 100 mM of L-lysine were required to produce a significant increase in the maximum rate of germination. L-glutamine was effective at concentrations > 25 mM, and interestingly, while 100 mM L-glutamine induced a lower maximum rate of germination than that of 50 and 200 mM (Fig. 6A). Most surprising was the pH dependence of these novel germinants.

Interestingly, L-glutamine and L-lysine induced faster germination under acidic conditions pH < 6 (Fig. 6B). L-threonine had two pH optimums (pH ~ 6 and ~ 8) and triggered germination less efficiently at physiological pH.

## 3.5 Discussion

*C. perfringens* spores are ubiquitously found in the environment, and therefore are easily aquired by the host. Once inside the host, these spores must germinate in order to cause disease. The wide variety of diseases caused by *C. perfringens* in different hosts suggests that the germinant specificity of *C. perfringens* spores has adapted to better fit the host. Indeed, previous studies have highlighted the differential germinant requirements between FP and NFB spores (42, 53, 54). In this context, the results presented in this study contribute to our understanding of the germinant specificity of spores of *C. perfringens* AI.

A major conclusion offered by this work is that L-threonine and to a lesser extent L-lysine and L-alanine might be universal germinants for *C. perfringens* AI spores. The fact that selected AI germinated poorly in BHI broth but yielded similar titers to those of FP and NFB spores in BHI agar plates suggest that the main germinants might be present at suboptimal concentrations in BHI broth. Indeed, the optimal concentrations of the novel germinants are ~ 25 mM for L-threonine and L-glutamine, and > 100 mM for L-lysine. Most interestingly was the fact that L-lysine and L-threonine are mostly active under more acidic conditions, while L-glutamine was most active at siglty acidic (pH 6.0) and slightly basic (pH 8.0) but not under physiological condition. However, it is unclear how these germinants might be related to host-specificity and mechanisms of pathogenesis. It was most interesting that *C. perfringens* AI spores did not germinate with any of the FP-associated germinants typically found in meat products (i.e., KCl and NaPi), with the exception of 06-10654

spores. The *cpe*-carrying plasmid has been reported to be conjugable between *C. perfringens* isolates (5), allowing *C. perfringens* isolates from, for example, animal sources to become potential pathogenic FP or NFB isolates. In this particular study, the ability of 06-10654 spores to germinate with germinants typically found in meat products suggest that 06-10654 spores might have an advantage to germinate and outgrow in meat products. Consequently, it might become a FP isolate if it harbors a *cpe*-carrying plasmid. However, to cause FP, 06-10654 spores should also possess high heat resistance properties that will enable survival of these spores to conventional food processing regimes. Unfortunately, we did not measure heat resistant properties of 06-10654 spores in order to provide stronger support to this scenario. In addition to the conjugable transfer of *C. perfringens* toxins between isolates, this highlights the importance of the fitness of the spore to specific niches to efficiently cause diseases, or in this particular case to cause FP. It is also worth noting that the germination of spores of *C. perfringens* AI might proceed as fast as that of FP and NFB isolates in presence of the correct germinant mixture as observed when AI spores were germinated with tissue culture media but not in BHI broth. It is unclear to us why RPMI 1640 media could trigger better germination of *C. perfringens* spores than DMEM and EMEM. However, AI spores require the mixture of the amino acid, salts and vitamins components to trigger germination, since no significant germination was observed when each group of components (i.e., amino acids, salts and vitamins) was tested independently or in pairs (data not shown). Further studies to dissect the germinant(s) component(s) are being conducted in our lab.

A second major conclusion of this work is that the germination specificity of AI spores is independent to their source of isolation. The germinant specificity of AI spores did not follow a specific pattern with respect to their source of isolation with the exception of spores of two poultry isolates (i.e., JGS4125 and JGS4126) germinating, albeit to a small extent, with the majority of nonpolar and aliphatic amino acids. This suggests the presence of great divergence in germinant specificity of AI spores. Further studies to correlate the phylogeny of GRs and housekeeping genes of AI will be required to fully understand how germinant-specificity correlates with the different lineages in *C. perfringens*.

Another conclusion from this work, is that L-glutamine and L-aspartic acid, and to a lesser extent L-lysine and L-arginine are novel germinants of FP and NFB spores. Previous studies in germination of *C. perfringens* spores of FP and NFB isolates did not thoroughly screen all amino acids (42, 53, 54). Therefore, the results provided by this study also contribute in our understanding of the germinant requirements of FP and NFB spores, and in contrast to previous identified germiannts (i.e., KCl and NaPi) the role of these novel germinants in *C. perfringens* pathogenesis remains unclear. In addition, the GR responsible for recognizing these germinants remains unclear, and studies with knockout mutants in FP and NFB isolates are being conducted to identify the their cognate receptor.

# Figures

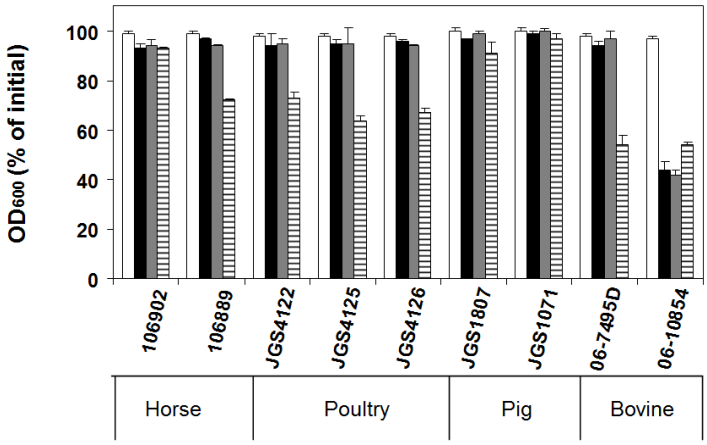


**Fig. 3.1. Germination of spores of *C. perfringens* AI with L-Asn, KCl and AK.** Heat activated spores of *C. perfringens* isolates were germinated at 37°C with: distilled water, white bars; 100 mM KCl, black bars; 100 mM L-asparagine, grey bars; and 100 mM AK, horizontal-line bars. Change in $OD_{600}$ was monitored as determined in Materials and Methods. The source of isolation is indicated.

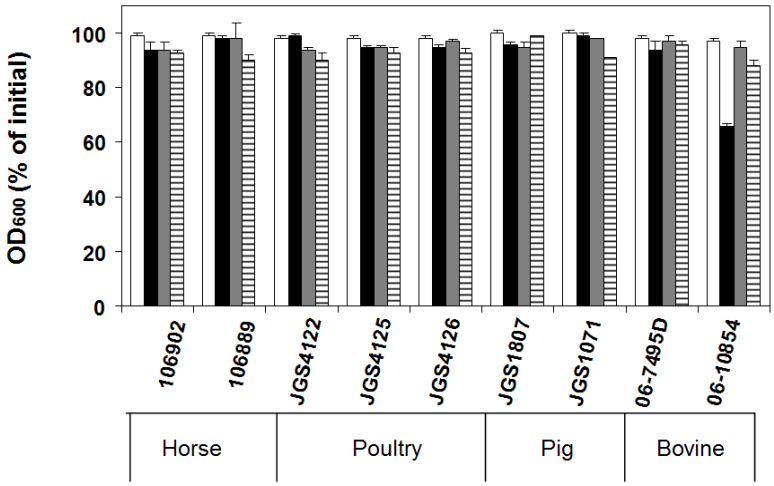


**Fig. 3.2. Germination of spores of *C. perfringens* AI with NaPi and Ca-DPA.** Heat activated spores of *C. perfringens* isolates were germinated at 37°C with: distilled water, white bars; 100 mM NaPi (pH 6.0), black bars; 100 mM NaPi (pH 7.0), grey bars; and 50 mM Ca-DPA, horizontal-line bars. Change in $OD_{600}$ was monitored as determined in Materials and Methods. The source of isolation is indicated.

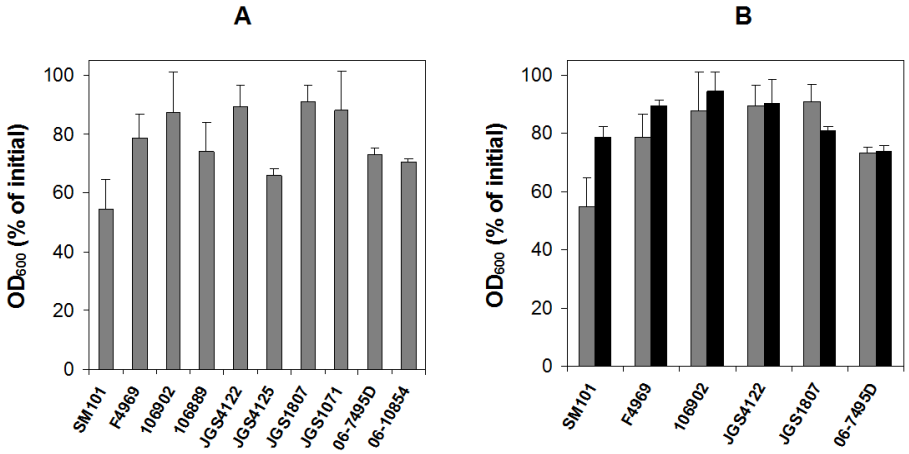


**Fig. 3.3A-B. Germination of spores of *C. perfringens* animal isolates with nutrient broth and bile salts.** Heat activated spores of *C. perfringens* isolates were incubated at 37°C with: A) BHI broth; B) BHI broth (grey bars) and BHI broth supplemented with 1% ST and 1% glycine (black bars). Change in $OD_{600}$ was monitored as determined in Materials and Methods. Data for BHI broth germination of selected C. perfringens isolates in panel B is from panel A.

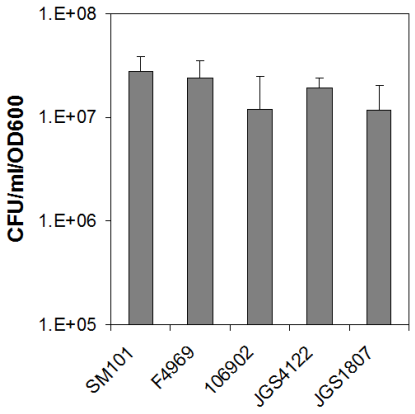


**Fig. 3.4. Colony forming efficiency of *C. perfringens* spores.** Heat activated spores of representative *C. perfringens* isolates at an $OD_{600}$ of 1.0 were plated onto BHI agar plates and incubated overnight under anaerobic conditions at 37°C. Colonies were counted and reported as determined in Materials and Methods.

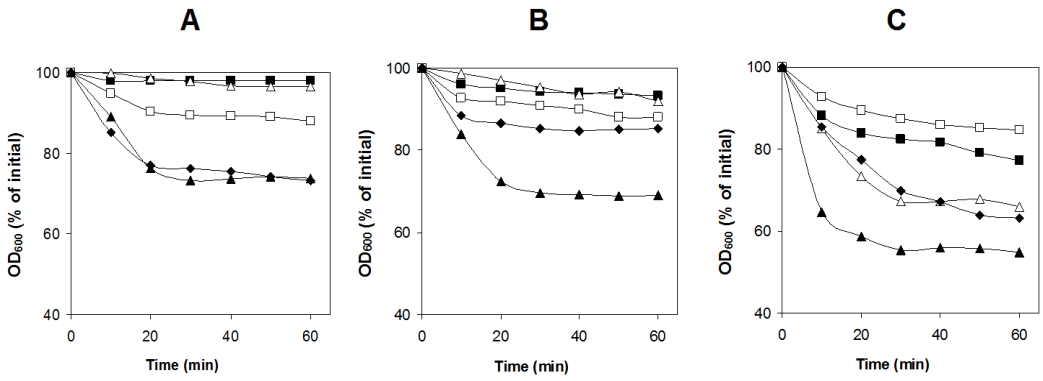


**Fig. 3.5A-C. Germination of *C. perfringens* spores in tissue culture media.** Heat activated spores of *C. perfringens* SM101 (black squares), F4969 (white squares), 106902 (white triangles), JGS4122 (black diamonds), JGS1807 (black triangles) were incubated at 37°C with: A) DMEM; B) EMEM; C) RPMI 1640. Change in $OD_{600}$ was monitored as determined in Materials and Methods.

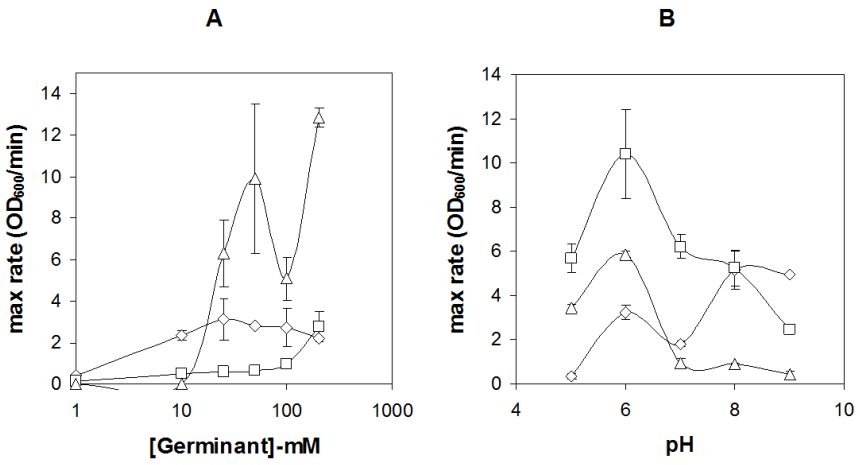


**Fig. 3.6A-B. Effect of pH (A) and germinant concentration (B) on *C. perfringens* spore germination.** Heat-activated spores of strains JGS4125 (white diamonds), JGS4126 (white squares), 06-10854 (white triangles) were incubated: A) at pH 7.0 with various concentrations of L-threonine (white diamonds), L-lysine (white squares), L-glutamine (white triangles); B) with 100 mM of L-threonine (white diamonds), L-lysine (white squares), L-glutamine (white triangles) at various pHs. The maximum rate of germination was calculated as described in materials and methods.

# Tables

## Table 3.1. *C. perfringens* isolates used in this study

| Strain | Relevant characteristics | Source/Reference |
|---|---|---|
| SM101 | Electroporatable derivative of human food poisoning type A isolate, NCTC8798, chromosomal *cpe*$^+$ | (85) |
| F4969 | Human isolate, plasmid *cpe*$^+$ | (10) |
| 106902 | Horse isolate, *cpb2*$^+$ | (83) |
| 106889 | Horse isolate, *cpb2*$^+$, *tpeL*$^+$ | (83) |
| JGS4122 | Poultry isolate, *cpb2*$^+$, *tpeL*$^+$, *netB*$^+$ | Paredes-Sabja, D. unpublished results |
| JGS4125 | Poultry isolate, *cpb2*$^+$, *tpeL*$^+$, *netB*$^+$ | Paredes-Sabja, D. unpublished results |
| JGS4126 | Poultry isolate, *cpb2*$^+$, *tpeL*$^+$, *netB*$^+$ | Paredes-Sabja, D. unpublished results |
| JGS1807 | Pig isolate, *cpb2*$^+$ | (84) |
| JGS1071 | Pig isolate, *cpb2*$^+$ | (84) |
| 06-7495-D | Bovine isolate, *cpb2*$^+$ | Paredes-Sabja, D. unpublished results |
| 06-10854 | Bovine isolate, *cpb2*$^+$, *tpeL*$^+$ | Paredes-Sabja, D. unpublished results |

**Table 3.2. Germination of spores of *C. perfringens* animal isolates with nonpolar and aliphatic amino acids.**

| Source/Strains | | Mean % decrease $OD_{600}$ ±SD[a] in 60 min at 40°C[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Ala | Val | Gly | Leu | Ile | Met |
| Human FP | SM101 | 30 ± 0.5 | 11 ± 0.4 | 23 ± 2.7 | 10 ± 2.3 | 12 ± 3.4 | 7 ± 1.6 |
| Human NFB | F4969 | 5 ± 0.3 | 4 ± 1.5 | 11 ± 2.4 | 10 ± 3.7 | 1 ± 1.7 | 0 ± 0.4 |
| Horse | 106902 | 21 ± 0.3 | 3 ± 3.9 | 0 ± 0.4 | 3 ± 2.0 | 2 ± 3.0 | 3 ± 4.2 |
| | 106889 | 12 ± 15.9 | 18 ± 1.6 | 13 ± 4.3 | 17 ±1.9 | 7 ± 1.9 | 9 ± 4.2 |
| Poultry | JGS4122 | 6 ± 2.1 | 11 ± 4.9 | 7 ± 4.6 | 12 ± 3.5 | 17 ± 0.6 | 15 ± 1.8 |
| | JGS4125 | 24 ± 1.5 | 25 ± 0.4 | 10 ± 2.1 | 28 ± 1.6 | 5 ± 4.0 | 25 ± 4.2 |
| | JGS4126 | 21 ± 6.2 | 21 ± 0.4 | 20 ± 0.4 | 13 ± 5.5 | 16 ± 4.4 | 21± 4.6 |
| Pig | JGS1807 | 4 ± 5.1 | 3 ± 2.4 | 13 ± 4.9 | 1 ± 0.6 | 4 ± 4.4 | 1 ± 1.1 |
| | JGS1071 | 0 ± 0.0 | 7 ± 1.4 | 0 ± 0.0 | 0 ± 2.3 | 1 ± 1-0 | 7 ± 1.4 |
| Bovine | 06-10854 | 21 ± 0.9 | 7 ± 0.2 | 13 ± 4.9 | 6 ± 0.4 | 5 ± 2.2 | 4 ± 2.3 |
| | 06-7495D | 13 ± 2.9 | 18 ± 0.3 | 8 ± 3.7 | 19 ± 4.7 | 9 ± 5.0 | 21 ± 2.1 |

[a]Values are average of duplicate experiments from two different spore preparations; SD, standard deviation.

[b]All compounds were used at 100 mM in 25 mM tris-HCl (pH 7.0).

**Table 3.3. Germination of spores of *C. perfringens* animal isolates with aromatic amino acids.**

| Source/Strain | | Mean % decrease $OD_{600}$ ±SD[a] in 60 min at 40°C[b] | | |
|---|---|---|---|---|
| | | Tyr | Trp | Phe |
| Human FP | SM101 | 9 ± 0.1 | 18 ± 0.6 | 4 ± 2.4 |
| Human NFB | F4969 | 6 ± 1.5 | 15 ± 2.1 | 9 ± 1.4 |
| Horse | 106902 | 1 ± 0.5 | 5 ± 1.5 | 3 ± 1.3 |
| | 106889 | 3 ± 0.6 | 10 ± 4.9 | 5 ± 4.5 |
| Poultry | JGS4122 | 1 ± 1.6 | 4 ± 1.9 | 0 ± 0.6 |
| | JGS4125 | 13 ± 1.5 | 19 ± 1.3 | 5 ± 3.8 |
| | JGS4126 | 13 ± 0.3 | 12 ± 2.6 | 5 ± 6.6 |
| Pig | JGS1807 | 2 ± 2.8 | 3 ± 0.0 | 0 ± 0 |
| | JGS1071 | 10 ± 3.6 | 5 ± 4.9 | 7 ± 3.8 |
| Bovine | 06-10854 | 6 ± 0.6 | 9 ± 0.9 | 5 ± 1.3 |
| | 06-7495D | 0 ± 0.0 | 8 ± 0.8 | 3 ± 3.3 |

[a]Values are average of duplicate experiments from two different spore preparations; SD, standard deviation.

[b]Phe was used at 100 mM in 25 mM Tris-HCl (pH 7.0); Tyr and Trp were used at 3 mM in 25 mM Tris-HCl (pH 7.0).

**Table 3.4. Germination of spores of *C. perfringens* animal isolates with polar and uncharged amino acids**

| Source/Strain | | Mean % decrease $OD_{600}$ ±SD[a] in 60 min at 40°C[b] | | | | |
|---|---|---|---|---|---|---|
| | | Ser | Thr | Pro | Asn | Gln |
| Human FP | SM101 | 9 ± 5.4 | 15 ± 3.2 | 4 ± 1.5 | 45 ± 1 | 43 ± 2.3 |
| Human NFB | F4969 | 7 ± 4.0 | 13 ± 3.4 | 2 ± 2.1 | 5 ± 2.2 | 27 ± 9.7 |
| Horse | 106902 | 2 ± 0.8 | 16 ± 0.9 | 1 ± 0.1 | 6 ± 2.4 | 3 ± 4.0 |
| | 106889 | 13 ± 4.6 | 43 ± 1.5 | 5 ± 6.6 | 6 ± 1.2 | 10 ± 14.7 |
| Poultry | JGS4122 | 11 ± 7.2 | 25 ± 5.8 | 1 ± 1.5 | 5 ± 1.8 | 2 ± 3.3 |
| | JGS4125 | 9 ± 12.3 | 44 ± 3.8 | 5 ± 7.1 | 5 ± 6.1 | 8 ± 10.9 |
| | JGS4126 | 11 ± 14.3 | 39 ± 7.5 | 10 ± 7.4 | 6 ± 1.7 | 6 ± 2.1 |
| Pig | JGS1807 | 0 ± 0.0 | 23 ± 3.6 | 0 ± 0.6 | 1 ± 1.0 | 0 ± 0.6 |
| | JGS1071 | 6 ± 6.7 | 14 ± 2.2 | 0 ± 0.6 | 12 ± 17.7 | 1 ± 0.6 |
| Bovine | 06-10854 | 9 ± 4.7 | 43 ± 3.4 | 3 ± 3.6 | 57 ± 1.8 | 14 ± 0.7 |
| | 06-7495D | 1 ± 0.1 | 23 ± 1.4 | 1 ± 1.8 | 3 ± 1.7 | 11 ± 1.1 |

[a]Values are average of duplicate experiments from two different spore preparations; SD, standard deviation.

[b]All compounds were used at 100 mM in 25 mM Tris-HCl (pH 7.0).

**Table 3.5. Germination of spores of *C. perfringens* isolates with positively charged amino acids.**

| Source/Strain | | Mean % decrease $OD_{600}$ ±SD[a] in 60 min at 40°C[b] | | |
|---|---|---|---|---|
| | | Lys | Arg | His |
| Human FP | SM101 | 25 ± 0.3 | 22 ± 0.9 | 13 ± 0.1 |
| Human NFB | F4969 | 20 ± 0.2 | 48 ± 2.1 | 7 ± 0.0 |
| Horse | 106902 | 4 ± 4.1 | 14 ± 3.6 | 5 ± 1.0 |
| | 106889 | 10 ± 7.1 | 0 ± 0.0 | 5 ± 2.8 |
| Poultry | JGS4122 | 9 ± 4.3 | 1 ± 0.6 | 6 ± 0.9 |
| | JGS4125 | 23 ± 8.9 | 7 ± 3.1 | 17 ± 9.8 |
| | JGS4126 | 46 ± 0.6 | 6 ± 1.2 | 27 ± 0.5 |
| Pig | JGS1807 | 0 ± 0.7 | 13 ± 7.9 | 3 ± 1.4 |
| | JGS1071 | 6 ± 6.2 | 10 ± 2.7 | 1 ± 0.5 |
| Bovine | 06-10854 | 9 ± 4.2 | 23 ± 3.9 | 2 ± 0.1 |
| | 06-7495D | 10 ± 8.1 | 6 ± 2.5 | 14 ±5.5 |

[a]Values are average of duplicate experiments from two different spore preparations; SD, standard deviation.

[b]All compounds were used at 100 mM in 25 mM Tris-HCl (pH 7.0).

**Table 3.6. Germination of spores of *C. perfringens* isolates with negatively charged amino acids.**

| Source/Strain | | Mean % decrease $OD_{600}$ ±SD[a] in 600 min at 40°C[b] | |
|---|---|---|---|
| | | Asp | Glu |
| Human FP | SM101 | 39 ± 0.6 | 9 ± 9.2 |
| Human NFB | F4969 | 50 ± 10.4 | 36 ± 4.4 |
| Horse | 106902 | 25 ± 2.7 | 20 ± 0.3 |
| | 106889 | 4 ± 5.1 | 3 ± 5.0 |
| Poultry | JGS4122 | 9 ± 7.8 | 0 ± 0.6 |
| | JGS4125 | 2 ± 1.1 | 3 ± 4.1 |
| | JGS4126 | 1 ± 1.1 | 13 ± 6.5 |
| Pig | JGS1807 | 26 ± 18.4 | 0 ± 0.0 |
| | JGS1071 | 4 ± 5.2 | 0 ± 0.0 |
| Bovine | 06-10854 | 31 ± 12.5 | 35 ± 4.6 |
| | 06-7495D | 13 ± 5.5 | 1 ± 1.7 |

[a]Values are average of duplicate experiments from two different spore preparations; SD, standard deviation.

[b]All compounds were used at 100 mM in 25 mM Tris-HCl (pH 7.0).

# Chapter 4

# Conclusion

*Clostridium perfringens* is a Gram-positive, anaerobic, spore-forming pathogenic bacterium ubiquitously found in the environment and as part of the intestinal flora of humans and animals. The ability to produce at least 17 toxins allows *C. perfringens* to cause different diseases, with the most common being *C. perfringens* type A food poisoning (FP), antibiotic-associated diarrhea, gas gangrene, necrotic enteritis in poultry, enterotoxemia in food animals. Spore germination of pathogenic bacteria is an essential and early step in the biology of their pathogenesis. Consequently, an understanding of the molecular mechanism of *C. perfringens* spore germination is essential for understanding the pathogenesis of the variety of illnesses caused by *C. perfringens* isolates. In this study, we analyzed the specificity of germinants to trigger spores germination of various isolates.

The first study was to further compare germination of spores of FP versus NFB isolates. Result from this study found novel differences in non-nutrient germination. While spores of both FP and NFB isolates germinated well with the cationic surfactant, dodecylamine, only spores of FP isolates did germinate with exogenous Ca-DPA. Our analyses of the *csp-sleC* locus involved in PG cortex hydrolysis during spore germination of FP and NFB isolates also showed significant differences.

The second study was to investigate the germinant requirements of spores of *C. perfringens* animal isolates. Result showed great variability in the germination specificity of spores of AI, and that some novel germinants for spores of AI including

L-threonine, L-lysine, and L-alanine. In addition, FP and NFB spores were found to germinate with the novel germinants L-glutamine and L-aspartic, and to a lesser extent L-lysine and L-arginine.

In sum, the results showed a great divergence in germinant specificity of AI spores. However, the germinant specificity observed among spores of *C. perfringens* isolates can be linked to the ability of *C. perfringens* to adapt different host and cause wide variety of diseases.

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