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Review Article

Clostridium perfringens: Sporulation, Spore Resistance and Germination

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Clostridium perfringens **are Gram-positive, endospore-forming, anaerobic bacteria with the ability to cause enteric diseases both in human and domesticated animals. As one of the leading cause of food-borne illness in the United States, certain** ***C. perfringens*** **type A isolates exert their action through the production of** ***C. perfringens*** **enterotoxin (CPE), which is expressed only during spore formation. In addition,** ***C. perfringens*** **spores are highly resistant to heat and other environmental factors. Since genome sequences of three** ***C. perfringens*** **strains have been annotated and made public, efforts have been made towards understanding the initiation of sporulation and identifying the key differences between** ***Clostridium*** **and** ***Bacillus*** **sporulation phosphorelay. Small, acid soluble spore proteins (SASPs) have been shown to be required for resistance of** ***C. perfringens*** **spores to heat. Work is also underway to identify nutrient signals required for** ***C. perfringens*** **spore germination.**

Keywords: ***Clostridium perfringens*****, Endospore, Small, acid soluble spore protein (SASP), Heat resistance, Germination**

Introduction

Clostridium perfringens is a Gram-positive, spore-forming anaerobic bacterium. It is ubiquitous in nature, including soil, insects, and the intestinal tract of animals and humans[1]. Spores of *C. perfringens* are highly resistant to stress factors such as heat, cold, UV radiation, desiccation, and many other environmental factors[1]. *C. perfringens* are capable of producing at least 15 different toxins although each individual isolates do not produce all 15 of these toxins. These isolates are categorized into five types, A through E, based upon their ability to produce four major toxins (alpha, beta, epsilon, and iota)[2-3]. Alpha toxin, a phospholipase C, is the causative agent of gas gangrene[1]. The beta toxin induces hemorrhagic necrosis of the intestinal mucosa. Epsilon toxin is a pore forming toxin and iota toxin belongs to the family of binary toxins[4].

C. perfringens associated food poisoning (FP) and non-food-borne gastrointestinal diseases (NFBGID) in humans are typically caused by type A isolates that are capable of producing a 35-kDa protein called *C. perfringens* enterotoxin (CPE)[1]. CPE encoded gene (*cpe*) can be located either on the chromosome or on a plasmid[1]. *C. perfringens* isolates carrying *cpe* on the chromosome are associated with FP, while isolates carrying *cpe* on the plasmid are associated with NFBGID[5]. CPE is capable of binding to and interact with tight junction proteins, resulting in diarrhea and cramping[1].

C. perfringens type A food poisoning is the third most common food-borne disease in the USA[6]. Typically, human acquire the bacteria as a result of ingesting improperly prepared food (meat products) that are contaminated with vegetative cells of *C. perfringens*. Small number of bacteria can survive the passage to the small intestine where in the presence of certain signals or in competition with intestinal flora they will undergo sporulation[1]. Once the spores are matured, the mother cell lyses and CPE is released into the intestinal lumen to exert its action. Previous studies provided the genetic evidence that CPE is the essential virulence factor for *C. perfringens* type A FP and NFBGID[7]. Several studies have demonstrated that CPE production is highly regulated by sporulation[7-9]. In addition to producing CPE, *C. perfringens* FP isolates have the ability to form heat-resistance spores, which facilitates their survival in primary food vehicles (meat and poultry products) for *C. perfringens* type A FP[1]. In the presence of appropriate nutrients, dormant spores germinate and multiply, leading to food-borne illness after consumption of these contaminated foods[1].

The study of the properties of spores then, is crucial in understanding the pathogenesis and prevention of *C. perfringens* type A associated human diseases. This review will summarize recent advances in our understanding of the: (1) mechanism of sporulation in *C. perfringens*, (2) spore resistance to heat and other environmental factors, and (3) germination of spores.

1. Sporulation: Overview

In order for the bacteria to sense changes in their environment, many sensory regulatory systems have evolved that allow

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bacteria to alter their gene expression in order to survive. Many bacteria, including *C. perfringens*, have the ability to sense certain environmental stress and undergo a series of morphological changes to produce endospores that are highly stress resistant[10]. The mechanism of sporulation has been most extensively studied in the other Gram-positive bacilli, *Bacillus subtilis*.

In *B. subtilis*, the initiation of sporulation is turned on through a phosphorelay system consisted of sensory histidine kinases, the phosphotransferase Spo0F, the single-domain response regulator Spo0B and the sporulation transcription factor Spo0A[11-13]. Five sporulation specific histidine kinases KinA-E have been identified in *B. subtilis* that are associated with the initiation of sporulation. The structure of histidine kinases is comprised of a sensory domain in the N-terminal region, and activity domain in the C-terminal region. In response to environmental and cellular signals, sporulation specific histidine kinases phosphorylate Spo0F and Spo0B. When Spo0A is activated through the acceptance of a phosphoryl group from Spo0B, it regulates genes under its control through binding to a consensus sequence termed 'OA' box in the promoter region[10,14-16]. Morphologically, when a *B. subtilis* cell undergoes sporulation, it is asymmetrically divided by a septum into a larger compartment termed mother cell, and a smaller compartment termed prespore. Four sporulation and compartmental specific sigma factors (σ^E, σ^F, σ^G, and σ^K) regulate the developmental process of spore maturation[13]. The entire sporulation cascade in *B. subtilis* has been divided into stages 0 through VII, in which the last stage is associated with the release of the mature spore into the environment when the mother cell undergoes programmed cell death (Figure 1). Free spores are highly resistant to various environmental stress factors such as heat, cold, UV, and other factors[17]. Spores are metabolically dormant, however, when appropriate nutrients such as alanine or a mixture of L-asparagine, glucose, fructose and K^+ (AGFK) are present in the environment, receptors on the surface of the spore can trigger germination, and vegetative cells are formed[18].

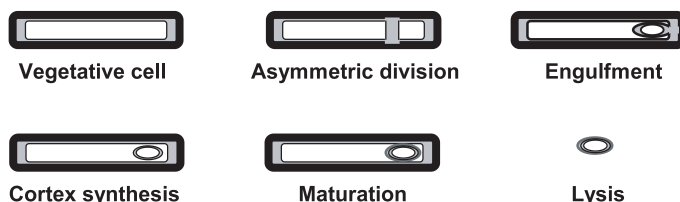

Figure 1. *Cartoon illustrating the process of sporulation. Starting with asymmetric division of the vegetative cell, leading to prespore formation, engulfment, maturation, and eventual release of the spore in which the mother cell autolysis.*

1.1. *Clostridium perfringens* Sporulation

Genome sequences of three *C. perfringens* strains and other *Clostridium* genomes have been completed[19-22]. Homologs of Spo0A are present in all sequenced *Clostridium* genomes. Interestingly, after *C. perfringens* strain 13 genome was first completed, it was discovered that a premature termination codon is present in the *spo0A* homologue[9]. *spo0A* sequencing from four additional *C. perfringens* isolates (two FP and two NFGBID isolates) identified no premature termination codon within the open reading frame (ORF)[9]. When *spo0A* knock-out mutant of *C. perfringens* SM101 was constructed and sporulation efficiency was tested, the *spo0A* mutant IH101 was unable to form endospores. The lack of spore formation in IH101 was restored when the mutant was complemented with a recombinant plasmid carrying wild-type *spo0A*, indicating that the expression of *spo0A* is essential for formation of spores in *C. perfringens*. In addition, CPE production was absent in the *spo0A* mutant, further confirming the previous observation that CPE is produced only in sporulating cultures of *C. perfringens*[9]. A similar study performed in *C. acetobutylicum* also demonstrated that *spo0A* is required for spore formation and solvent formation. Comparison of the amino acid sequence of *spo0A* homologue from *C. perfringens*, *C. acetobutylicum, C. botulinum, C. difficile*, and *C. tetani* revealed a significant homology with each other. To evaluate whether *C. perfringens* can be used as a model organism for studying sporulation process in other clostridia, *C. perfringens spo0A* mutant IH101 was complemented with wild-type *spo0A* from four different *Clostridium* species[8]. *spo0A* from *C. acetobutylicum* and *C. tetani* were able to restore spore formation (Figure 2) and CPE production[9] in the *C. perfringens spo0A* mutant IH101. Interestingly, *spo0A* from *C. botulinum*[9] and *C. diffiicle* (Figure 2) failed to complement the phenotypes of IH101. Chimeric fusion constructs containing *C. perfringens spo0A* receiver domain and upstream region fused with the response domain of *C. botulinum* and *C. difficile spo0A* also were unable to restore spore formation[9]. It is tempting to suggest that additional proteins are involved in the activation of Spo0A from *C. botulinum* and *C. difficile*. Similar studies have been performed using *C. botulinum spo0A* and *B. subtilis spo0A* mutant[23]. Wild-type *C. botulinum spo0A* was unable to complement *B. subtilis spo0A* but a chimeric construct similar to the one described above restored spore formation albeit at lower level when compared to wild-type. Results from these studies suggested that although *spo0A* is required for spore formation in all *Clostridium* species, the difference in environmental niches each species resides in might have resulted in different signals required for sporulation initiation.

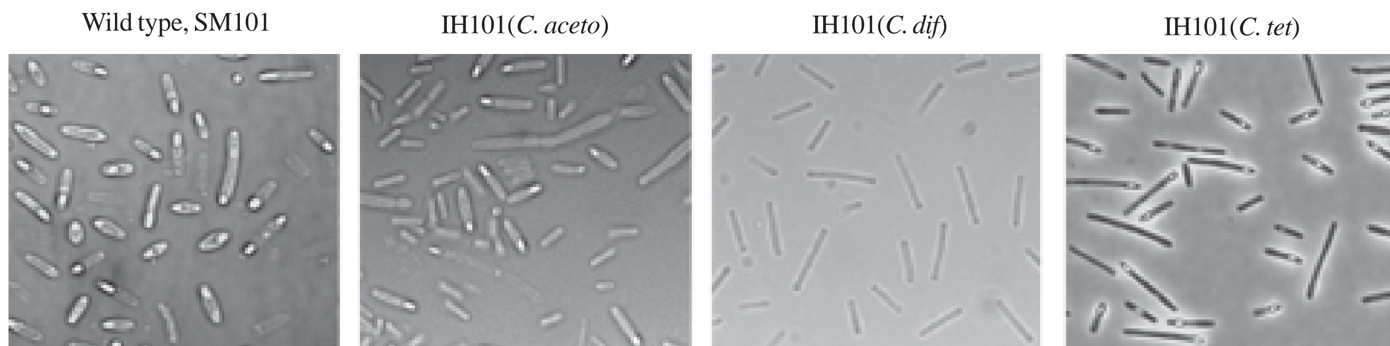

Figure 2. *Phase-contrast microscopic analysis of sporulating Clostridium perfringens wild-type and complemented strains. C. perfringens wild-type SM101, complemented strains IH101(C. aceto), IH101(C. dif), and IH101(C. tet) were grown in sporulating medium at 37ºC for 8-24 h and refractile spores were visualized using a phase-contrast microscope (Zeiss, Germany) with 1000x magnification. Representative fields were photographed at 1000x magnification. C. aceto, C. dif, and C. tet represent complementing plasmid containing wild-type spo0A from C. acetobutylicum, C. difficile, and C. tetani, respectively. Reproduced from Huang et al.*[8].

1.2. *Clostridium perfringens* Phosphorelay System

Activation of Spo0A requires the protein to be phosphorylated. In *B. subtilis*, Spo0A is phosphorylated through a phosphorelay[12]. Since no obvious homologues of Spo0F and Spo0B have been identified in all completed *Clostridium* genomes, it has been suggested that the initiation of clostridial sporulation utilize a different pathway than that of *B. subtilis*[14]. Several hypotheses for this phenomenon can be envisioned. First, perhaps phosphorylation of Spo0A is induced through a different phosphorelay system. Second, it is possible that in *Clostridium* species Spo0A is directly phophorylated by a sensor histidine kinase[10,14,16].

If a different phopshorelay system exists in *C. perfringens*, then the members of this system must share some conserved residues with Spo0F and Spo0B of *B. subtilis* because such system will have to interact with the receiver domain of *C. perfringens* Spo0A. However, searches through the genome sequence of *C. perfringens* have not identified any orthologs of Spo0F and Spo0B. Given the evidence that the genus *Clostridium* arise as a separate group more than 2.7 billion years ago, before the arise of aerobic bacilli such as the genus *Bacillus*[24], it is conceivable that the phosphorelay system in *B. subtilis* developed from a simpler system in *Clostridium*. In *B. subtilis*, all five sensor histidine kinases are orphan kinases, meaning that they do not have an adjacent response regulator[11]. All five sensor kinases have an N-terminal signaling region and a C-terminal activity domain. In addition, three of the sporulation kinases contain transmembrane domains suggesting that they might be capable of receiving environmental signals in order to activate the sporulation cascade[25]. Bioinformatics and microarray studies identified six orphan histidine kinases in *C. acetobutylicum*[24,26]. Transcriptome profile of *C. acetobutylicum* revealed four kinases as candidate that might phosphorylate Spo0A. In *C. botulinum*, a candidate histidine kinase was transformed into a *B. subtilis spo0A* mutant along with wild-type *C. botulinum spo0A*[23]. The construct was lethal for *B. subtilis* but results suggest *C. botulinum spo0A* was phosphorylated by the candidate kinase. Bioinformatics analyses of *C. perfringens* genomes identified seven orphan kinases and five of which have putative transmembrane domain (I-hsiu Huang, personal communications). Works are currently underway in our laboratory to introduce knock-out mutations in each putative *C. perfringens* kinase gene to evaluate its role in sporulation.

1.3. Signal(s) Required for *C. perfringens* Sporulation

Environmental signals that trigger sporulation in *Bacillus* and *Clostridium* are not well understood. In *C. acetobutylicum*, efforts have been made in identifying the signals for solvent formation, which is linked with sporulation. Signals that have been identified include pH, butyrate, ATP, and NADPH[24,27]. In *C. perfringens*, it was found that inorganic phosphate (Pi) is required for initiation of sporulation[28]. The discovery was made by noting that in the commonly using Duncan Strong Medium (DS)[29]**,** the level of Pi was ~35 m*M*, which is much higher than the level (m*M*) required for optimum growth. Various concentration of Pi ranging from 0 to 100 m*M* was tested to evaluate its effect on sporulation efficiency of *C. perfringens* strain SM101[30]. At low concentration (<3 m*M*), little or no sporulation was observed. Maximum sporulation efficiency was observed in culture that was grown in the presence of 30 to 40 m*M* Pi. Interestingly, there appear to be a deleterious effect on both cell growth and sporulation efficiency if the level of Pi continues to increase. As a control, sporulation efficiency was also tested in modified DS medium with Tris or MOPS addition as a buffering agent. Similar cellular load and final pH was observed when compared to DS medium with Pi addition, however, no spore formation was detected in these two mediums. Universality of Pi as a signal for *C. perfringens* spore formation was also tested by the addition of 35 m*M* Pi in two other growth medium commonly used for vegetative growth, trypticase glucose yeast extract (TGY) and fluid thioglycollate (FTG)[5,7]. Previous studies have shown that glucose can inhibit sporulation[31]. To prevent the inhibitory effect of glucose, it was omitted from the modified TY and FTG medium. Spore formation was only observed in TY and FTG medium supplemented with 35 m*M* Pi albeit at less efficiency than observed in DS medium. It was shown that Pi was required for efficient CPE production as well[30]. Cell morphology comparison and *spo0*A northern blot analysis suggested that Pi is required at the onset of sporulation before asymmetric division[30]. It has been suggested that since an average of 15 to 30 m*M* concentration of Pi is normally present in a human intestinal lumen, *C. perfringens* might have evolved to sense m*M* concentration of

Pi as a signal for sporulation[28]. Further work involving the characterization of Pi receptors on the surface of *C. perfringens* should help in understanding this phenomenon.

Although genetic tools for clostridial research requires further development, attempts in understanding the mechanism of sporulation in *C. perfringens* and other *clostridium* species have bought about numerous discoveries. With the completion of the annotated *C. perfringens* strain SM101 genome, comparative genomics should aid in the elucidation of the mechanism of *C. perfringens* sporulation. For example, microarray works are currently underway in our laboratory to identify genes involved in Pi induced sporulation.

2. Spore Resistance

In addition to producing CPE, there are several other factors that contribute to *C. perfringens* type A FP. The ability to form heat-resistant spores is one of the most important factors[2]. One of the major causes for FP is the inadequate cooking or warming of food which allows spores to survive in the food. Hence, spore heat resistance is an important virulence factor for *C. perfringens* type A FP. It has also been shown in a recent study that most cases of FP are caused by strains carrying the *cpe* gene on the chromosome while NFBGID are caused by isolates carrying *cpe* on a large plasmid. The association of FP to chromosomal *cpe* isolates can be attributed to the fact that spores of these isolates are highly resistant to heat which enhances their survival in improperly prepared foods[5]. Spores of some FP isolates can survive boiling for over an hour[5]. However, the molecular basis for this high heat resistance of FP isolates and the basis for the differences in heat resistance between spores of FP versus the NFBGID isolates have not been studied in detail.

The ability of spores to survive in extreme environments is common to all sporulating Gram-positive bacteria and has been studied extensively in *B. subtilis*. However, it is important to understand these mechanisms in pathogenic Gram-positive bacilli such as *C. perfringens* to better design and develop a protective measure against *C. perfringens* type A FP.

2.1. Role of Small, Acid Soluble Spore Proteins (SASPs) in Resistance of *Clostridium perfringens* Spores to Heat

Spores of *Bacillus* and *Clostridium* species contain a number of small, acid soluble spore proteins (SASPs), which comprise 10-20% of the total spore protein. Extensive studies have been conducted on these proteins in *B. subtilis* to evaluate their role in spore resistance[17,32]. SASPs in *B. subtilis* have been classified into two types, the α/β type and the γ type. The *B. subtilis* α/β type SASP are encoded by multiple genes and comprise a large protein family whose amino acid sequences are very highly conserved between and within species. In *B. subtilis*, α/β type SASP have been shown to bind to DNA and change the conformation of spore DNA from B to A[33-34]. *In vitro* experiments using DNase I treatments have shown the ability of α/β type SASP to bind DNA and protect DNA from damage[35-36]. Strains lacking α/β SASP have been shown to have higher susceptibility to heat[30,37], UV radiation[38], desiccation and chemicals such as, hydrogen peroxide[39].

SASPs have also been identified in a variety of other sporulating bacteria including *Clostridium* spp.[40-42]. Previous studies[40-42] identified 3 α/β type SASP in *C. perfringens* and two α/β type SASP in *C. bifermentans*. The genes (*ssp*) encoding α/β SASP have been cloned and sequenced from laboratory strains of these species[41]. Recent genome sequencing of pathogenic strains of *C. perfringens*, strain 13 (a gas gangrene strain) and SM10 (A FP strain)[20,22], have identified the three genes coding for the α/β type SASP which have been named *ssp1, 2* and *3*.

Recent studies[43] have shown that all three *ssp* genes are present in most, if not all, FP isolates and are highly conserved. The expression of *ssp* genes was examined by fusing the putative promoter regions of *ssp1*, *2*, or *3* from a *C. perfringens* FP strain SM101 to *Escherichia coli gusA*. These *ssp-gusA* fusion constructs were introduced into wild-type SM101 and β-glucuronidase (GUS) activity was measured during vegetative and sporulation growth (Figure 3). Results from the GUS assays showed that the three *ssp* genes were expressed during sporulation and not during vegetative growth (Figure 3) and that expression of these genes is dependent on the master regulator of sporulation, Spo0A[43].

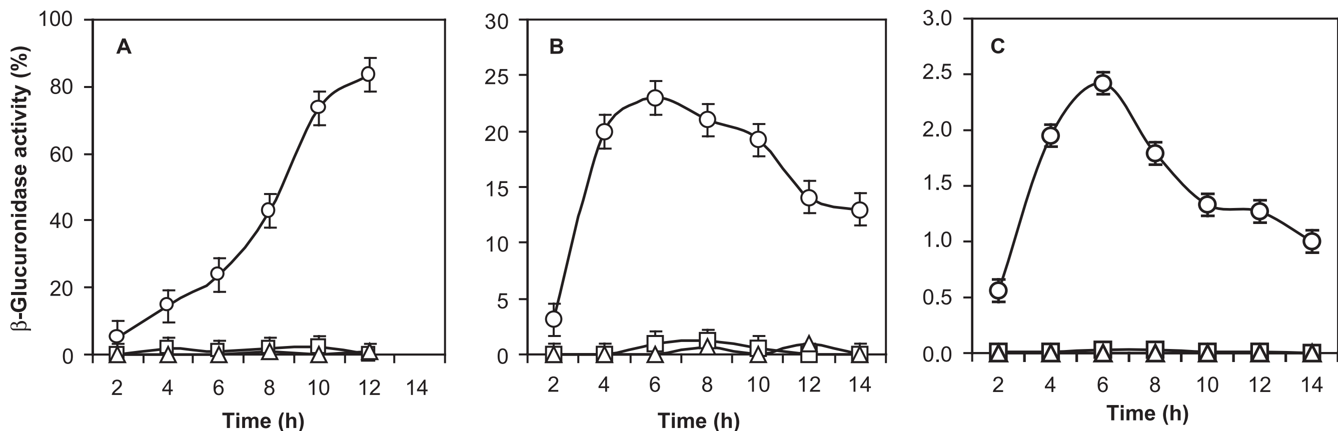

Figure 3. *Expression of ssp1-gusA (A), ssp2-gusA (B) and ssp3-gusA (C) fusions in C. perfringens wild-type SM101 grown in vegetative (□) and sporulation (○) medium, and in spo0A mutant IH101 grown in sporulation medium (△). β-Glucuronidase activity was calculated as previously described and is expressed in Miller units[43]. Reproduced from Raju et al.* [43].

To evaluate the role of α/β type SASP in spore heat-resistance, knock-out mutation was introduced into *ssp3* using the double-antibiotic selection strategy[43]. Evaluation of the heat sensitivities of spores produced by *C. perfringens* wild-type and *ssp3* mutant showed that spores of *ssp3* mutant exhibited slightly lower heat resistance than the spores of wild type (Figure 4). This phenotype could be restored by complementing the *ssp3* mutant with a wild type *ssp3*, suggesting that *C. perfringens* α/β type SASP play a similar role in spore heat resistance.

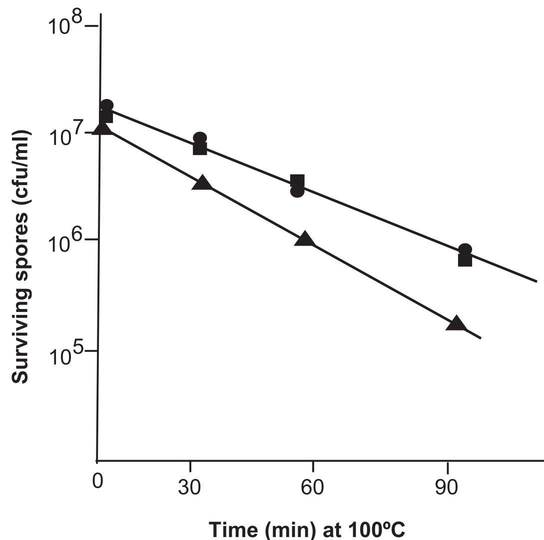

Figure 4. *Thermal death curves for sporulating cultures of wild-type SM101 (●), ssp3 mutant DR101 (▲), and complemented DR101(pDR18) (■) strains. Heat-shocked DS medium cultures of SM101, DR101 and DR101(pDR18) were heated at 100°C for specific times, and the number of viable spores per milliliter of each culture was determined*[8,43]. *The data are the results of representative experiments; these results were highly reproducible. Reproduced from Raju et al.*[43].

However, complete deletion of α/β type SASP is required to conclusively prove that α/β type SASP play a major role in *C. perfringens* spore resistance. In our recent study, we successfully down regulated the production (~90% reduction) of α/β type SASP in *C. perfringens* SM101 using *ssp2*-asRNA (Deepa Raju, personal communication). The heat resistance of spores expressing *ssp2*-asRNA was then compared with that of wild-type spores. The major conclusion from our recent work is that our previous hypothesis[43] that *C. perfringens* α/β-type SASP play a significant role in the resistance of *C. perfringens* spores to moist heat is correct, as spore resistance to moist heat decreased ~6-fold when levels of α/β-type SASP in spores decreased ~10-fold ($D_{100°C}$ value going from 80 to 13 min). This is similar to the magnitude of the decrease in the moist heat resistance of *B. subtilis* spores in which up to ~85% reduction in the level of α/β-type SASP in these spores[38].

2.2. Expression of *ssp* in Non-food-borne Gastrointestinal Disease (NFBGID) Isolates

As mentioned earlier, FP isolates of *C. perfringens* carry the *cpe* gene on the chromosome while the NFBGID isolates carry the gene on a large plasmid. NFBGID isolates are less resistant to moist heat when compared to FP isolates[5]. Two hypotheses can be envisioned for this lower heat resistance of NFBGID isolates. First, the *cpe* plasmid present in these isolates might encode factor(s) that contribute to lower heat resistance. Second, NFBGID isolates produced less or no α/β type SASP, which play a major role in heat resistance of spore of FP isolates[43].

To test the first hypothesis, the *cpe* plasmid was cured from a NFBGID isolate and heat sensitivity of plasmid-cured strain was compared with that of its parent strain[44]. No differences in heat resistance were observed between the vegetative cells and spores of *cpe* plasmid-containing versus plasmid-cured strain (Table 1), suggesting that *cpe* plasmid does not encode factors that confer heat sensitivity to NFBGID isolates[44].

Table 1. *Heat resistance of vegetative cells and spores*[a]

Strain	Genotype[b]	D values (min) for		Source
		Veg[c] cells at 55°C	Spores at 100°C	
SM101	WT	11 ± 0.7	62 ± 5.0	43
MRS101	*cpe*⁻	12 ± 2.0	61 ± 2.1	43
F4969	WT	8.0 ± 0.5	0.5 ± 0.1	44
MRS4969	*cpe*⁻	7.0 ± 1.0	0.6 ± .05	43
MRS4970	*cpe* plas⁻	7.0 ± 0.5	0.6 ± 0.1	40

[a]Results shown are based on at least 3 determinants for each experimental parameter for each strain.
[b]WT = Wild type; *cpe*⁻ = *cpe* knockout derivative of SM101 or F4969; *cpe* plas⁻ = *cpe* plasmid-cured derivative of MRS4969
[c]vegetative cells of each strain
Adopted from Raju *et al.*[44]

The second hypothesis was tested by evaluating the presence and expression of *ssp* genes in NFBGID isolates and then compared the level of α/β type SASP production in a NFBGID versus FP isolate. These results demonstrated that NFBGID isolates (i) carry functional *ssp* ORFs identical to that in FP isolates, and (ii) produce α/β type SASP at a level similar to that of FP isolates. Collectively, these results suggest that the differences in heat-resistance between spores of FP versus NFBGID isolates is not the result of impaired expression of *ssp* genes and/or decreased production of SASPs in NFBGID isolates (Deepa Raju, personal communication).

2.3. Future Studies on Mechanism of Spore Resistance

Studies on spore resistance have shown that α/β type SASPs play a major role in spore resistance to moist heat and UV radiation. Further characterization of the role of α/β type SASP in resistance to other stress factors such as chemical treatments and study their mechanism of action will provide a better understanding of the importance of α/β type SASP. However, it is also evident that there are factors other than α/β type SASP that are involved in spore resistance. It has been shown in *B. subtilis* that the spore water content affects the moist heat resistance of spores[30]. The

higher the spore water content, the lower the resistance to moist heat for the spores. Therefore, identifying the factors determining the level of spore water content in *C. perfringens* and their role in spore heat resistance should help in understanding the mechanism of resistance of *C. perfringens* spores to heat and other stress factors.

3. Spore Germination

Spore germination is a key step in the survival cycle of spore-former bacteria. Dormant bacterial endospores are highly resistant to environmental stress, and can recover metabolic characteristics of vegetative cells when they sense the appropriate nutrient germinant. In recent years, there has been an increased interest to understand the molecular basis of spore germination mechanism of pathogenic spore-formers (*i.e*., *B. cereus*, *C. botulinum* and *C. perfringens*). Elucidation of the mechanism of germination should help in designing and developing inhibitors that can be used to control spore germination in food and animal feed industry.

3.1. Spore Germination in *C. perfringens*

For germination to occur, spores must sense specific compounds, termed germinant, present in the environment that indicates optimal growing conditions. Spores of *B. subtilis* germinate in the presence of L-alanine, L-valine, and the mixture of AGFK[18]. Germinant that trigger spore germination were identified for *C. perfringens* type A[45]. Potassium ion initiates spore germination only in heat resistant strains that produce CPE, whereas a mixture of L-alanine, inosine, and $CaCl_2$ in the presence of CO_2 initiated spore germination only in heat sensitive strains that did not produce detectable amounts of CPE[45].

In *B. subtilis*, the nutrient receptors, termed GerA homologs, are responsible for the transduction of the germination signal upon binding of the germinant[18]. They are encoded by three tricistrionic operons where each ORF encodes for one of the three A, B, and C proteins forming a multiple subunit nutrient receptor[46-47]. These nutrient receptors are located in the inner membrane of the spore[18,48]. The recently sequenced genomes of *C. perfringens*[20,22] identified GerA homologs encoded by a monocistronic gene and a bicistrionic operon. GerA homologs were also identified in the sequenced genomes of *C. acetobutylicum*[21] and *C. tetani*[19]. From genetic studies, homologs have been detected in *C. botulinum* and *C. sporogenes*[49]. All clostridial GerA homologs share the same tricistrionic architecture of *gerA*-type operons found in *B. subtilis*, with the exception of *C. perfringens* where no studies regarding these putative nutrient receptors have been performed.

Transduction of germination signals from the nutrient receptor is followed by an increase in pH inside the germinating spore core. Up to 80% of the spore's Na^+ and K^+ are released from within the spore[50], which are believed to be pumped by an Na^+/K^+-H^+ antiporter termed GerN in *B. cereus*[51-52]. This latter event leads to the release of Ca^{2+}-DPA complex from the spore core. Proteins encoded by the *spoVA* hexacistrionic operon are believed to play a role in Ca^{2+}-DPA release in spores of *B. subtilis* (Figure 5)[20,22]. GerN and SpoVA homologs have been found to be present in the genomes of *C. perfringens*[20,22] as well as in other *Clostridium* species[19,21]. This suggests a similar pathway for ion and Ca^{2+}-DPA release after transduction of germination signal by the nutrient receptor.

In *B. subtilis*, two germination-specific cortex-lytic enzymes (CwlJ and SleB), where CwlJ is known to be activated by Ca^{2+}-DPA, are responsible for peptidoglycan degradation[53-55]. In contrast, studies carried out in *C. perfringens* strain S40, a type A heat sensitive strain that does not produce *C. perfringens* enterotoxin (CPE), identified at least three germination-specific cortex-lytic enzyme that act in a cooperative manner: spore cortex-lytic enzyme (SCLE), cortical fragment-lytic enzyme (CFLE), and germination-specific protease (GSP)[56]. SCLE is encoded by *sleC* and is synthesized in the mother cell as a precursor with a molecular mass of 50-kDa and 4 domains, but during sporulation it is processed to a 35-kDa inactive proenzyme[57-58]. SCLE has substrate specificity towards intact peptidoglycan[59], and it is activated when the N-terminal proregion is cleaved by germination serine protease (GSP) during spore germination[58,60]. Three GSP homologues, synthesized in the mother cell compartment[57], have been found in spore fraction extracts of *C. perfringens* strain S40. They are encoded by *cspA*, *cspB* and *cspC* and located outside the cortex of dormant spores of *C. perfringens* strain S40[61]. CFLE is encoded by *sleM* and is synthesized as a mature enzyme that degrades the polysaccharide moiety of SCLE-modified peptidoglycan which would act as the activation signal[62]. Interestingly, homologs of these three germination-specific cortex-lytic enzymes identified in *C. perfringens* strain S40 have been found to be present in the genomes of other *C. perfringens* strains[20,22] as well as in other *Clostridium* species, (*i.e*., *C. tetani* and *C. acetobutylicum*)[19,21]. This suggests that the mechanism of peptidoglycan degradation studies in *C. perfringens* strain S40 might be universal for *Clostridium* species.

Further studies to understand the molecular basis of spore germination should involve identification and characterization of nutrient receptors and their signal transduction mechanism. A better understanding of spore germination would allow for the development of safer food processing protocols as well as inhibitors that could be used as sporicidal agents or food additives in the food and feed industry.

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