Sporulation and enterotoxin regulation by sigma factors in *Clostridium perfringens*

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Abstract

Clostridium perfringens is a leading cause of food poisoning annually in the United States. Ingested *C. perfringens* vegetative cells respond to the acidic conditions of the stomach by initiating sporulation. The process of sporulation is essential in the formation of an enterotoxin (CPE) that is responsible for the symptoms of acute food poisoning. During sporulation, the cell must differentiate into the mother cell and the forespore. Studies in *Bacillus subtilis* have shown that gene expression during sporulation is compartmentalized, with different genes expressed in the mother cell and the forespore. The cell-specific RNA polymerase sigma factors coordinate the development of the differentiating cell. These sigma factors are σ^F, σ^E, σ^G, and σ^K. The *C. perfringens cpe* gene, encoding the enterotoxin CPE, is transcribed from three promoters, P1, P2, and P3. P2 and P3 were previously proposed to be σ^E-dependent, and P1 was proposed to be σ^K-dependent based on consensus recognition sequences. In this study, mutations were introduced into the *sigE* and *sigK* genes of *C. perfringens*. In the *sigE* and *sigK* mutants, promoter fusion assays indicated that there was no transcription of *cpe* in either mutant. We also determined through transcriptional analyses that σ^E-associated RNA polymerase and σ^K-associated RNA polymerase co-regulate the transcription of each other. RT-PCR analyses indicated that σ^K is a very early acting

sigma factor. The evidence provided here shows that the regulation of sporulation in *C. perfringens* is not the same as it is in *B. subtilis*, as previously proposed.

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Chapter I. General overview

General overview of *Clostridium perfringens*

The genus *Clostridium* consists of Gram-positive, anaerobic, endospore forming rods (99, 127) first described from a tissue infection in 1892 (128). Clostridia are commonly found in soils and in the gastrointestinal tract of humans and animals (69, 76, 111, 134). *Clostridium perfringens* has been detected in the gastrointestinal tract of healthy individuals at levels up to 10^3 cfu/g of feces (85).

C. perfringens produces 13 toxins whose genes are either located on the chromosome or on plasmids. Each strain of *C. perfringens* produces only a subset of these toxins (94). Four major toxins, α-toxin (phospholipase C), β-toxin, ε-toxin, and ι-toxin, are used to classify *C. perfringens* into five major toxinotypes (94) (Table 1-1). The different toxinotypes are associated with different diseases in humans and/or animals (94) (Table 1-2). In addition to the major toxins, *C. perfringens* produces nine accessory toxins, β2-toxin, δ-toxin, θ-toxin (perfringolysin O), κ-toxin (collagenase), λ-toxin, μ-toxin, ν-toxin, sialidases, and CPE (100). *C. perfringens* does not use a type III secretion system to infect cells with toxins, but secretes them into the environment (94).

One of the major diseases produced by *C. perfringens* is gas gangrene, also known as clostridial myonecrosis. Vegetative cells or spores of *C. perfringens* contaminate a wound and grow. Disease results when *C. perfringens* releases toxins which destroy tissue by causing membrane damage and by degrading the extracellular matrix of tissue (116).

Another major disease caused by *C. perfringens* is food poisoning due to the production of the enterotoxin, CPE, during sporulation. The acquisition and mechanism of disease caused by CPE is discussed in a later section.

Table 1-1. Major toxins produced by *C. perfringens* toxinotypes.

Toxinotype	Major toxin			
	α	β	ε	ι
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Based on: (94, 112).

Table 1-2. Diseases caused by *C. perfringens* toxinotypes.

Toxinotype	Associated diseases
A	Gas gangrene and gastrointestinal diseases such as food poisoning and antibiotic-associated diarrhea in humans, diarrhea in foal and pigs, necrotic enteritis in fowl
B	Dysentery in lambs and hemorrhagic enterotoxemia in sheep, hemorrhagic enteritis in calves and foals
C	Necrotic enteritis (Pigbel or Darmbrand disease) in humans, enterotoxemia in sheep, necrotic enteritis in piglets, lambs, calves, and foals
D	Enterotoxemia in lambs (pulpy kidney disease) and calves, enterocolitis in goats
E	Enterotoxemia in calves and lambs, enteritis in rabbits

Based on: (94, 112).

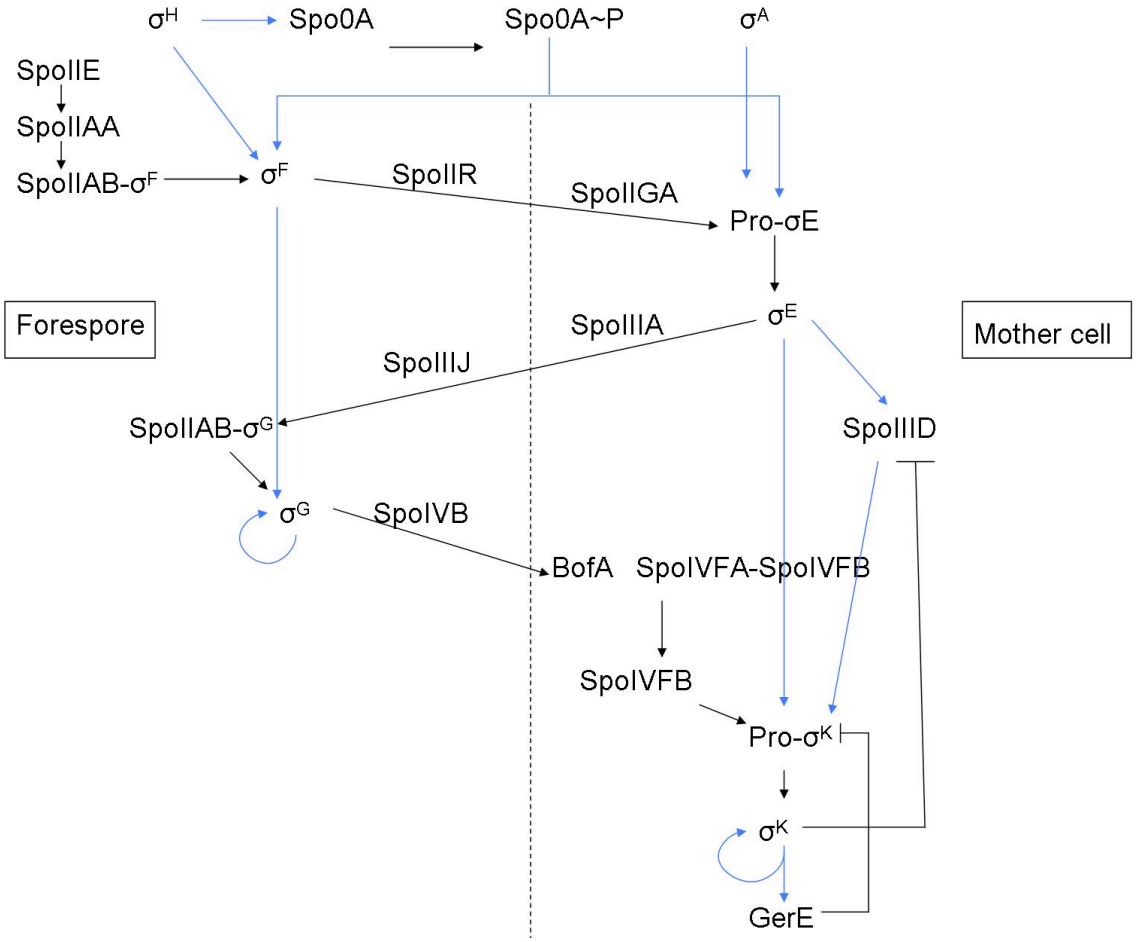
Regulation of sporulation in *Bacillus subtilis*

There is limited information available on the regulation of *C. perfringens* sporulation. Therefore, sporulation in the Gram-positive organism *Bacillus subtilis* is used as a model for *C. perfringens* sporulation. When *B. subtilis* reaches a nutrient starved state, it undergoes sporulation, resulting in the formation of a dormant spore. At the onset of sporulation by *B. subtilis*, a polar septum forms to divide the sporangium into two unequal compartments that have different developmental fates (96). The smaller compartment, the forespore, will eventually become the spore, while the larger compartment, the mother cell, will lyse once the spore is mature. The mature spore is dormant and resistant to high heat, UV radiation, lytic enzymes, and chemicals (87).

The differentiation of the cell into a forespore and a mother cell is controlled by four sporulation specific sigma factors that associate with RNA polymerase to direct transcription of sporulation specific genes whose gene products are necessary for spore formation (Fig. 1-1). Two of the sigma factors (σ^F and σ^G) are active only in the forespore while the two others (σ^E and σ^K) are active only in the mother cell. These sigma factors are regulated in a sequential order with σ^F being active first, followed by σ^E, then σ^G, and finally σ^K (72). *B. subtilis* sporulation involves the coordination of over 125 essential genes (119).

Sporulation in *B. subtilis* is activated by the phosphorylated form of Spo0A initiating the transcription of several genes. The phosphorylation of Spo0A is the result of a phosphorelay system that relies on environmental cues such as nutrient starvation (7). These cues activate up to five kinases (7, 42) that phosphorylate the response

Figure 1-1. Regulation of sporulation in *B. subtilis*.



The major proteins needed for sporulation regulation in *B. subtilis*. Blue arrows represent transcriptional control. Black arrows represent protein activation. Black lines with barred ends represent negative regulation. The dashed center line represents the polar septum.

Based on: (42, 90).

regulator Spo0F (43). Phosphorylated Spo0F then donates its phosphate to Spo0B, which then transfers the phosphate to Spo0A (7). Phosphorylated Spo0A activates or represses promoters used by the vegetative sigma factor σ^A- and the alternative sigma factor σ^H-associated RNA polymerase (42). σ^H-dependent transcription of *spo0A* (108) and the *spoIIA* operon, which consists of *spoIIAA*, *spoIIAB*, and *spoIIAC*, is essential for sporulation and the activation of σ^F (31, 133). Spo0A and σ^A-associated RNA polymerase transcribes the *spoIIG* operon which has gene products involved in the activation of σ^E (1, 6).

The operon containing the σ^F encoding gene, *spoIIA*, is transcribed by σ^H -associated RNA polymerase activated by Spo0A (132) prior to polar septum formation in *B. subtilis* (35). σ^F is encoded by the *spoIIAC* gene (122) and is only active in the forespore after polar septation (41).

Three proteins, SpoIIAB, SpoIIAA, and SpoIIE, regulate σ^F activity (16, 74, 83, 104). Initially, σ^F is held inactive by the anti-σ factor SpoIIAB (23). Release of σ^F from SpoIIAB occurs when SpoIIE interacts with phosphorylated SpoIIAA, an anti-anti-σ factor, to dephosphorylate it, allowing SpoIIAA to become active (22). SpoIIAA then forms a complex with SpoIIAB, releasing it from σ^F and allowing σ^F to be active (16). σ^F -controlled genes are necessary for engulfment of the forespore cell by the mother cell (60).

The *spoIIG* operon which includes *spoIIGA* and *spoIIGB* (48, 53) is transcribed by σ^A-dependent RNA polymerase (1, 6) prior to and after polar septation (35, 44). Pro-σ^E is transcribed as the second gene in the *spoIIG* operon, *spoIIGB,* while *spoIIGA* encodes a protease that cleaves pro-σ^E (124).

The processing of pro-σ^E to active σ^E occurs through cleavage of 29 amino acids from the N-terminus (66) and only happens after polar septation has been completed (117). Processing is directed by SpoIIGA, which is predicted to be a membrane-bound protein (93). One of the gene products controlled by σ^F, SpoIIR, is active in the forespore and essential in the activation of SpoIIGA in the mother cell (50, 71). SpoIIR interacts with the integral membrane protein SpoIIGA to initiate the processing of pro-σ^E to σ^E (44). The interaction of SpoIIR and SpoIIGA may serve as a checkpoint to regulate the processing of pro-σ^E until after σ^F is activated (34).

Mutations in both *spoIIGA* and *spoIIGB* have a dramatic effect on sporulation. When SpoIIGA is mutated, σ^E is never activated, a result that in *B. subtilis* is characterized by the lack of heat resistant spores (53). Mutations in the gene encoding σ^E results in *B. subtilis* cells that cannot develop beyond polar septation and results in cell that have a disporic phenotype, characterized by asymmetric septa formed at both poles of the cell (47, 67).

σ^E-associated RNA polymerase is responsible for the gene transcription of more than 260 genes in the mother cell (17, 27). Of these 260+ genes, only 45 are essential for sporulation (27). Functions of these gene products include regulating engulfment of the forespore, metabolism control, synthesis and assembly of the spore cortex and spore coat, and to prepare the cell for future stages of sporulation (27). σ^E-associated RNA polymerase transcribes genes encoding σ^K and SpoIIID (61, 62, 121, 123).

σ^G is the product of the *spoIIIG* gene (49), whose transcription is σ^F- and σ^G-directed (121). However, there does appear to be some transcription of *spoIIIG* due to read-through from the upstream *spoIIG* operon, which encodes SpoIIGA and σ^E (75).

Transcription of *spoIIIG* occurs only in the forespore (49) and near the end of engulfment of the forespore by the mother cell (91).

σ^G is not active until the end of engulfment. SpoIIAB, the anti-σ factor that regulates σ^F, is also responsible for holding σ^G inactive (32, 52). Activation also involves SpoIIIJ, which is active in the forespore, and the products of *spoIIIA*, which are active in the mother cell (105). It is not yet fully understood how SpoIIAB, SpoIIIJ, and the products of *spoIIIA* interact with each other to control the activation of σ^G.

SpoIIID is transcribed by σ^E-associated RNA polymerase and is expressed in the mother cell (61, 123). SpoIIID functions as a DNA binding protein that activates transcription of *sigK* (62, 118) and regulates specific genes under σ^E-associated RNA polymerase control (37). A feedback loop regulates SpoIIID in which an increase of σ^K causes a decrease of SpoIIID levels (36).

sigK is created when two genes, *spoIVCB* and *spoIIIC*, are brought together into one cistron by a chromosomal rearrangement (118). SpoIIID then activates the promoter for *sigK* (38, 62, 118) and the transcription of the σ^K-encoding gene is driven by σ^E and σ^K (62, 89).

sigK encodes the inactive pro-σ^K (118). The activation of pro-σ^K to σ^K is a complicated process relying on proteins in the forespore and in the mother cell. First, in the forespore, σ^G-associated RNA polymerase transcribes *spoIVB* whose product is SpoIVB (11). SpoIVB then interacts with a complex of proteins consisting of BofA, SpoIVFA, and SpoIVFB located in the mother-cell (101). The processing of pro-σ^K to active σ^K occurs through the cleavage of 20 amino acids from the N-terminus (59, 73) and is directed by the metalloprotease SpoIVFB (58, 136, 141). SpoIVFB is regulated by

SpoIVFA (12) and BofA (141), which are inhibitors of SpoIVFB activity. How SpoIVB interacts with SpoIVFA and BofA to allow SpoIVFB to be active and process pro-σ^K is not yet understood.

σ^K-associated RNA polymerase directs the transcription of over 100 genes whose products are involved in synthesis of the spore cortex and coat (26, 115, 137, 140). GerE, a DNA-binding protein (139), is under σ^K-associated RNA polymerase control and is a negative regulator of *sigK* (46).

C. perfringens sporulation

C. perfringens has many genes which are homologous to sporulation associated genes in *B. subtilis*, and therefore *B. subtilis* serves as a model for *C. perfringens* sporulation (90). However, there are some differences between the two systems. For example, the phosphorelay pathway that activates Spo0A in *B. subtilis* does not exist in clostridia (24, 106). Spo0A is essential for sporulation in *C. perfringens*, but it is not known what activates it (45).

There has been much research into increasing *C. perfringens* sporulation efficiency through media components. It is known that starch levels play an important role in the formation of spores (65). The level of inorganic phosphate that is present has also been shown to affect the percentage of sporulation in *C. perfringens*. Media that contains inorganic phosphate levels between 30 and 40 mM have the highest levels of sporulation (95). Exposure to hydrochloric acid has been shown to increase sporulation in some strains of *C. perfringens* (131). The addition of raffinose (63), caffeine,

theophylline, and isobutylmethylxanthine to sporulation media also increases sporulation (102).

C. perfringens enterotoxin

C. perfringens enterotoxin (CPE) is a common cause of food poisoning. A study conducted from 1983 to 1997 determined there were an estimated 250,000 cases per year of *C. perfringens* food poisoning (81). CPE is also linked to non-food-borne illness, such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD) (30, 86). AAD is a particular concern for the elderly taking antibiotics (85).

The formation of endospores is essential for the ability of *C. perfringens* to cause food-borne illness in humans and animals (13, 21). Unlike the other toxins produced by *C. perfringens*, CPE is only produced when the cell is undergoing sporulation and not during vegetative growth (8, 99). During sporulation, CPE accumulates in the cytoplasm of the mother cell, forming large crystals that are visible by electron microscopy (19, 70). When the sporangium lysis, the toxin is released (18). The presence of CPE in feces is used to identify *C. perfringens* as the infectious agent in food-poisoning outbreaks (3, 5).

The *cpe* gene is either located on the chromosome or on a plasmid. Most of the enterotoxigenic *C. perfringens* type A strains involved in human food poisoning carry *cpe* on the chromosome while those strains involved in AAD, SD, or animal diseases carry *cpe* on a plasmid (9, 114). It has been found that *C. perfringens* with *cpe* located on the chromosome can survive longer at colder temperatures than strains with *cpe* located on plasmids (68), while *C. perfringens* vegetative cells and spores with *cpe* located on the chromosome are more heat resistant than strains which carry *cpe* on plasmids (103).

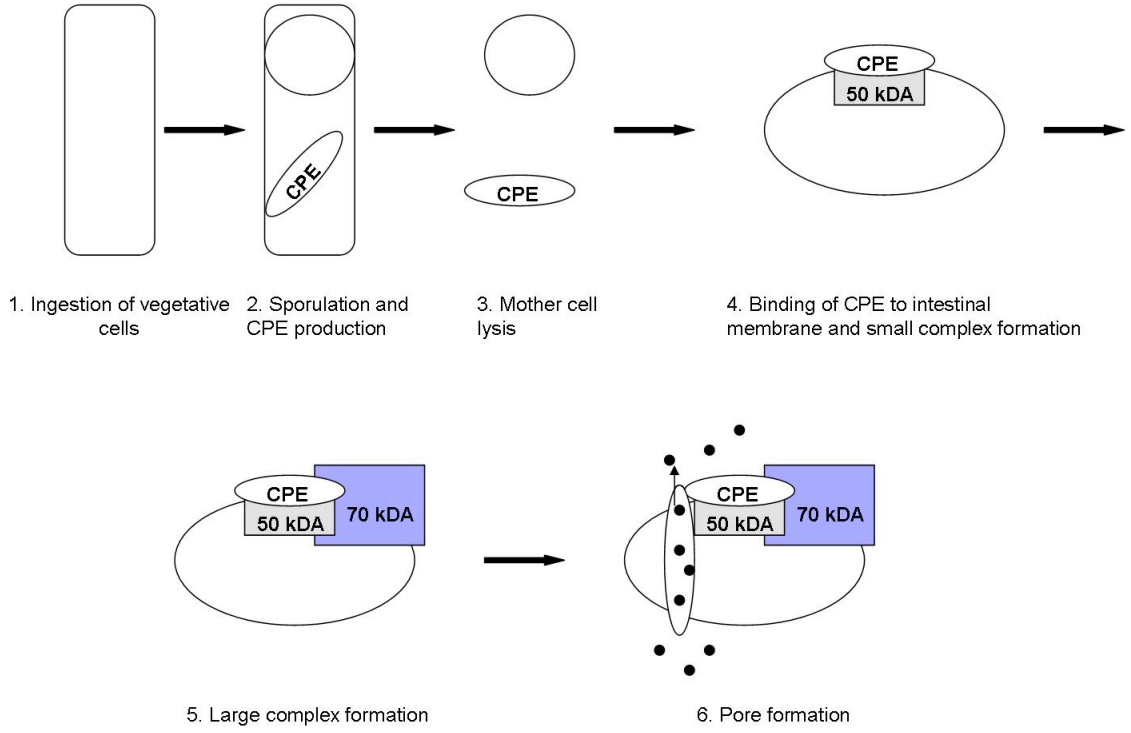
CPE synthesis is compartment specific, only being produced in the mother cell, the same compartment in which σ^E and σ^K are active. There are three promoters involved in CPE synthesis (P1, P2, and P3). P1 appears to be σ^K-dependent, while P2 and P3 appear to be σ^E-dependent based on consensus recognition sequences (138). Using reporter genes, it was shown that transcription of these promoters strongly correlates with sporulation (138).

Pathogenesis of disease caused by food-borne CPE positive type A strains

Due to the prevalence of this bacterium, it is a common problem for the food industry, especially when large amounts of food is prepared in advance and allowed to cool overnight, such as is common in restaurants, nursing homes, and hospitals (10, 15, 88, 92). Generally the contaminated food is a meat product, but *C. perfringens* has been isolated from vegetable based foods as well (84).

CPE is produced in the small intestine after ingestion of at least 10^7 *C. perfringens* vegetative cells (84, 85, 92). Symptoms begin eight to twenty hours from ingestion of contaminated food and include abdominal pain, nausea, and diarrhea (3, 80). Deaths may occur in a low number of cases in the elderly and children due to dehydration (92). After the vegetative cells are ingested, sporulation is stimulated by the acidic pH of the stomach (131). The cells then proceed to the small intestine where the sporangium lysis, releasing CPE as well as the mature spore (21, 120). The mechanism of how CPE causes food poisoning is diagramed in figure 1-2.

Figure 1-2. The major steps in CPE mechanism of action.



Based on: (55).

CPE is a 35 kDa toxin (14) that recognizes and binds to claudin, a ~45-50 kDa membrane polypeptide located on the apical surface of intestinal epithelial cells (78). This results in a ~90 kDa CPE containing complex, known as the small complex (54, 77, 129). The last 30 amino acids of the C-terminal region of CPE are necessary for binding (40). Claudins are structural and functional components of tight junctions (40, 56). Claudin-3, -4, -6, -7, -8, and -14 have been found to be receptors for CPE binding (33, 51, 97, 107, 113, 125).

The small complex interacts with a ~65-70 kDa protein present on the eukaryotic plasma membrane to form a ~160 kDa CPE-containing complex, known as the large complex (129, 130). The ~65-70 kDa has been identified as the structural tight junction protein occludin, in which the N-terminal half of native CPE directly or indirectly interacts with after binding (107). An increase in activity of CPE occurs when the first 44 amino acids are deleted from the N-terminus (56). The amino acids necessary for large complex formation are number 45 through 53 (54, 56)

The formation of the large complex is necessary for the cytotoxic effects of CPE (110, 113). CPE associated with the large complex becomes inserted into the membrane forming a channel that leads to loss of solutes (78, 79). CPE binding to these cell proteins located in the tight junctions of the villi causes tissue damage and triggers fluid and electrolyte transport into the lumen of the intestines in humans and rabbits (20, 29). This causes the symptoms of diarrhea and intestinal cramping.

Chapter II. Sporulation and enterotoxin (CPE) production in σ^E and σ^K mutants of *Clostridium perfringens*

Abstract

In order for the cell to differentiate into the mother cell and the forespore, gene expression must be coordinated and tightly controlled. Studies in *Bacillus subtilis* have shown that gene expression during sporulation is compartmentalized, with different sporulation specific sigma factors expressed in the mother cell and forespore. *Clostridium perfringens* produces an enterotoxin, CPE, which is the causative agent of food poising in humans and animals. Synthesis of CPE is dependent on sporulation and is only produced in the developing mother cell. This is the same compartment in which σ^E and σ^K are presumed to be active. There are three promoters involved in *cpe* synthesis (P1, P2, and P3). P1 appears to be σ^K-dependent, while P2 and P3 appear to be σ^E-dependent. In this study, mutations were made in the *sigE* and *sigK* genes and it was found that *cpe* is not transcribed in these mutants. In addition, we demonstrate that unlike the system observed in *B. subtilis*, the *C. perfringens sigE*- mutant appears to be blocked in sporulation at a later stage than does the *sigK* -mutant.

Introduction

Clostridium perfringens is a common cause of food poisoning, responsible for approximately 250,000 cases in the United States each year (81). After the ingestion of vegetative cells in contaminated food, an enterotoxin, CPE, is produced by sporulating cells in the gastrointestinal tract (20, 21). The enterotoxin binds to receptors on the surface of intestinal epithelial cells and interacts with tight junctions forming a pore (77). This triggers the loss of fluids and electrolytes, leading to the symptoms of diarrhea and intestinal cramping (20, 29).

CPE is only produced in the cytoplasm of the mother cell during sporulation of *C. perfringens* (19, 70). After sporulation is completed the mother cell lysis to release the mature spore, subsequently releasing CPE (18). In laboratory conditions, extracellular enterotoxin is first detected 9 to 10 h after inoculation into sporulation media and increases during the next 14 h as more CPE is released (18). *C. perfringens* strains that do not sporulate, do not produce the enterotoxin (21).

In *Bacillus subtilis* sporulation is broken down into specific stages. Stage I is characterized by the gathering of two nucleoids into an axial filament (96). During stage II an asymmetric cell division septum is formed, separating the cell into the forespore and the mother cell (96). Stage III is characterized by engulfment of the forespore by the mother cell (96). Stage IV is defined by spore cortex formation, while stage V is characterized by spore coat formation, and stage VI is defined by spore maturation (96). The final stage, VII, is characterized by the release of the spore from the mother cell (96). Duncan (19) found that *C. perfringens* type A mutants blocked at stages III, IV and V still produced CPE, but in reduced amounts. Although CPE is only produced in

sporulating cells, CPE is not necessary for sporulation, as many strains of *C. perfringens* produce spores but not CPE (18).

C. perfringens strains containing the *cpe* promoters fused to the *E. coli* reporter gene *gusA* on a plasmid indicated that *cpe* is transcribed in very early stationary phase, about the same time that CPE protein levels were detected (82). It has been shown that only sporulating cells and not vegetative cells produce *cpe* mRNA (13). These results demonstrate that CPE expression is controlled at the transcriptional level during sporulation. Zhao and Melville (138) found that there are three promoters that regulate the transcription of *cpe*. The upstream region for P1 is similar to σ^K consensus recognition sequences and the regions upstream of P2 and P3 are similar to σ^E consensus recognition sequences (138). In this study, mutations made in the σ^E and σ^K encoding genes resulted in the loss of transcription of *cpe*, demonstrating that transcription of *cpe* is dependent on σ^E and σ^K.

A mutation in the gene encoding σ^E in *B. subtilis* results in a disporic phenotype, in which asymmetric septa form at both cell poles (47). A mutation in the *B. subtilis* σ^K gene is characterized by the completion of engulfment, but spore development stops before spore cortex and spore coat can be added (96). Our results demonstrate that a *sigE*- mutant in *C. perfringens* is blocked at the same stage of development as a *B. subtilis sigE*- mutant (stage II). Unexpectedly, a *sigK*- mutant in *C. perfringens* appears to be blocked in sporulation at an earlier stage of development (stage 0) than the *sigE*- mutant.

Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 2-1. *E. coli* strains were grown in Luria Bertani (LB) medium (10 g tryptone, 10 g NaCl, 5 g yeast extract and 15 g agar when needed per liter) at 37°C on plates or in broths with shaking. As needed, 300 μg/ml erythromycin and 20 μg/ml chloramphenicol were added to the medium.

C. perfringens strains were grown anaerobically in PGY medium (30 g proteose peptone #3, 20 g glucose, 10 g yeast extract, 1 g of sodium thioglycolate per liter) or BHI (brain-heart infusion) (Difco) at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) with 30 μg/ml erythromycin and 20 μg/ml chloramphenicol added as needed.

Construction of *sigK* and *sigE* mutants

To create the *sigK* mutant, KM1, an internal 382 bp gene fragment of *sigK* was PCR amplified from the *C. perfringens* SM101 chromosomal DNA template using oligonucleotide primers OSM172 (5'-GTTGAG*GAGCTC*TTAAGACTAGTAGCAC-3') and OSM173 (5'-GGACTT*GGTACC*TCTGACATCTTTTTATAA-3'). OSM172 had a SacI restriction site and OSM173 had a KpnI restriction site designed into the primer sequence. The resulting PCR product was then digested with SacI and KpnI and ligated into the *C. perfringens* suicide vector pSM300 (126), resulting in pNLDK (Table 2-1).

To create the *sigE* mutant, KM2, a *C. perfringens* suicide vector containing a 433 bp internal fragment of the *sigE* gene was constructed. This fragment was PCR amplified using the oligonucleotides OSM168 (5'-GAAGCT*GAGCTC*AGTATTCTTATTGAGAG-3') and OSM169 (5'-

Table 2-1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
E. coli		
DH10B	F- *mcrA* Δ(*mrr-hsdRMSmcrBC*) F80d *lacZ Δ*M15 *lacX74 deoR recA1 araD139* D (*ara*, *leu*)7697 *galU galK λ-rpsL endA1 nupG*	Gibco/BRL
JM107	F' *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA+/B+*/e14-(McrA)Δ(*lac-proAB*) *thi gyrA96*(Nal^r) *endA1 hsdR17* (r_K-m_K+) *relA1 glnV44*	(135)
C. perfringens		
SM101	High efficiency of electroporation derivative of NCTC 8798	(138)
KM1	*sigK*- mutant of SM101	This study
KM2	*sigE*- mutant of SM101	This study
Plasmids		
pSM300	*E. coli* origin of replication, erythromycin resistance	(126)
pNLDK	pSM300 with the *sigK* gene internal fragment	This study
pNLDE	pSM300 with the *sigE* gene internal fragment	This study
pJV5	*E. coli-C. perfringens* shuttle vector, chloramphenicol and kanamycin resistance	(127)
pSM305	pBluescriptSK- with a 3 Kb HindIII insert containing complete *sigK* and *pilT* genes and gene fragments of *CPR_1739* and *ftsA* from *C. perfringens* strain 8798, the parent strain of SM101	(Insert sequence Accession No. AF218835)
pKM2	pJV5 with complete *sigK* gene from plasmid pSM305	This study
pKM3	pJV5 with complete *spoIIG* operon	This study
pKM12	pJV5 with a frameshift mutation leading to a stop codon in the *sigK* gene	This study
pJV7	pJV5 with complete *sigK* and *pilT* genes from plasmid pSM305	This study
pJIR750	*E. coli-C. perfringens* shuttle vector, chloramphenicol resistance	(2)
pSM100	pBluescript SK- with the NCTC 10240 *cpe* promoter	(82)
pSM104	pJIR750 with Φ($cpe_{\text{NCTC 10240}}$-*gusA*) (P1, P2, and P3)	(82)
pSM127	pSM104 with Φ($cpe_{\text{NCTC 10240}}$-*gusA*) (P1and P2)	(138)
pSM170	pSM104 with Φ($cpe_{\text{NCTC 10240}}$-*gusA*) (P3)	(138)

CCACAT*GGTACC*AATCTTAATTCAACAATTTC-3'). OSM168 had a SacI restriction site and OSM169 had a KpnI restriction site designed into the primer sequence. The resulting PCR product was then digested with SacI and KpnI and ligated into pSM300, resulting in the plasmid pNLDE (Table 2-1).

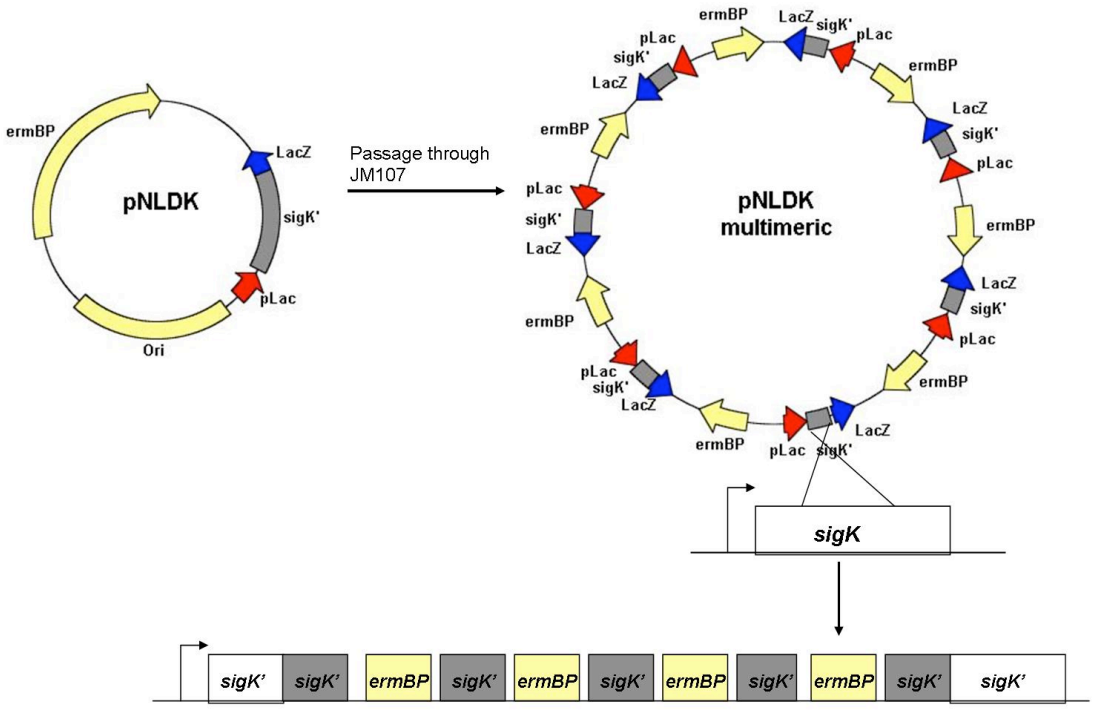
pNLDK and pNLDE were electroporated into *E. coli* strain JM107 (*recA*+) to produce a multimeric form of the plasmid. Multimeric plasmids have been found to insert into the chromosome via homologous recombination more efficiently than monomeric forms (Fig. 2-1) (127). After passage through JM107, the plasmids were purified via a cesium chloride gradient.

Sixteen μg of purified pNLDK and 24.3 μg of purified pNLDE were transformed into *C. perfringens* strain SM101 by electroporation using 4 mm-gap cuvettes. The cells were allowed to recover for 3 h in BHI broth in the absence of antibiotics, and then plated on BHI with 30 μg/μl erythromycin.

Southern blot analysis

To confirm the insertion of the multimeric pNLDE and pNLDK into the *sigE* or *sigK* genes, Southern blotting was performed on the erythromycin-resistant transformants. Chromosomal DNA of *C. perfringens* strain KM1 (*sigK*-) was digested with PstI and EcoRI and the digested DNA separated by electrophoresis on a 0.8% agarose gel. The DNA was then transferred to nitrocellulose by blotting. Hybridization with a biotinylated labeled probe specific to the internal fragment of *sigK* was used to detect the *sigK* gene in *C. perfringens* SM101 and in *C. perfringens* KM1 (*sigK*-). A shift from 2 kb to above 10 kb indicated the recombination of pNLDK into the *sigK* gene

Figure 2-1. Schematic diagram showing the insertion of the multimeric form of pNLDK into the wild type copy of the *sigK* gene.



The monomeric form of pNLDK was passed through *E. coli* JM107 (*recA*+) to create a multimeric form of the plasmid. The multimeric form of pNLDK was electroporated into *C. perfringens* SM101, and through homologous recombination of the internal fragment of *sigK'* on pNLDK and the *sigK* gene on the chromosome, resulted in the *sigK* mutant *C. perfringens* strain KM1 (*sigK*-). A similar procedure was used for the mutagenesis if the chromosomal *sigE* gene.

(data not shown). A similar procedure was performed for confirmation of a *sigE* mutant, except that chromosomal DNA from the erythromycin-resistant mutant, *C. perfringens* strain KM2 (*sigE*-), was digested with XbaI. A shift from a wild type size of 2.1 kb to above 10 kb indicated the recombination of pNLDE into the *sigE* gene (data not shown).

C. perfringens electroporation procedure

C. perfringens strain SM101 was grown anaerobically in 5 ml of liquid PGY for 14-16 hours. Cells were then centrifuged in an IEC clinical centrifuge at setting number 5 for 10 minutes at room temperature. The cell pellet was the washed twice in 5 ml of ice cold electroporation buffer (5 ml 1 mM sucrose, 1 ml 100 mM sodium-phosphate, 15 µl 1M $MgCl_2$, and 10 ml water) and resuspended in 3 ml of electroporation buffer. 400 µl of resuspended cells were added to a 4 mm-electrode gap cuvette and transformed with 1-100 ug of plasmid DNA using a BTX ECM 630 electroporator set at 2500 V with a resistance of 125 ohms and a capacitance of 50 µF.

Construction of complementation plasmids for _C. perfringens_ KM1 (_sigK_-) and _C. perfringens_ KM2 (_sigE_-)

Complementation of *C. perfringens* KM1 (*sigK*-) was attempted by digesting the complete *sigK* gene and promoter from pSM305 with PstI and HincII. This fragment was then ligated into the *E. coli-C. perfringens* shuttle vector, pJV5, digested with SmaI and PstI, to produce the plasmid pKM2 (Table 2-1). To determine if the downstream gene from *sigK*, *pilT*, had an effect on sporulation, a plasmid was constructed by digesting a region containing 120 bp upstream of *sigK* through *pilT* from pSM305 using PvuII. This fragment was then ligated into pJV5 cut with SmaI, resulting in pJV7 (Table 2-1).

Complementation of *C. perfringens* KM2 (*sigE*-) was attempted by PCR amplification of the entire operon consisting of *spoIIGA*, *sigE,* and 400 bp upstream of *spoIIGA* from the *C. perfringens* SM101 chromosome using the oligonucleotide primers OKM9 (5'-GCTGAG*GGATCC*GTTGCTGCGTCTTC-3'), which had a BamHI restriction site designed into the primer, and OKM11(5'-CTAATCTC*GAATTC*CCCTTAATAAATTTCTCTCTAG-3'), which had an EcoRI restriction site designed into the primer. The PCR product was then digested with BamHI and EcoRI and ligated into pJV5, digested with BamHI and EcoRI, resulting in the plasmid pKM3 (Table 2-1).

In an attempt to complement *C. perfringens* KM1 (*sigK*-), primers were designed to PCR amplify an 1,079 bp region from the *C. perfringens* SM101 chromosome that contains the entire *sigK* gene and the upstream promoter region using the oligonucleotide primers OKM12 (5'-GATAGA*GGATCC*GGAGGTGGAAATACTG-3'), which had a BamHI restriction site designed into the primer, and OKM13 (5'-GCAAATCA*GAATTC*CCTTCTTTAACTGTTAACTC-3'), which had an EcoRI restriction site designed into the primer. The PCR product was cloned into pJV5, resulting in pKM12. Sequencing of the insert revealed that a thymine base was inserted 364 bp into the *sigK* gene and caused a frameshift mutation resulting in a termination codon. Upon translation this would result in a truncated σ^K.

Sporulation assay

C. perfringens strains were grown 14-16 h in 5 ml fluid-thioglycolate (FTG) with the appropriate antibiotic if needed. Two hundred fifty µl was subcultured into 5 ml prewarmed sporulation medium, DSSM (15 g proteose peptone #3, 10 g sodium

phosphate, 4 g raffinose, 4 g yeast extract, 1 g of sodium thioglycolate per liter, pH adjusted to 7.8) and grown anaerobically for 24 h at 37°C. Samples were diluted and plated in duplicate on PGY agar in the absence of antibiotics to determine the number of viable cells per ml of culture. Three hundred fifty µl samples were heated at 75°C for 15 minutes, diluted, and plated in duplicate on PGY agar to determine the number of spores per ml of culture. All assays were performed in triplicate. The following formula was used to determine the sporulation efficiency:

Sporulation efficiency = [cfu heated inoculum / (cfu heated inoculum + cfu vegetative inoculum)] * 100

Extraction of RNA

C. perfringens SM101 and *C. perfringens* KM1 (*sigK*-) strains were grown 14-16 h in FTG with erythromycin if needed. A 1% inoculum was used to inoculate prewarmed DSSM, and growth of the culture was measured at an optical density of 600 nm using a Genesys 10 uv scanning spectrophotometer. Fifty ml of culture was collected at optical densities of 0.1 nm and 1.0 nm representing early log and early stationary phase of growth, respectively. Cells were concentrated and stored at -80°C. RNA was extracted using TRIzol reagent (Invitrogen) and contaminating DNA was removed with RQ1 DNase (Promega), as per manufacturer's instructions. RNA was stored in liquid nitrogen until use.

Reverse transcription PCR (RT-PCR)

To determine the transcriptional activity of genes in the *sigK* region, primers were designed to amplify an internal fragment of *pilT* and upstream and downstream of the intergenic spaces of *sigK*, *pilT*, and *ftsA*. A primer was designed 57 bp upstream of the *CPR_1739* stop codon (OKM12, 5'- GATAGAGGATCCGGAGGTGGAAATACTG-3') and 502 bp downstream of the *sigK* start codon (OJV13, 5'-

CCTGTATTGAATTTTCAACAGCC-3') to determine if *sigK* is cotranscribed with *CPR_1739*, annotated as a penicillin binding protein transpeptidase domain protein. To determine if there is a transcript extending between *sigK* and *pilT* a primer was designed 113 bp upstream of the *sigK* stop codon (OJV12, 5'-GGTGATATAAAAACACAGAGGG-3') and 626 bp downstream of the *pilT* start codon (OJV22, 5'-GTCTCCAAATCTCTCATTTCCCC-3'). To determine if *pilT* is transcribed during sporulation in *C. perfringens* SM101 and if its transcription is affected by a mutation in the *sigK* gene, primers were designed internal to *pilT* (OJV11, 5'-GGTTAGAACATGAAATATTGCGCCC-3' and OJV22). In order to determine if *pilT* and *ftsA* are cotranscribed and if a mutation in *sigK* blocks this transcription, OJV11 was used which is 942 bp upstream of the *pilT* stop codon and a primer was designed 53 bp downstream of the *ftsA* start codon (OJV18, 5'-CGATGCACAAATATTTCTGTTTCC-3').

cDNA synthesis was performed by reverse transcription of 2 μg RNA with 50 pmol of each primer using the Access RT-PCR kit (Promega), following the manufacturer's instructions. PCR reactions set up without reverse transcriptase made it possible to verify the absence of contaminating DNA. All RT-PCR reactions were performed in duplicate.

Transmission electron microscopy (TEM) sample preparation

Samples were grown 14-16 h in 5 ml FTG with the appropriate antibiotic, then 750 μl was inoculated into 75 ml of DSSM. The optical density at 600 nm was measured every hour for 8 hours. One ml samples were obtained at 3, 5, and 8 h post-inoculation into DSSM, corresponding to mid-log phase, early stationary phase and stationary phase.

Samples were centrifuged, and the pellet suspended in 84 µl of 0.5 M $NaPO_4$, pH7 and 5 µL of 25% EM grade glutaraldehyde, gently vortexed, and stored at 4°C overnight. The samples were washed four times with ice cold 0.1 M sodium phosphate buffer, pH 6.7, suspended in 1% osmium in phosphate buffer (0.1 M, pH 6.7) and stored at 4°C overnight. The samples were then washed in 0.5 M NH_4Cl, suspended in 2% melted, warm agar and immediately spun at 10,000 x g for 3-5 minutes while cooling. A standard ethanol dehydration was performed and the samples were embedded in Spurr's resin. Samples were cut and collected on grids and stained with 1% uranyl acetate for 5 minutes and Reynold's lead for 1 minute.

Cell phenotypes

The phenotypes of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) in mid-log phase, early stationary phase, and stationary phase after inoculation into DSSM, as well as *C. perfringens* KM1(pKM2) (complemented *sigK*-) and *C. perfringens* KM2(pKM3) (complemented *sigE*-) in stationary phase after inoculation into DSSM were quantified by determining the stage of development present in a population of sporulating cells. Only complete longitudinal sections of cells were assessed. The number of cells counted varied for each sample from 17 to 49, except for *C. perfringens* KM2 (*sigE*-) at 3 h in which 6 cells were counted.

***cpe* promoter plasmid constructs**

The construct containing all three *cpe* promoters fused to the *E. coli gusA* gene (pSM104) was described previously (82), as was construction of pSM127 and pSM170 (138). Briefly, pSM104 was created by fusing the promoter of *C. perfringens* NCTC 10240 in frame to the *E. coli* reporter gene *gusA* and then ligating this construct into the

E. coli-C. perfringens shuttle vector pJIR750 (82). Deletions of the *C. perfringens* NCTC 10240 *cpe* promoter (pSM100) were ligated into pSM104 that had been digested, therefore replacing the full *cpe* promoter with ones containing deletions, resulting in pSM127 and pSM170 (138). Promoters for *C. perfringens* NCTC 8798, the parent strain of *C. perfringens* SM101, and *C. perfringens* NCTC 10240 had the same patterns of *cpe* promoter induction (82).

β-glucuronidase assays

Cultures were grown overnight in 5 ml fluid thioglycolate (FTG) with the appropriate antibiotic(s), and then 750 µl was subcultured into 75 ml prewarmed DSSM. The optical density at 600 nm was measured hourly for 8 h and 1 ml cell aliquots removed at these time points and stored at -20^{o}C. Cells were resuspended in 0.8 mL buffer containing DTT and 8 µl toluene. Samples were incubated on ice for 10 minutes and then at 37^{o}C for 30 minutes prior to the addition of 160 µl 6mM p-nitrophenyl β-D-glucuronidate (PNPG). The samples were incubated until a yellow color developed, at which time the reaction was terminated by the addition of 400 µl 1M sodium carbonate. The absorbance at 405 nm was measured and the specific activity (in units) calculated with the following equation:

Specific activity = (1000 x A_{405})/(Time [in minutes] x OD_{600} x culture volume [in milliliters])

Results

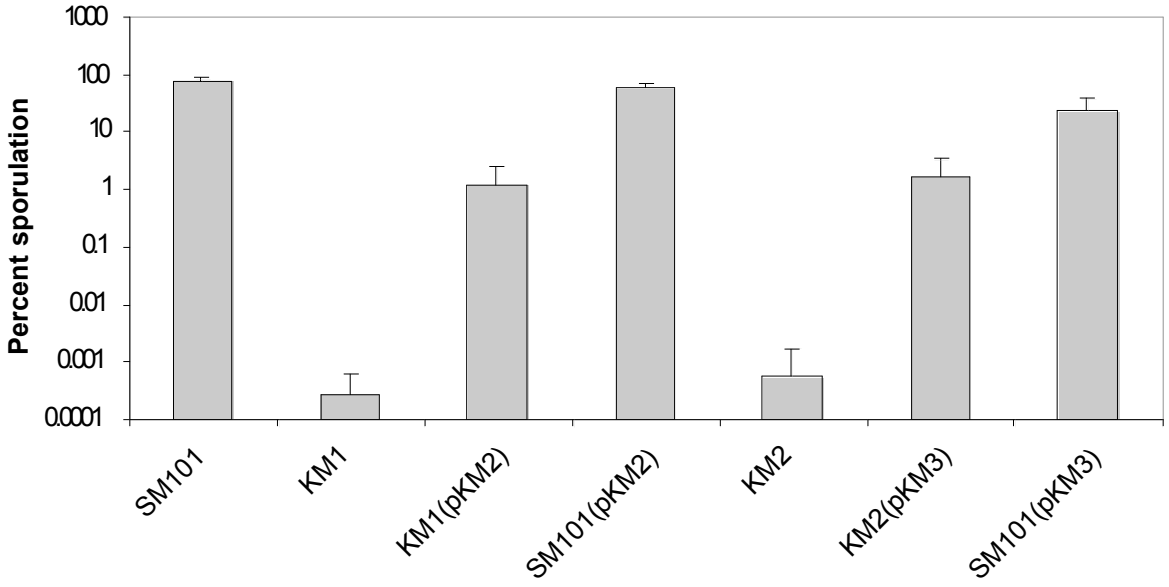
C. perfringens KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) do not produce detectable numbers of spores.

Both *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) formed less than 10 heat-resistant spores per ml after incubation in DSSM for 24 h, whereas the wild type strain, *C. perfringens* SM101, formed an average of 4.01 x 10^7 spores/ml. The sporulation efficiencies of *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) were 0.0003% and 0.0006%, respectively, while *C. perfringens* SM101 had a sporulation efficiency of 79.8% (Fig. 2-2). These results indicate that the σ^E and σ^K are essential for formation of heat resistant spores.

Complementation of *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) resulted in low levels of spores.

To complement *C. perfringens* KM2 (*sigE*-), the entire operon of *spoIIG*, consisting of *spoIIGA* and *sigE*, and the upstream promoter was cloned into an *E. coli-C. perfringens* shuttle vector, resulting in the plasmid pKM3. pKM3 was then electroporated into *C. perfringens* KM2 (*sigE*-). Sporulation assays were performed and it was determined that *C. perfringens* KM2(pKM3) (complemented *sigE*-) sporulates at a level of about 1.7%, producing an average of 3.12 x 10^5 spores/ml. pKM3 was also electroporated into *C. perfringens* SM101 to determine if copy number would have an effect on sporulation efficiency. *C. perfringens* SM101(pKM3) had an average sporulation efficiency of about 22.6%, producing an average of 6.29 x 10^6 spores/ml (Fig. 2-2). The sporulation efficiency of complemented *C. perfringens* KM2(pKM3) (complemented *sigE*-) was 3,000 fold higher than *C. perfringens* KM2 (*sigE*-).

Figure 2-2. Sporulation efficiencies of *C. perfringens* SM101 and the sigma factor mutants, *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-), as well as the sporulation efficiencies of complementation vectors in mutants and *C. perfringens* SM101.



Cells from an overnight FTG culture were inoculated into DSSM and allowed to grow for 24 h before being plated to determine the number of viable cells and the number of heat resistant spores.

To complement *C. perfringens* KM1 (*sigK*-), the entire *sigK* gene, including the upstream promoter was ligated into an *E. coli-C. perfringens* shuttle vector, resulting in the plasmid pKM2. pKM2 was then electroporated into *C. perfringens* KM1 (*sigK*-). Sporulation assays were performed and it was determined that *C. perfringens* KM1(pKM2) (complemented *sigK*-) sporulates at a level of about 1.3%, producing an average of 2.63 x 10^5 spores/ml. pKM2 was also electroporated into *C. perfringens* SM101 to determine if copy number would have an effect on sporulation. *C. perfringens* SM101(pKM2) had an average sporulation efficiency of about 60% producing an average of 2.28 x 10^7 spores/ml (Fig. 2-2). These results indicate that sporulation increases about 4,000 fold from *C. perfringens* KM1 (*sigK*-) to the complemented *C. perfringens* KM1. *C. perfringens* KM1 (*sigK*-) complemented with pJV7 had a sporulation efficiency of about 1% (data not shown), indicating that *pilT* does not affect the complementation of *C. perfringens* KM1 (*sigK*-).

Due to the fact that the sporulation efficiencies of *C. perfringens* SM101 with the complementing plasmids were not lowered to around 1%, we determined that the lack of complementation in *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) is not due to a copy number problem.

A *sigK* mutation appears to have a dominant negative phenotype.

In an attempt to complement *C. perfringens* KM1 (*sigK*-), primers were designed to PCR amplify the *sigK* gene and the upstream promoter region. The product was cloned into an *E. coli-C. perfringens* shuttle vector, resulting in the plasmid pKM12. Upon sequencing the insert, it was found that a thymine base insertion after base pair 363

resulted in a frameshift mutation, changing the 122nd amino acid from an arginine to a stop codon (Fig. 2-3). This truncated σ^K is missing 112 amino acids from the C-terminus.

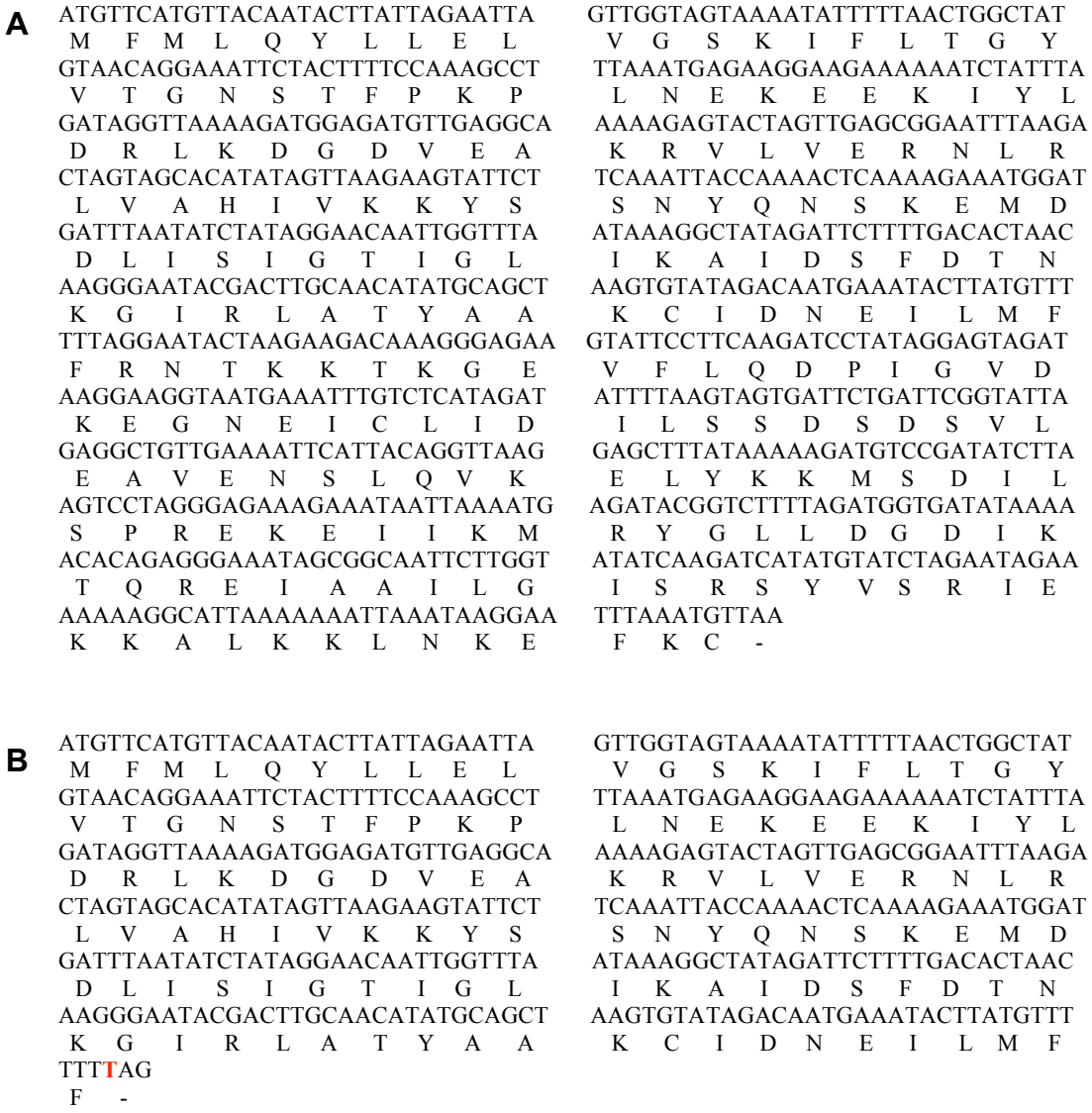
When pKM12 was electroporated into *C. perfringens* SM101 and a sporulation assay performed, it was found that the sporulation efficiency was below the limit of detection (data not shown), similar to what was observed in *C. perfringens* KM1 (*sigK*-). This suggests that a *sigK*- mutation exerts a dominant negative effect.

***C. perfringens* KM1 (*sigK*-) is blocked earlier in the sporulation cascade than *C. perfringens* KM2 (*sigE*-).**

Samples of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) were analyzed by TEM to determine how mutations in the *sigK* and *sigE* genes affected the development of the spore (Fig. 2-4). Cells were observed during mid-log phase, early stationary phase, and stationary phase. The phenotypes observed were quantified based on the stage of sporulation the cells were in (Fig. 2-5).

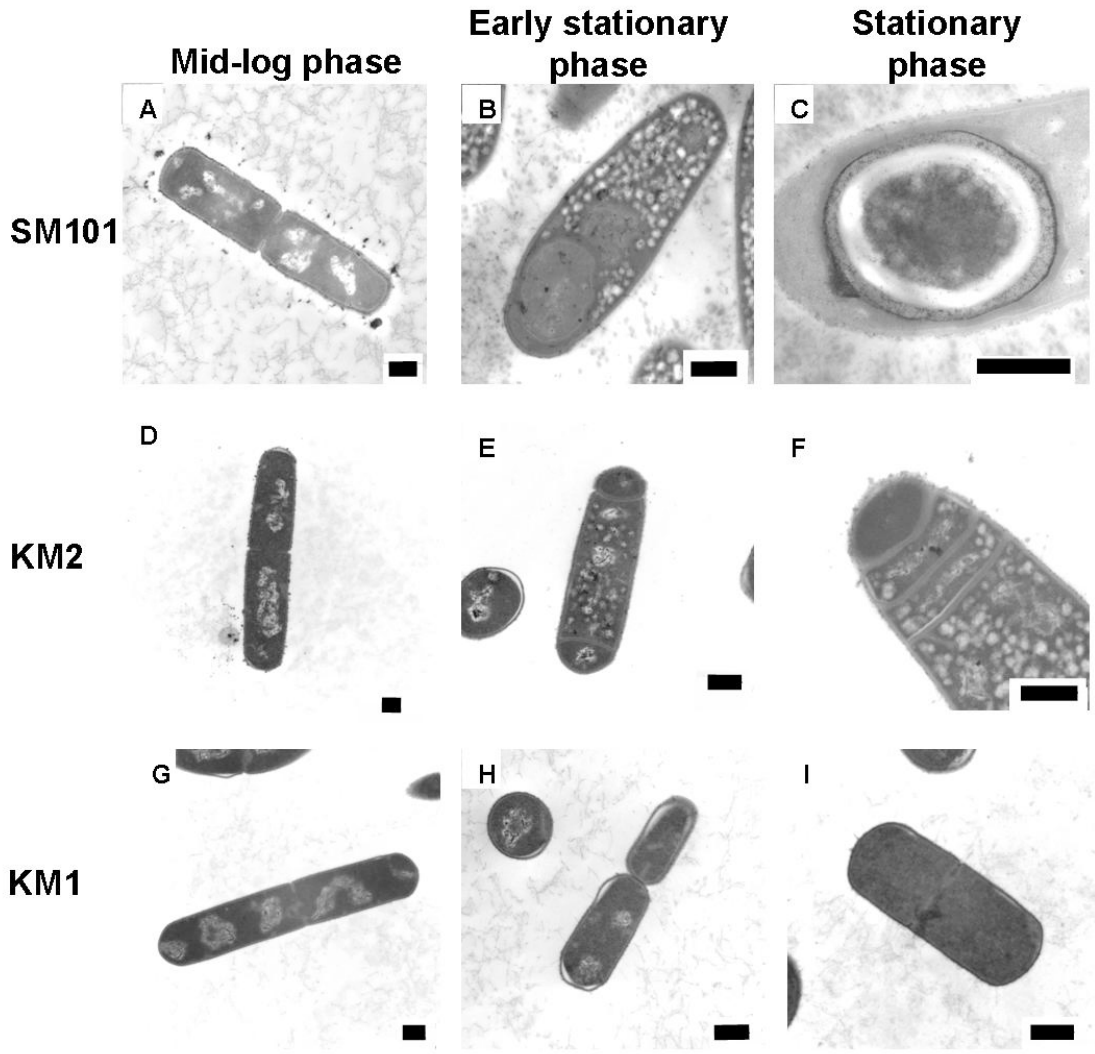
Samples of *C. perfringens* SM101 were analyzed during mid-log phase, early stationary phase, and stationary phase after inoculation into sporulation media, DSSM, to determine what stage of sporulation the cells were in (Fig. 2-4A-C and Fig. 2-5A). In the mid-log phase sample, the large majority of the cells were vegetative, although low numbers of cells had formed the asymmetric septa (stage II) and had initiated engulfment (stage III). In early stationary phase most cells were undergoing the process of engulfment, however some remained in the vegetative state. By stationary phase, the majority of cells had formed mature endospores (stage VI). While the cells were undergoing sporulation, granules were present in the mother cell (Fig. 2-4B) that we believe are either starch granules or polyhydroxybutyrate (PHB) inclusions.

Figure 2-3. Nucleotide and amino acid sequences of the wild-type and mutated *sigK* genes.



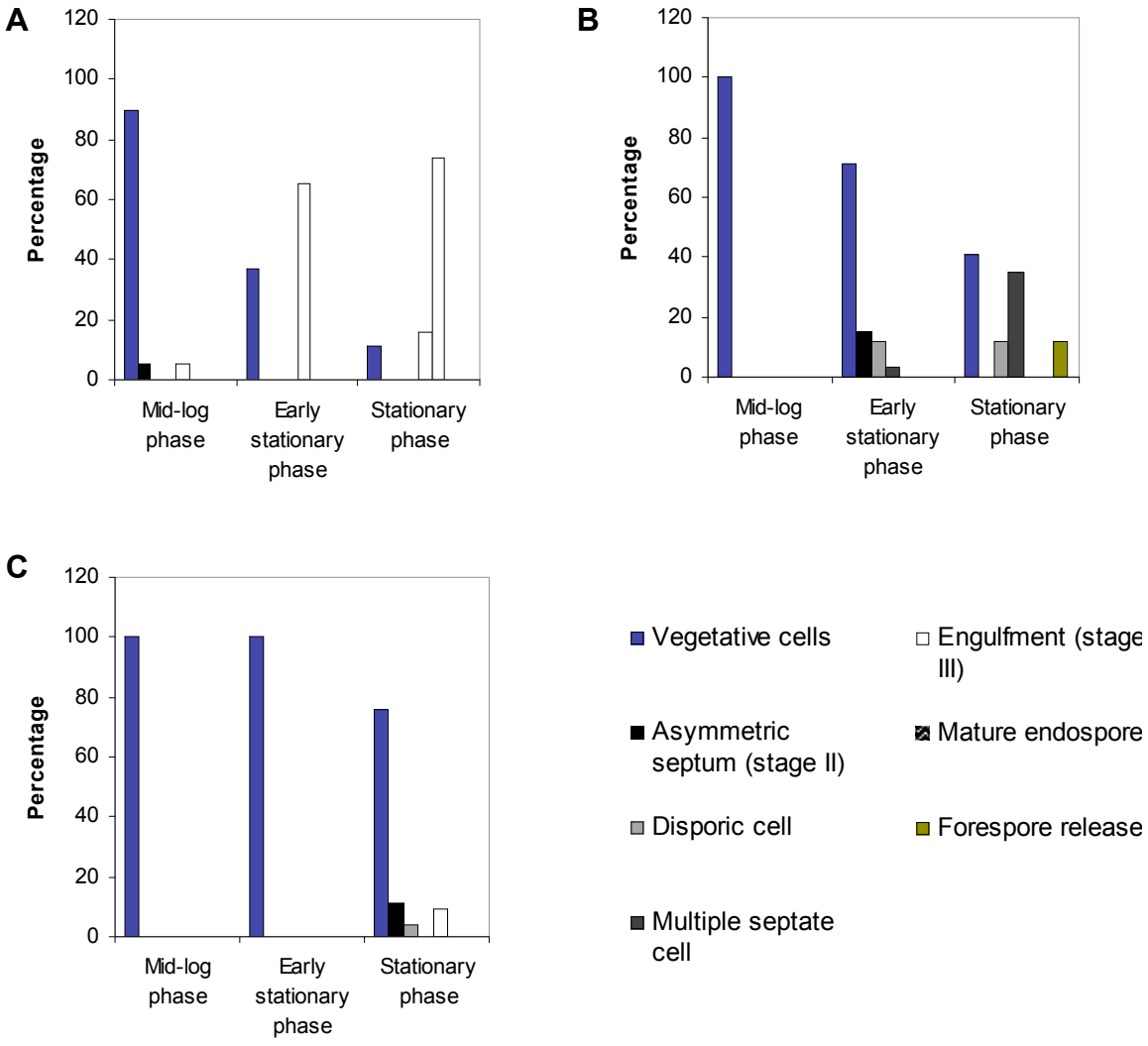
The nucleotide sequence of the *sigK* gene and the amino acid sequence of σ^K (A). The nucleotide sequence up to the premature stop codon in the mutated *sigK* gene and the amino acid sequence of the truncated σ^K (B). The red "T" indicates the inserted thymine that resulted in a frame shift mutation and a premature stop codon. The "-" sign denotes the stop codon.

Figure 2-4. Transmission electron micrographs comparing sporulation in *C. perfringens* strains SM101, KM2 (*sigE*-), and KM1 (*sigK*-).



Samples of sporulating cultures of *C. perfringens* strains SM101 (A-C), KM2 (*sigE*-) (D-F), and KM1 (*sigK*-) (G-I) were analyzed during mid-log phase (A, D, G), early stationary phase (B, E, H), and stationary phase (C, F, I) of growth. Bars in bottom right corners of electron micrographs represent 500 nm.

Figure 2-5. Comparison of cell morphologies of *C. perfringens* wild-type SM101 and the mutant strains, *C. perfringens* KM2 (*sigE*-) and *C. perfringens* KM1 (*sigK*-).



The phenotypes of *C. perfringens* SM101 (A), *C. perfringens* KM2 (*sigE*-) (B), and *C. perfringens* KM1 (*sigK*-) (C) during mid-log phase, early stationary phase, and stationary phase after inoculation into DSSM were quantified by observing only cells in which complete longitudinal sections were present.

Similar granules have been reported in other sporulating organisms and serve as an energy source for the cell during sporulation (28, 57, 64, 109).

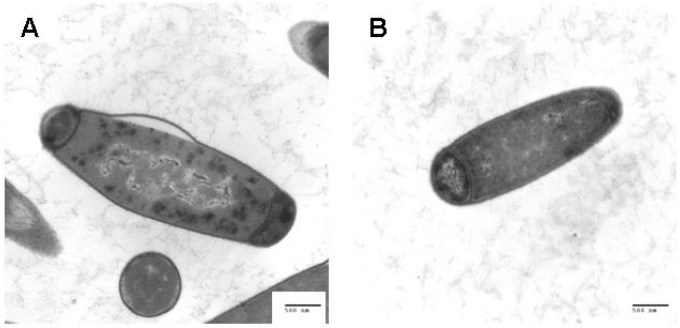
C. perfringens KM2 (*sigE*-) cells never developed beyond the asymmetric septa formation (Fig. 2-4D and 2-4F). During mid-log phase all of the cells viewed were in a vegetative state (Fig 2-5B). In the early stationary phase, cells were forming the asymmetric septa and some had the disporic phenotype characteristic of *sigE* mutants in *B. subtilis* (47). By the stationary phase, many cells had formed multiple asymmetric septa or were still vegetative. A few underdeveloped forespores appeared to be separating from the mother cell (Fig. 2-5B). Cells undergoing sporulation also had granules (Fig. 2-4E and 2-4F).

A mutation in the *sigK* gene prevented sporulation in the majority of cells (Fig. 2-4G-I). In *C. perfringens* KM1 (*sigK*-), during mid-log phase and early stationary phase, all of the cells were in a vegetative state (Fig. 2-5C). Most of the cells were still vegetative at stationary phase; however, a low number of cells had formed an asymmetric septum (stage II), were disporic, or had completed engulfment (stage III) (Fig. 2-5C). Although some cells observed during this time point were sporulating, very few had granules (Fig. 2-4G-I).

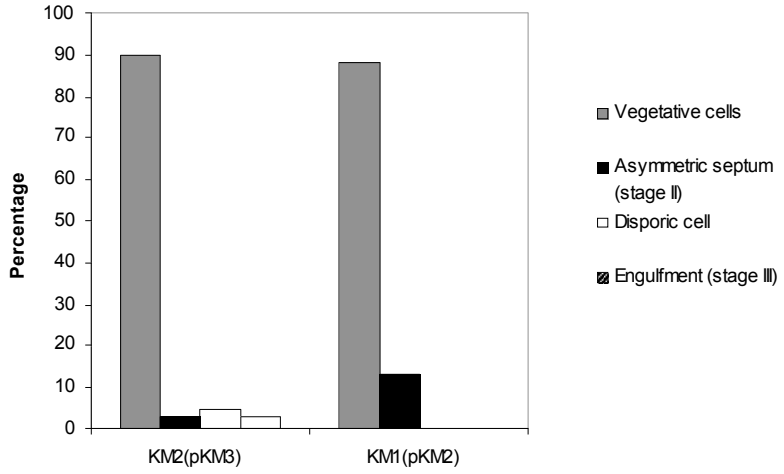
Complemented *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) are mostly vegetative cells and do not form granules when sporulating.

C. perfringens KM1(pKM2) (complemented *sigK*-) and *C. perfringens* KM2(pKM3) (complemented *sigE*-) were observed using transmission electron microscopy during stationary phase (Fig. 2-6A and 2-6B). The majority of cells viewed were vegetative cells. In *C. perfringens* KM2(pKM3) (complemented *sigE*-) low

Figure 2-6. Transmission electron micrographs depicting sporulation in the complemented strains of *C. perfringens* KM2 (*sigE*-) and *C. perfringens* KM1 (*sigK*-) and comparison of cellular morphologies of these strains.



C



Sporulating *C. perfringens* KM2(pKM3) (complemented *sigE*-) (A) and *C. perfringens* KM1(pKM2) (complemented *sigK*-) (B) were observed during stationary phase. Bars in bottom right corners represent 500 nm. The phenotypes of cells were quantified by observing only cells in which complete longitudinal sections were present (C).

numbers of cells had formed the asymmetric septum (stage II), were disporic, or had completed engulfment (stage III) (Fig. 2-6C). In *C. perfringens* KM1(pKM2) (complemented *sigK*-) 13% of cells viewed had formed the asymmetric septum (stage II) (Fig. 2-6C). No granules were present in any of the cells observed (Fig. 2-6A and 2-6B).

Reconstruction of the *sigK* mutant had similar sporulation results to *C. perfringens* KM1 (*sigK*-).

Because *C. perfringens* KM1 (*sigK*-) does not appear to be blocked at the same stage of sporulation (stage III) as a *B. subtilis sigK* mutant and that, when complemented, only a low percentage of cells formed spores, there may be a second spontaneous mutation affecting *C. perfringens* KM1 (*sigK*-). Therefore, the *sigK* mutant was regenerated by electroporation of 65 μg pNLDK into *C. perfringens* SM101. Transformants were selected by growth on BHI containing erythromycin. Erythromycin resistant colonies were screened by Southern blot analysis to confirm that pNLDK had inserted into the *sigK* gene.

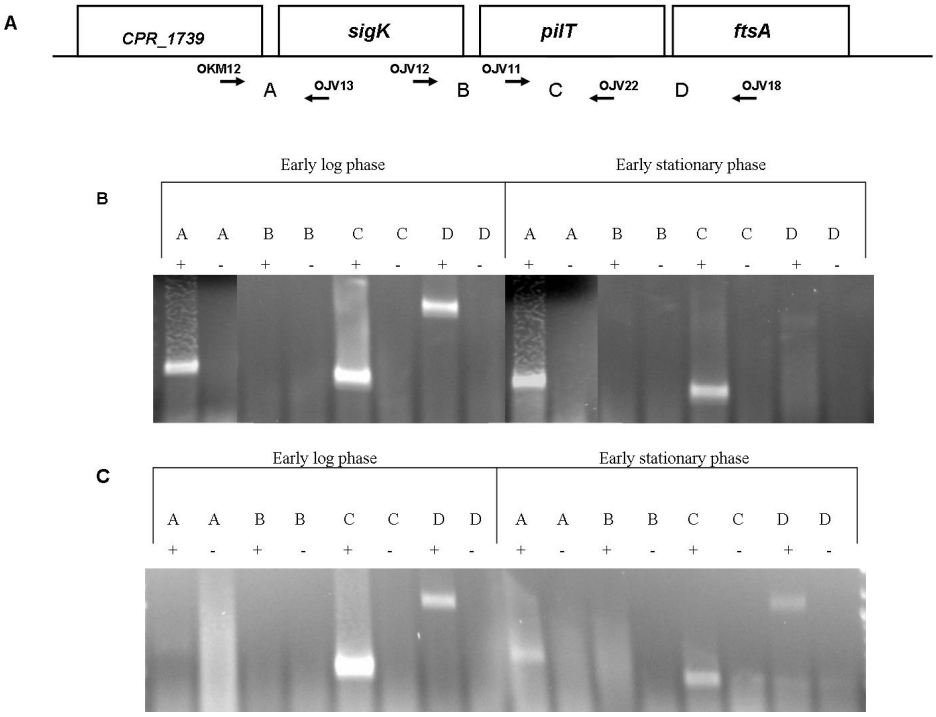
Nine *sigK* mutants from two electroporations were constructed. In all nine, sporulation was below the limits of detection after incubation in DSSM for 24 h (data not shown). The nine *sigK* mutants complemented with pKM2 were found to have an average sporulation efficiency of 2.8% (data not shown), similar to the original *sigK* mutant sporulation efficiency of 1.3%. These results indicate that the *C. perfringens* KM1 (*sigK*-) phenotype is either a true *sigK*- mutant phenotype and not the result of a secondary mutation or the mutagenesis procedure is causing the same secondary mutation in all the *sigK* mutants.

A mutation in the *sigK* gene of *C. perfringens* KM1 (*sigK*-) does not have polar effects on downstream genes.

To characterize the transcription of genes surrounding *sigK* (Fig. 2-7A), RT-PCR was performed using RNA extracted from sporulating *C. perfringens* SM101 cultures during early log phase and early stationary phase (Fig. 2-7B). A product indicated that a transcript is present in that region. It was found that in *C. perfringens* SM101 there is a transcript between *CPR_1739* and *sigK*. There is no transcript between *sigK* and *pilT*, indicating that *pilT* is under the control of its own promoter. *pilT*, whose gene product is an ATPase necessary for motility (127), is transcribed in sporulating cells of *C. perfringens* SM101. It was also determined that *pilT* and *ftsA* are cotranscribed in *C. perfringens* SM101. *ftsA* is upstream of *ftsZ* and the two genes are located in an operon in *B. subtilis* (4). *ftsZ* mutants in *B. subtilis* do not form the asymmetric septum (4). If the mutagenesis of *sigK* had an effect of the downstream genes, we hypothesized that the lack of transcription of *ftsA* could be a reason for the asporic phenotype.

RNA was extracted from sporulating cultures of *C. perfringens* KM1 (*sigK*-) during early log phase and early stationary phase and the transcription of genes in the *sigK* region was analyzed (Fig. 2-7A and 2-7C). There was a product present between *CPR_1739* and *sigK* indicating that there is a transcript present, although it was very faint in the early log phase sample. As in *C. perfringens* SM101, there is no transcript between *sigK* and *pilT*. A product was also present in *pilT* and between *pilT* and *ftsA*, indicating that a mutation in the *sigK* gene does not have a polar effect on the downstream genes and that σ^K-associated RNA polymerase is not required for transcription of *pilT* and *ftsA*.

Figure 2-7. Transcription of genes in the *sigK* region in *C. perfringens* SM101 and *C. perfringens* KM1 (*sigK*-).



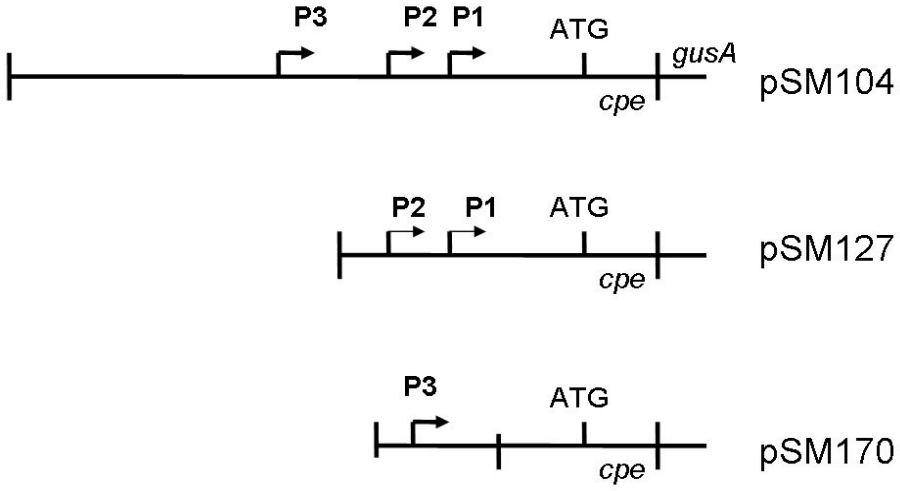
The location of genes surrounding the *sigK* gene (A). Letter "A" indicates the region between primers OKM12 and OJV13, "B" between OJV12 and OJV22, "C" between OJV11 and OJV22, and "D" between OJV11 and OJV18. RNA from *C. perfringens* SM101 (B) and *C. perfringens* KM1 (*sigK*-) (C) was harvested during early log and early stationary phases after inoculation into DSSM. Letters indicate which primer pairs were used in the reaction. "+" signs indicate reactions in which reverse transcriptase was added to the reaction while "-" signs indicate negative controls.

Both σ^E and σ^K are necessary for transcription of the *cpe* gene.

In previous studies, Zhao and Melville (138) found that there are three promoters for *cpe*, and based on consensus recognition sequences for σ^E- and σ^K-associated RNA polymerase in *B. subtilis*, are possibly σ^E- and σ^K-dependent in *C. perfringens*. Plasmids containing *cpe* promoter region(s) fused to the *E. coli* reporter gene *gusA* are described previously (Fig. 2-8) (82, 138) . These plasmids were electroporated into *C. perfringens* strains SM101, KM1 (*sigK*-) and KM2 (*sigE*-). Sporulation was induced by growing the cells in DSSM and samples removed to measure β-glucuronidase activity.

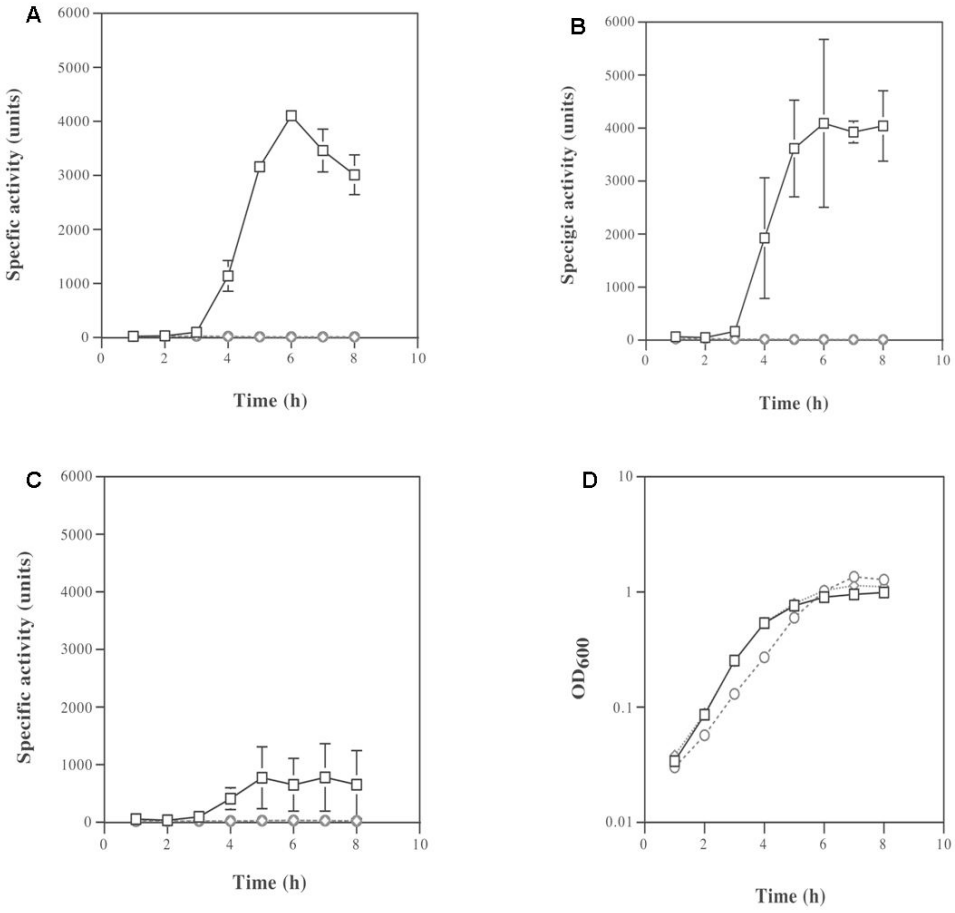
The activities of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) with plasmids containing *cpe-gusA* fusions are shown in figure 2-9. The activities of the construct containing all three *cpe* promoters (pSM104) and the construct containing P1 and P2 (pSM127) had similar levels of β-glucuronidase activity in *C. perfringens* SM101 (Fig. 2-9A and 2-9B). The construct containing only P3 (pSM170) had 19% the activity of *C. perfringens* SM101(pSM104) (Fig. 2-9C). In all three *cpe-gusA* constructs, an induction of β-glucuronidase activity occurred between 3 h and 4 h after inoculation into sporulation medium DSSM, when cells were between mid-log phase and early stationary phase (Fig. 2-9D). These results are similar to previous results that suggest that most of *cpe* is transcribed from P1 and P2 (138). There was no β-glucuronidase activity detected for pSM104, pSM127, or pSM170 in *C. perfringens* KM1 (*sigK*-) or *C. perfringens* KM2 (*sigE*-). These results indicate that σ^E and σ^K are needed for transcription of the three *cpe* promoters.

Figure 2-8. Schematic diagram of the *C. perfringens cpe* promoter fusions to *E. coli gusA*.



Based on: (138).

Figure 2-9. Expression of the *cpe* promoter-*gusA* fusions in wild-type *C. perfringens* SM101 and *C. perfringens* KM1 (*sigK*-) and KM2 (*sigE*-) mutant strains.



Transcription from the *cpe* promoters was measured by the specific activity of β-glucuronidase. Expression of pSM104 (A), pSM127 (B), and pSM170 (C) in wild-type *C. perfringens* SM101 (squares), *C. perfringens* KM1 (*sigK*-) (diamonds), and *C. perfringens* KM2 (*sigE*-) (circles). Representative growth curves (D) of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) in DSSM.

Discussion

C. perfringens produces an enterotoxin when sporulating that is a major causative agent for food poisoning (81). Previous work has indicated that there are three promoters upstream of *cpe* that are σ^E- and σ^K-associated RNA polymerase specific (138). σ^E and σ^K are active in the mother cell of *B. subtilis* and are essential for the formation of spores. Mutations were introduced into *sigE* (*C. perfringens* KM2) and *sigK* (*C. perfringens* KM1) genes of *C. perfringens* SM101. Before the transcription of *cpe* could be studied, sporulation of *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) had to be characterized.

We have found that KM2 (*sigE*-) and KM1 (*sigK*-) strains of *C. perfringens* do not produce detectable amounts of spores. When complemented, sporulation is not fully restored, but the sporulation efficiency does increase 3,000 to 4,000 fold to about 1%. When a mutated *sigK* gene that resulted in a truncated σ^K protein upon translation was electroporated into *C. perfringens* SM101, sporulation was inhibited. These results indicate that the truncated σ^K acts antagonistically to wild-type *sigK*, a phenotype typical of a dominant negative effect. We can apply this to *C. perfringens* KM1 (*sigK*-) and hypothesize that the mutation made to create *C. perfringens* KM1 (*sigK*-) has a partial-dominant negative effect over the wild-type copy of the gene present in pKM2, which is why sporulation is not fully restored. Because pKM2 is a multicopy number plasmid, a low percentage of cells are able to overcome the dominant negative effect and sporulate.

Electron microscopy allowed for a phenotypic analysis of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-). *C. perfringens* SM101 begins sporulation during mid-log phase, very early when compared to *B. subtilis*. *B. subtilis* cells do not

initiate sporulation until stationary phase (42). By early stationary phase in *C. perfringens* SM101, the majority of cells were already undergoing engulfment (stage III). The phenotype of *C. perfringens* KM2 (*sigE*-) is similar to that of a *B. subtilis spoIIGB*- mutant, which is characterized by multiple septa formed at the poles of the cell. In *B. subtilis* this phenotype is caused because three σ^E-directed genes, *spoIID, spoIIM*, and *spoIIP*, whose gene products are responsible for blocking the formation of multiple septa are not transcribed (25). Unexpectedly, the phenotype of *C. perfringens* KM1 (*sigK*-) showed a block in spore formation before the block characterized by σ^E mutants. A possible explanation for the phenotype of *C. perfringens* KM1 (*sigK*-) may be due to an artifact of a mutation of a gene upstream in the sporulation cascade that is not 100% effective, which is why 9% of the cells had developed a forespore and appeared to be blocked at a similar stage as *sigK*- mutants in *B. subtilis*.

C. perfringens KM1 (*sigK*-) also has very few cells in which granules were present, which may indicate an early block on sporulation. The formation of granules in sporulating cells has been observed in *Bacillus cereus*, *Clostridium botulinum,* and *Clostridium butyricum* (28, 57, 64, 109). Starch and polyhydroxybutyrate (PHB) granules have been found to accumulate during sporulation but are degraded by the time the mature spore is formed and have been hypothesized to serve as an energy supply for the developing spore (57).

Due to the fact that *C. perfringens* KM1 (*sigK*-) appeared atypical when compared to *B. subtilis sigK* mutants, it was remade using pNLDK. The nine resulting transformants produced less than 10 spores/ml and when complemented had an average of 2.8% sporulation, similar to what was observed in the original *sigK* mutant, *C.*

perfringens KM1. Due to the low complementation levels, we believe that if there is a secondary mutation in an upstream gene in the sporulation cascade, it is common in all of the *sigK* mutants.

There was a concern that a mutation in the *sigK* gene was having a polar effect on the genes downstream of it, *pilT* and *ftsA*. Although PilT is necessary for motility in *C. perfringens* (127) and not necessarily a concern for the atypical phenotype exhibited by *C. perfringens* KM1 (*sigK*-), the downstream *ftsA-Z* operon is involved with asymmetric polar septation in *B. subtilis* (4) and could have been an explanation for the very early block in sporulation observed in *C. perfringens* KM1 (*sigK*-). RT-PCR results indicated that these genes are transcribed in *C. perfringens* KM1 (*sigK*-); therefore there is no polar effect downstream from the mutagenesis of the *sigK* gene. We also wanted to determine if there was an upstream polar effect on *CPR_1739* whose gene product is annotated as a penicillin binding protein transpeptidase domain protein. There appears to be reduced transcription of this product in *C. perfringens* KM1 (*sigK*-) when compared to *C. perfringens* SM101. This could be due to a polar effect, or possibly that there is read-through from *CPR_1739*, which may be a sporulation specific gene that is not being transcribed efficiently due to non-functional σ^K.

Results from the promoter fusions to *gusA* indicate that transcription of *cpe* is dependent on σ^E and σ^K. This coincides with the fact that there was no sporulation detected in *C. perfringens* KM1 (*sigK*-) and *C. perfringens* KM2 (*sigE*-) and that enterotoxin production is always associated with sporulation.

Acknowledgements

I would like to acknowledge John Varga, Yuling Zhao, and Stephen Melville for constructing many of the vectors used in this study and Kathy Lowe for assistance with the electron microscopy.

Chapter III. Regulation of σ^E and σ^K in *Clostridium perfringens*

Abstract

Clostridium perfringens and *Bacillus subtilis* are Gram-positive organisms that undergo differentiation from an actively growing vegetative cell into a dormant spore. The regulation of sporulation sigma factors in *B. subtilis* has been well studied and is known to be a very tightly controlled process. The regulation of sporulation has not been studied in depth in *C. perfringens* as it has been in *B. subtilis.* However, because *C. perfringens* and *B. subtilis* share many homologous genes associated with sporulation, *B. subtilis* has served as a model for the study of *C. perfringens* sporulation. Sporulation in *B. subtilis* is regulated by four sporulation specific sigma factors, σ^F, σ^E, σ^G, and σ^K. The results reported here provide evidence that sporulation regulation involving the mother cell sigma factors, σ^E and σ^K, may in fact be different in these two organisms.

Introduction

The molecular regulation of sporulation in *Clostridium perfringens* has not been studied in detail as it has in *Bacillus subtilis*, which serves as a model for sporulation in Gram-positive organisms. Spore formation in *B. subtilis* involves the formation of an asymmetric septum that divides the cell into the forespore and the mother cell after the organism reaches a nutrient starved state (96). It has been observed that in *C. perfringens* the morphological events, which are divided into seven stages (I-VII), during the formation of the spore are similar to those of *B. subtilis* (42, 64). In *B. subtilis* four sporulation specific sigma factors, σ^F, σ^E, σ^G, and σ^K, regulate the sporulation process (42, 72). Homologues of these genes have been found in *C. perfringens*.

In *B. subtilis*, σ^F and σ^G regulate gene expression in the forespore while σ^E and σ^K control gene expression in the mother cell. These sigma factors are activated in a linear fashion, with σ^F active first, followed by σ^E, σ^G, and lastly σ^K (72). In *B. subtilis* the σ^F encoding gene, *spoIIAC*, is transcribed by σ^H-associated RNA polymerase before initiation of sporulation (35, 132). The σ^E encoding gene, *spoIIGB*, is also transcribed before asymmetric septation, however transcription also occurs after septum formation in *B. subtilis* (35, 44). *spoIIGB* is transcribed by σ^A-associated RNA polymerase, a sigma factor mainly associated with the transcription of housekeeping genes (1, 39). Transcription of *spoIIIG*, the gene encoding σ^G, is transcribed by σ^F- and σ^G-associated RNA polymerase (121), while *sigK*, the structural gene encoding σ^K, is transcribed by σ^E- and σ^K-associated RNA polymerase (62, 89). The activation of *sigK* transcription is also regulated by the DNA binding protein SpoIIID (62, 118). *spoIIID* is transcribed in the mother cell by σ^E-associated RNA polymerase (61, 123).

In *B. subtilis* each of the four sporulation sigma factors are regulated post-transcriptionally. σ^F and σ^G are held inactive by the anti-sigma factor SpoIIAB (23, 32). σ^E and σ^K are first translated as inactive pro-proteins that only become active after proteases cleave amino acids from the N-terminal of the proteins (66, 118).

We have previously found that through electron microscopic examination of sporulating cultures of the *C. perfringens sigK* and *sigE* mutant strains (Chapter II) that sporulation regulation in *C. perfringens* may be different than in *B. subtilis*. Electron micrographs revealed that in the *sigK* mutant strain, *C. perfringens* KM1, sporulation was blocked before the formation of the asymmetric septum. In *B. subtilis*, a *sigK* mutant is blocked in sporulation after engulfment of the forespore by the mother cell (96). Sporulation in the *sigE* mutant, *C. perfringens* KM2, was halted at the formation of the asymmetric septum and exhibited a disporic phenotype, consistent with observations in *B. subtilis spoIIGB* mutants (47). If σ^K is an earlier acting sigma factor than σ^E in *C. perfringens*, this would have a dramatic effect on the regulation of sporulation when compared to *B. subtilis*. To investigate regulation of σ^E and σ^K, we observed the transcriptional activity of their promoters, as well transcription of the sporulation sigma factor encoding genes. The pro-protein processing of pro-σ^E and pro-σ^K was also investigated to determine when the mother cell sigma factors are activated. The regulation of SpoIIID was studied as it is an intermediate in sporulation regulation between σ^E and σ^K. Through these results we demonstrated that σ^K may be an early acting sigma factor and that the timing and regulation of sporulation sigma factors are different in *C. perfringens* when compared to *B. subtilis*.

Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 3-1. All *C. perfringens* strains were grown anaerobically in PGY medium (30 g proteose peptone #3, 20 g glucose, 10 g yeast extract, 1 g of sodium thioglycolate per liter) or BHI (brain-heart infusion) (Difco) at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) with 30 μg/ml erythromycin and 20 μg/ml chloramphenicol added as needed. Luria Bertani (LB) medium (10 g tryptone, 10 g NaCl, 5 g yeast extract, and 15 g agar as needed per liter) was used to grow *E. coli* at 37°C on plates or in broths with shaking. As needed, 300 μg/ml erythromycin and 20 μg/ml chloramphenicol were added to the media.

Plasmid constructs

To determine if the region upstream of *spoIIGA*, the promoter-proximal gene in the *spoIIG* operon, can function as a promoter, a PCR product containing 199 bp upstream of *spoIIGA* and the first 39 bp of the *spoIIGA* gene was ligated upstream of the β-glucuronidase encoding gene, *gusA*, in the *E. coli-C. perfringens* shuttle vector pSM240, to create pKM4 (Table 3-1). The region was amplified by PCR using the oligonucleotide primers OKM14 (5'-CTAATAT*CTGCAG*GTCAATATATACAG-3') and OKM15 (5'-GAACTTATA*GTCGAC*GAATACATTTTCATTG-3'). OKM14 had a PstI restriction site and OKM15 had a SalI restriction site designed into the primer.

To determine if there was a promoter located upstream of *sigK*, oligonucleotide primers OSM125 (5'-CGTTCCCCCT*CTGCAG*GCTTATTACTATAATATG-3') and OSM139 (5'-CCTATTTTCA*GTCGAC*TAGTTTTAGAAC-3') were used to amplify a

Table 3-1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
E. coli		
DH10B	F- *mcrA* Δ(*mrr-hsdRMSmcrBC*) F80d *lacZ* ΔM15 *lacX74 deoR recA1 araD139* D (*ara*, *leu*)7697 *galU galK* λ*-rpsL endA1 nupG*	Gibco/BRL
C. perfringens		
SM101	High efficiency of electroporation derivative of NCTC 8798	(138)
KM1	*sigK*- mutant of SM101	This study
KM2	*sigE*- mutant of SM101	This study
Plasmids		
pSM240	*E. coli*-*C. perfringens* shuttle vector containing *E. coil gusA* gene, chloramphenicol resistance	This study
pKM4	*spoIIG* promoter region fused to pSM240	This study
pSM242	*sigK* promoter region fused to pSM240	This study

78 bp region upstream of the *sigK* gene. OSM125 had a PstI restriction site and OSM139 had a SalI restriction site designed into the primer. This product was then digested and ligated into pSM240, resulting in the plasmid pSM242 (Table 3-1).

Construction of plasmids used in the complementation of the *sigE* mutant, *C. perfringens* KM2, and the *sigK* mutant, *C. perfringens* KM1, were described previously (Chapter II).

β-glucuronidase assays

β-glucuronidase assays in *C. perfringens* were performed as previously described (Chapter II). Briefly, the optical density at 600 nm of sporulating *C. perfringens* cultures were measured hourly for 8 h using a Genesys 10 uv scanning spectrophotometer as 1 ml samples were removed and stored at -20^{o}C. Cells resuspended in buffer and toluene were mixed with p-nitrophenyl β-D-glucuronidate (PNPG) until a yellow color developed. The reaction was then terminated with sodium carbonate and the absorbance at 405 nm measured. The specific activity (in units) was calculated using the following equation:

Specific activity = (1000 x A_{405})/(Time [in minutes] x OD_{600} x culture volume [in milliliters])

The means of triplicate samples are shown in order to represent the promoter activity of the *spoIIG* operon and the *sigK* gene.

Extraction of RNA

Total RNA was extracted from sporulating cultures of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) as previously described (Chapter II). The optical density at 600 nm of sporulating *C. perfringens* cultures were measured using a Genesys 10 uv scanning spectrophotometer and 50 ml of culture was collected at optical densities of 0.1 nm and 1.0 nm representing early log and early stationary phase of growth, respectively. RNA was extracted using TRIzol reagent (Invitrogen) according to the

manufacturer's protocol. Chromosomal DNA was removed from the RNA by the use of RQ1 DNase (Promega) following the manufacturer's instructions. RNA was stored in liquid nitrogen until use.

Reverse transcription PCR (RT-PCR)

RT-PCR was performed on RNA extracted from *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-). To determine the transcriptional activity of sporulation sigma factor encoding genes, oligonucleotide primers were designed internal to the genes (Table 3-2). Oligonucleotide primers OSM166 and OSM167 were used to amplify a 487 bp region of *sigF*. To amplify a 433 bp fragment of *sigE*, OSM168 and OSM169 were used. OSM170 and OSM171 were utilized to amplify a 431 bp fragment of *sigG* and OSM172 and OSM173 were used to amplify a 382 bp fragment of *sigK*. It is important to note that OSM 168 and OSM169 were used in the mutagenesis of the *sigE* gene in *C. perfringens* SM101 to create *C. perfringens* KM2 (*sigE*-) and that OSM172 and OSM173 were used in the mutagenesis of the *sigK* gene to create *C. perfringens* KM1 (*sigK*-) (Chapter II).

To determine if *spoIIID* was co-transcribed with the upstream gene, *CPR_2157*, the oligonucleotide primer OKM34 was designed 248 bp upstream of the stop codon in the *CPR_2157* gene and OKM23 was designed 49 bp downstream of the start codon in *spoIIID* (Table 3-2). A RT-PCR product would indicate that there is a transcript extending between these two genes.

cDNA synthesis was performed by reverse transcription of 2 μg RNA with 50 pmol of each primer using the Access RT-PCR kit (Promega), following the manufacturer's instructions. RT-PCR reactions set up without reverse transcriptase made

Table 3-2. Oligonucleotide primer sequences used in RT-PCR experiments.

Primer name	Sequence (5' – 3')	Gene Targeted
OSM166	GAGGGTGAGCTCATGAGGATATATTTCAG	*sigF*
OSM167	GAAATAGGTACCCATTATGATTTGTTTACCC	*sigF*
OSM168	GAAGCTGAGCGCAGTATTCTTATTGAGAG	*sigE*
OSM169	CCACATGGTACCAATCTTAATTCAACAATTTC	*sigE*
OSM170	GAGAGGAGCTCTTAAGGGGAATCTGAGATTAG	*sigG*
OSM171	CATATATTGCATCTCCACCATCATAATAAATAGG	*sigG*
OSM172	GTTGAGGAGCTCTTAAGACTAGTAGCAC	*sigK*
OSM173	GGACTTGGTACCTCTGACATCTTTTTATAA	*sigK*
OKM23	GCTGTTTTTCTTATAGTAGCCC	*spoIIID*
OKM34	GAAGGTTGCTTTGTTAAAATAGAGCATCAAAATGG	*spoIIID*

it possible to verify the absence of contaminating DNA. All RT-PCR reactions were performed in duplicate.

Antisense RNA detection

To determine if antisense RNA was transcribed in *C. perfringens* KM2 (*sigE*-) and *C. perfringens* KM1 (*sigK*-), RT-PCR was performed. First, one primer was added for reverse transcription of the mRNA template to cDNA. The reverse transcriptase was then heat killed and the second primer added for the second strand synthesis of cDNA and amplification of the product. Reactions were set-up using wild-type *C. perfringens* SM101 as a control. The antisense strand of RNA was detected by the addition of OSM168 for *sigE* and OSM172 for *sigK* during the reverse transcription reaction. The sense strand of RNA was detected by the addition OSM169 for *sigK* and OSM173 for *sigE* in the cDNA synthesis reaction.

Western Blot Analyses

Samples were grown overnight at 37^{o}C in 5 ml FTG medium. Cells were then subcultured at a 1% inoculum into 75 mls prewarmed DSSM in 100 ml serum bottle with rubber stoppers. Over a time period beginning 1 h after inoculation into DSSM 1 ml of sample was collected and the optical density at 600 nm measured. One ml of sample was also collected at these time points and stored at -80^{o}C until shipped on dry ice to Lee Kroos's lab at Michigan State University where western blots were performed as previously described (73).

Results

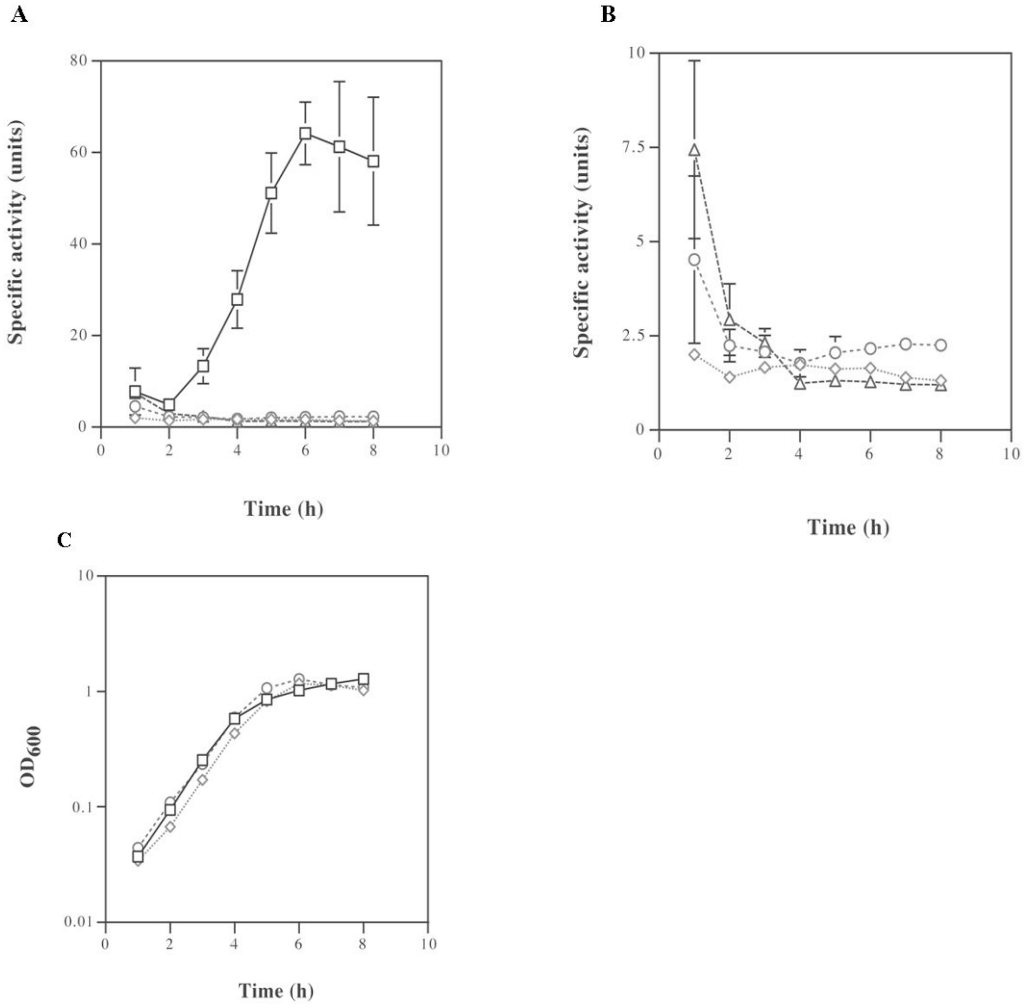
Transcription from the *spoIIG* promoter is eliminated in *C. perfringens* KM2 (*sigE*-) and *C. perfringens* KM1 (*sigK*-).

To investigate the activity of the *spoIIG* promoter, the upstream region of *spoIIGA* was fused to the promoterless *gusA* gene (pKM4). The activities of *C. perfringens* strains SM101, KM2 (*sigE*-), and KM1 (*sigK*-) with pKM4 are shown in figure 3-1. Strains were induced to sporulate by inoculation into DSSM and samples were removed to measure β-glucuronidase activity. In *C. perfringens* SM101 the specific activity increased drastically between 2 and 3 hours (Fig. 3-1A), which correlates with early to mid-log growth phase (Fig. 3-1C). The activity of the *spoIIG* promoter was not above background levels of pSM240 in *C. perfringens* KM2 (*sigE*-) and *C. perfringens* KM1 (*sigK*-) (Fig. 3-1B), indicating that σ^E- and σ^K-associated RNA polymerase are necessary for transcription of the *spoIIG* operon.

Transcription from the *sigK* promoter is reduced in *C. perfringens* KM1 (*sigK*-) and eliminated in *C. perfringens* KM2 (*sigE*-).

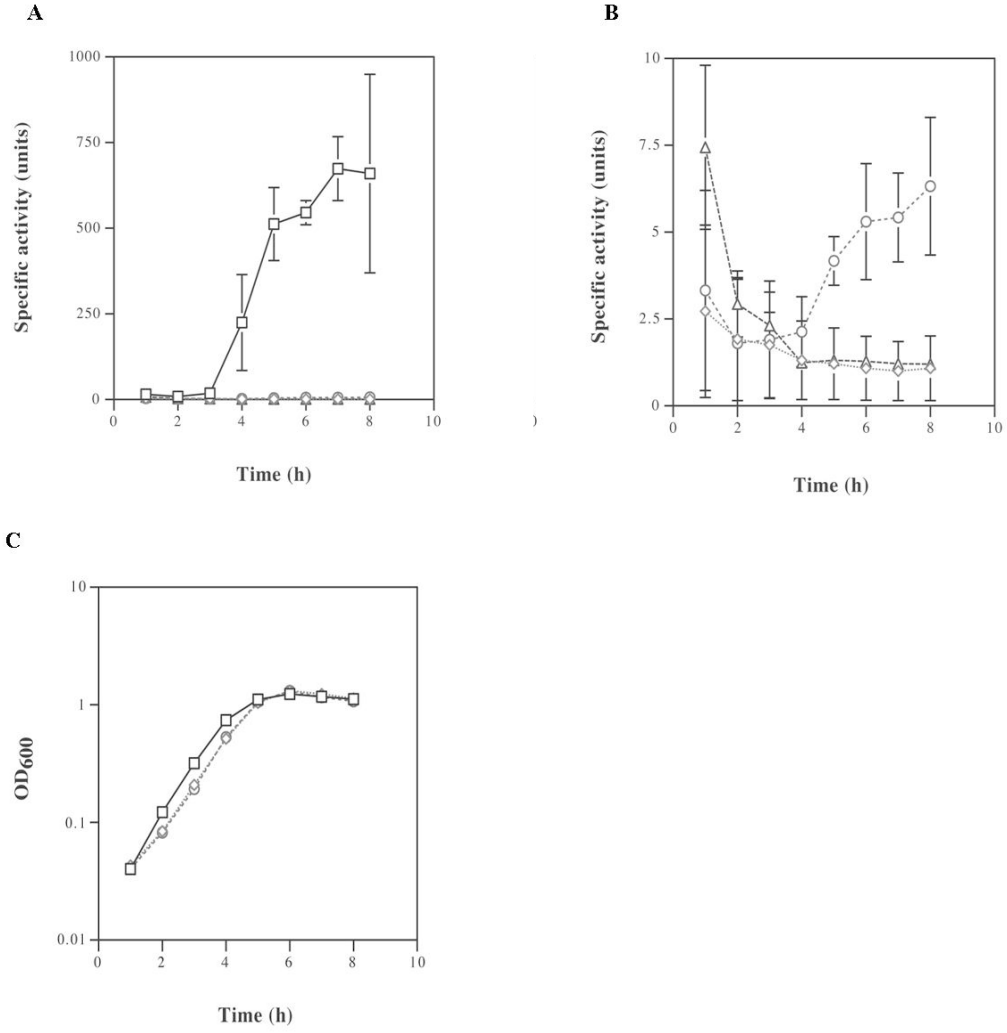
The promoter activity of *sigK* in *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) was investigated with regard to β-glucuronidase activity, expressed by the *gusA* reporter gene fused to the promoter region of *sigK* on a plasmid (pSM242) (Fig. 3-2). *C. perfringens* SM101(pSM242) showed induction of activity between 3 and 4 hours after inoculation into sporulation media (Fig. 3-2A), corresponding to late log to early stationary growth phase (Fig. 3-2C). The activity of pSM242 was reduced around 100 fold in *C. perfringens* KM1 (*sigK*-), indicating that σ^K-associated RNA polymerase does transcribe most of the *sigK* gene. The activity of pSM242 was not above background

Figure 3-1. Expression of the *spoIIG* promoter-*gusA* fusion in wild-type *C. perfringens* SM101 and *C. perfringens* KM1 (*sigK*-) and KM2 (*sigE*-) mutant strains.



β-glucuronidase assays were performed to determine when the *spoIIG* promoter is active in *C. perfringens* SM101 and if the promoter is active in *C. perfringens* KM1 (*sigK*-) and KM2 (*sigE*-) strains. (A) β-glucuronidase activities of *spoIIG-gusA* fusion in *C. perfringens* strains SM101 (squares), KM2 (diamonds), KM1 (circles), and SM101with the plasmid backbone, pSM240 (triangles). (B) β-glucuronidase activities of *spoIIG-gusA* fusion in *C. perfringens* strains KM2 (diamonds), KM1 (circles), and the plasmid backbone (pSM240) in SM101 (triangles). (C) Growth curves of *C. perfringens* strains SM101 (squares), KM2 (diamonds), and KM1 (circles) containing the *spoIIG-gusA* fusion.

Figure 3-2. Expression of the *sigK* promoter-*gusA* fusion in *C. perfringens* SM101, KM1 (*sigK*-), and KM2 (*sigE*-) strains.



β-glucuronidase assays were performed to determine when the *sigK* promoter is active in *C. perfringens* SM101 and if the promoter is active in *C. perfringens* KM1 (*sigK*-) and KM2 (*sigE*-) strains. (A) β-glucuronidase activities of the *sigK-gusA* fusion in *C. perfringens* strains SM101 (squares), KM2 (diamonds), KM1 (circles), and the SM101(pSM240) strain (triangles). (B) β-glucuronidase activities of the *sigK-gusA* fusion in *C. perfringens* strains KM2 (diamonds), KM1 (circles), and SM101(pSM240) (triangles). (C) Growth curves of *C. perfringens* strains SM101 (squares), KM2 (diamonds), and KM1 (circles) containing the *sigK-gusA* fusion.

levels in *C. perfringens* KM2 (*sigE*-) (Fig. 3-2B), indicating that σ^E-associated RNA polymerase is necessary for transcription of the *sigK* gene.

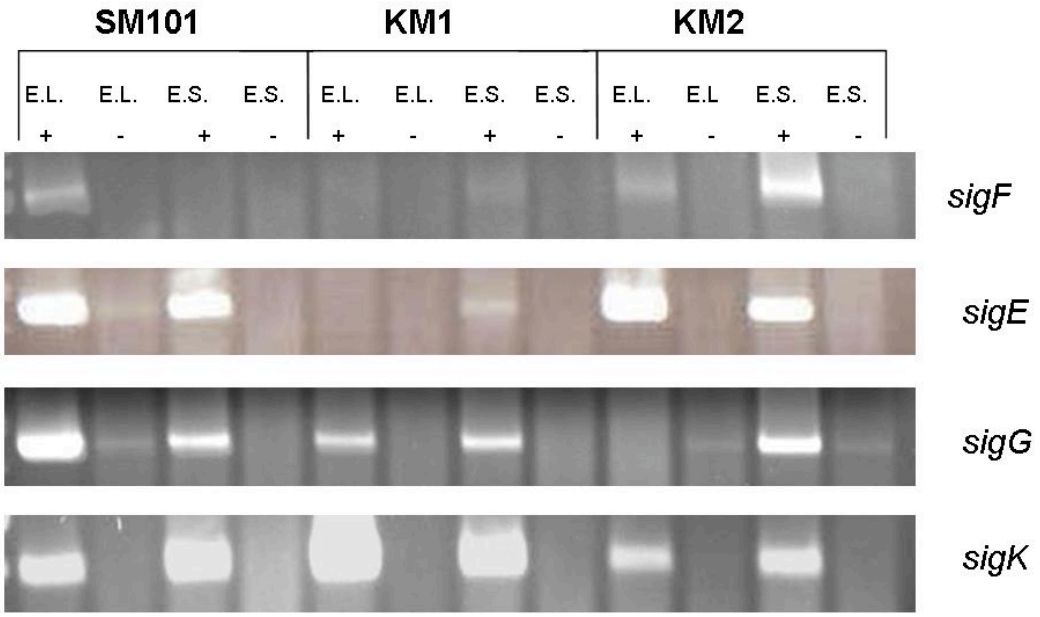
Transcription of sporulation sigma factor encoding genes during early log and early stationary phase in *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-).

Reverse transcription PCR (RT-PCR) was utilized to determine when *sigF*, *sigE*, *sigG*, and *sigK* are transcribed in *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) (Fig. 3-3). RT-PCR amplification of *sigE*, *sigG*, and *sigK* in *C. perfringens* SM101 determined that these three sporulating sigma factor genes are transcribed during early log and early stationary phases, while *sigF* is transcribed only during early log phase.

RT-PCR analysis of *C. perfringens* KM1 (*sigK*-) indicated that σ^K-associated RNA polymerase is necessary for normal transcription of *sigF* and *sigE*. There is no transcription of *sigF* in early log phase and it appeared that there is a faint transcript in early stationary phase. RT-PCR analysis of *sigE* in *C. perfringens* KM1 (*sigK*-) indicated that there was no transcription in early log phase and reduced transcription in early stationary phase when compared to wild-type *C. perfringens* SM101. Transcription of *sigG* did not appear to be affected by a mutation in the *sigK* gene. There was transcription of *sigK*, which was surprising given that there was a very low amount of transcription of *sigK* from analysis of the *sigK* promoter (Fig. 3-2B).

The analysis of transcription of sporulation sigma factor encoding genes in *C. perfringens* KM2 (*sigE*-) indicated that transcription of *sigF* and *sigG* is affected by a mutation in the *sigE* gene. Transcription of *sigF* in *C. perfringens* KM2 (*sigE*-) occurred in early stationary phase and a slight amount appeared to be transcribed in early log phase. There was no transcription of *sigG* during early log phase, but transcription does

Figure 3-3. Transcription of sporulation sigma factor encoding genes in *C. perfringens* SM101, KM1 (*sigK*-), and KM2 (*sigE*-) strains.



Reverse transcription PCR (RT-PCR) of the four sporulation sigma factor genes in *C. perfringens* SM101, KM1 (*sigK*-) and KM2 (*sigE*-) strains. Samples were collected during early log phase (E.L.) and early stationary phase (E.S.). "+" signs indicate reactions in which reverse transcriptase was added to the reaction, "-" signs indicate those reactions in which reverse transcriptase was not included in the reaction (negative control). Products were viewed on an ethidium-bromide-stained 2% agarose gel.

occur in early stationary phase. *sigE* and *sigK* were transcribed during early log and early stationary phase in *C. perfringens* KM2 (*sigE*-), which was surprising since there was no transcription of these two genes from early assays analyzing transcription from the promoter (Fig. 3-1B).

Antisense RNA may be down-regulating gene expression from the complementation plasmids.

Previous work has shown that *C. perfringens* strains KM2 (*sigE*-) and KM1 (*sigK*-) produced an undetectable number of spores (Chapter II). *C. perfringens* strains KM2 (*sigE*-) and KM1 (*sigK*-) were only partially complemented as indicated by sporulating at an efficiency of about 1%, while wild-type *C. perfringens* SM101 has an average sporulation efficiency of about 80% (Chapter II).

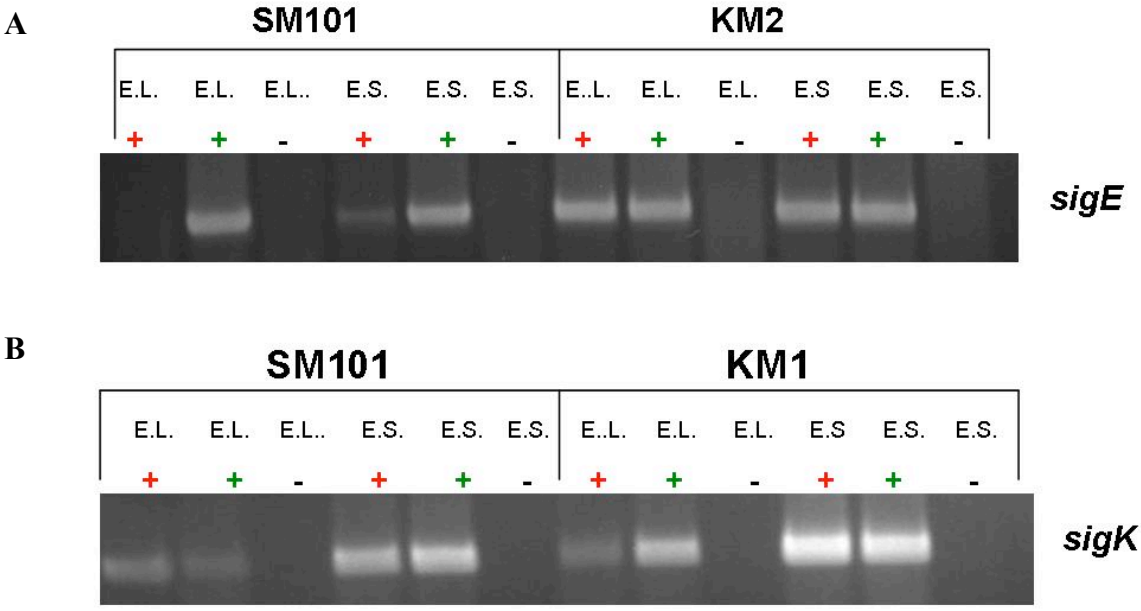
The RT-PCR results demonstrated that there was transcription of *sigK* in *C. perfringens* KM1 (*sigK*-) and *sigE* in *C. perfringens* KM2 (*sigE*-) (Fig. 3-3), which conflicts with the results from the β-glucuronidase assays performed earlier that indicated there was little to no transcription from the promoters of these genes in the mutants (Fig. 3-1B and 3-2B). The primers used for RT-PCR analysis were the same as the one used to clone the internal fragment of the gene for mutagenesis. We investigated the possibility that antisense RNA may be produced from an unknown promoter on the inserted multimeric plasmid (pNLDK or pNLDE). The sense and antisense RNA strands would form a duplex and cause the down-regulation of expression from the genes on the complementing plasmids. The system may not be 100% efficient, which would account for the restoration of sporulation to 1%.

To verify that antisense RNA may be down-regulating the gene expression in *C. perfringens* KM2(pKM3) (complemented *sigE*-) and *C. perfringens* KM1(pKM2) (complemented *sigK*-) strains, RT-PCR reactions were performed on *C. perfringens* KM2 (*sigE*-) and *C. perfringens* KM1 (*sigK*-) in which one primer was added to the reaction to synthesize the cDNA by reverse transcription. Then, the second primer was added for amplification of cDNA. This allowed us to determine what direction gene transcription was in (Fig. 3-4).

The results indicated that in *C. perfringens* SM101, during early log phase and early stationary phase the sense strand of RNA encoding *sigE* was present (Fig. 3-4A). There was a faint product indicating that antisense RNA is present during early stationary phase. When *C. perfringens* KM2 (*sigE*-) was analyzed, it was found that both sense and antisense RNA were present (Fig. 3-4A).

When RT-PCR was performed to determine the sense and antisense RNA strand transcription of the *sigK* gene, it was found that both strands were transcribed during early log and early stationary phases in wild-type *C. perfringens* SM101 (Fig 3-4B). The transcription of *sigK* in *C. perfringens* KM1 (*sigK*-) was analyzed, and it was found that both sense and antisense strands were being transcribed (Fig 3-4B).

Figure 3-4. Sense and antisense RNA transcript detection using RT-PCR in *C. perfringens* SM101 and mutant strains.



To detect which strand of RNA was being transcribed one primer specific for either the sense or antisense strand was added for the reverse transcription step of RT-PCR. Later the second primer was added for amplification of the product. Samples were taken during early log phase (E.L.) and early stationary phase (E.S.). Red "+" signs indicate reactions for detection of the antisense strands, while green "+" signs indicate reactions for the sense strands. "-" signs indicate negative control reactions. Products were viewed on an ethidium-bromide-stained 2% agarose gel.

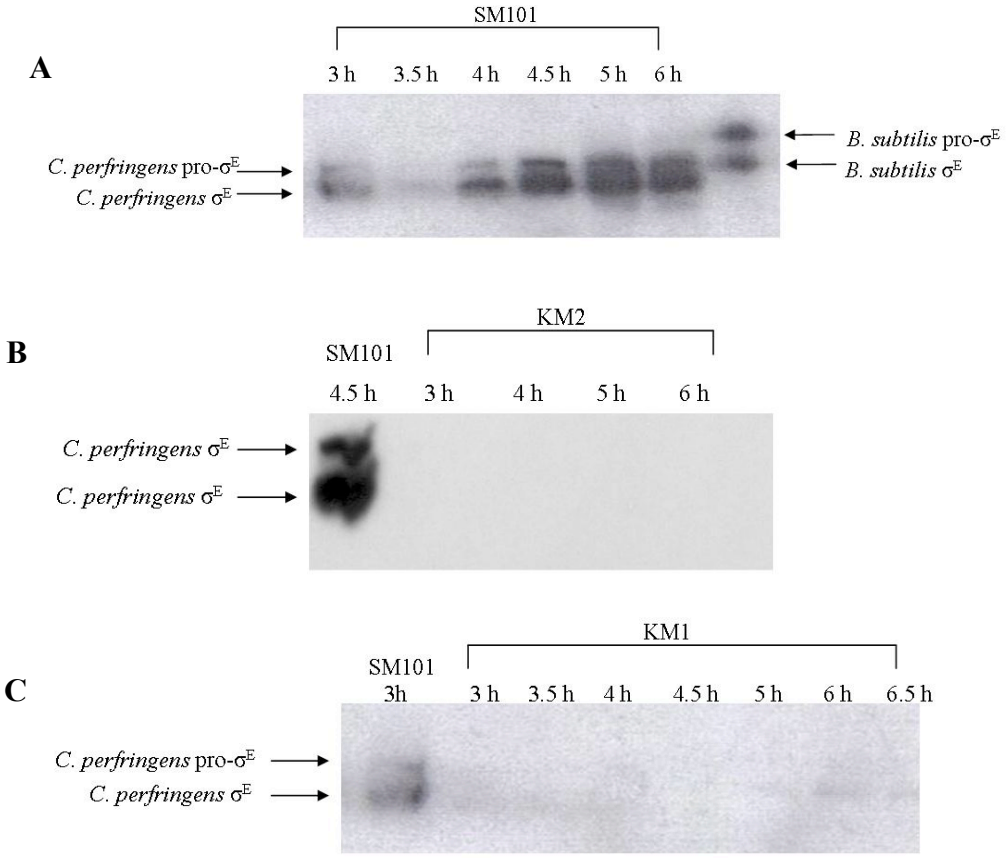
Pro-σ^E/σ^E was not detected in *C. perfringens* KM2 (*sigE*-) or *C. perfringens* KM1 (*sigK*-).

To test when pro-σ^E was processed to active σ^E, samples were taken from sporulating cultures and western blots were performed. Antibodies generated against *B. subtilis* pro-σ^E/σ^E were found to react against *C. perfringens* pro-σ^E/σ^E (Fig. 3-5A). Pro-σ^E/σ^E was present in the *C. perfringens* SM101 sample at 3 h post inoculation into DSSM (Fig. 3-5A), correlating to mid-log growth (data not shown). There was no pro-σ^E/σ^E detected in *C. perfringens* KM2 (*sigE*-) (Fig. 3-5B) or *C. perfringens* KM1 (*sigK*-) (Fig. 3-5C). These results were expected since there was no activity of the *spoIIG* promoter in *C. perfringens* KM2 (*sigE*-) or *C. perfringens* KM1 (*sigK*-) (Fig. 3-1B).

Pro-σ^K/σ^K was not detected in *C. perfringens* strains KM1 (*sigK*-) or KM2 (*sigE*-).

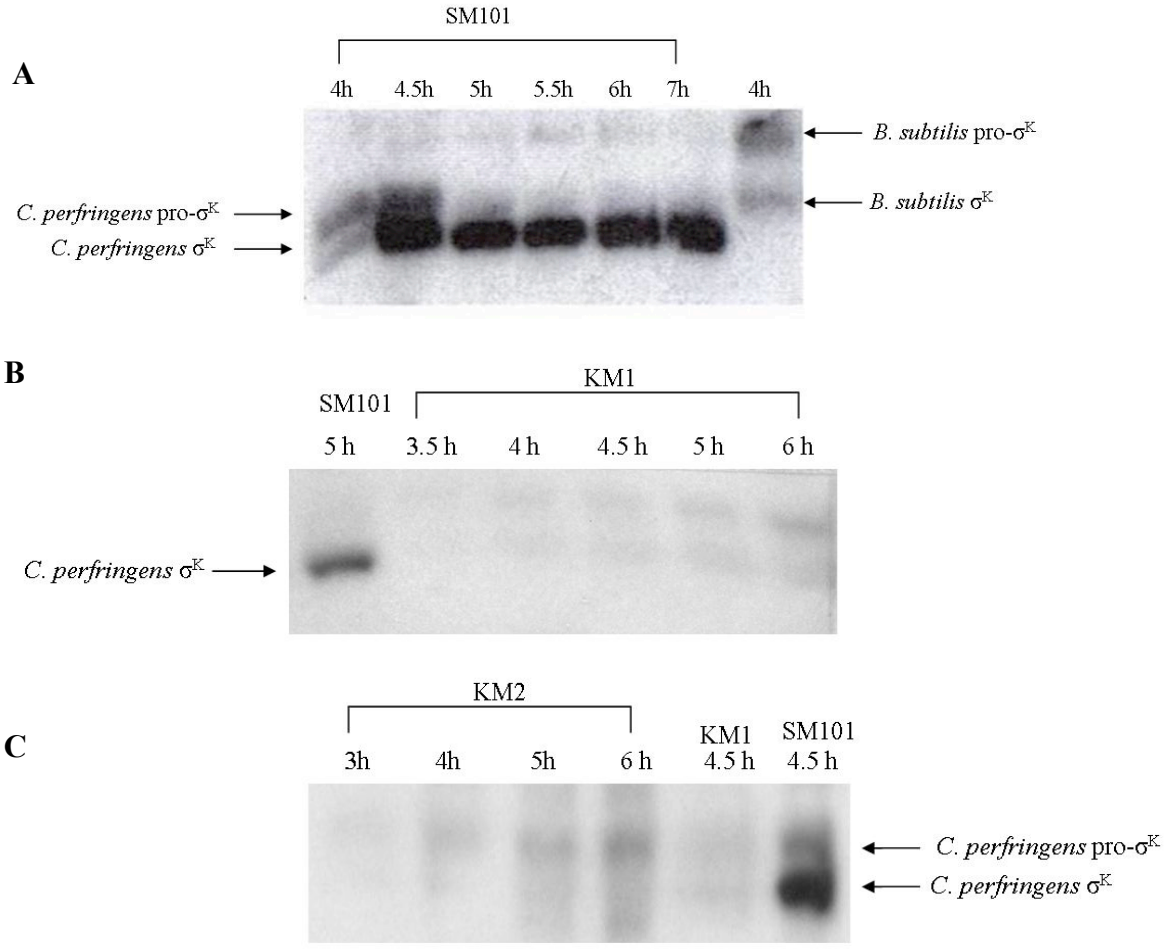
To determine when pro-σ^K was processed to active σ^K, samples were taken from cultures after inoculation into sporulation medium, DSSM, and western blots were performed. Antibodies generated against *B. subtilis* pro-σ^K/σ^K were found to react against *C. perfringens* SM101 pro-σ^K/σ^K (Fig. 3-6A). Pro-$\sigma^K/\ \sigma^K$ is present in the *C. perfringens* SM101 sample at 4 h post-inoculation into DSSM (Fig. 3-6A), correlating to late log growth (data not shown). By 4.5 h, almost all of the pro-σ^K had been processed to σ^K. There is no pro-σ^K/σ^K detected in *C. perfringens* KM1 (*sigK*-) (Fig. 3-6B) or *C. perfringens* KM2 (*sigE*-) (Fig. 3-6C).

Figure 3-5. Pro-σ^E and σ^E in *C. perfringens* wild-type and mutant strains.



Wild-type *C. perfringens* SM101, *C. perfringens* KM2 (*sigE*-), and *C. perfringens* KM1 (*sigK*-) were inoculated into sporulation media and samples were collected over an 8 h time course. Western blot analyses using anti-σ^E antibodies generated against *B. subtilis* σ^E were performed on *C. perfringens* strains SM101 (A), KM2 (B), and KM1 (C). *B. subtilis* served as a positive control when analyzing the *C. perfringens* SM101 blot. *C. perfringens* SM101 samples served as a positive control in blots analyzing σ^E in the mutant strains.

Figure 3-6. Pro-σ^K and σ^K in *C. perfringens* wild-type and mutant strains.



Wild-type *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) were inoculated into sporulation media and samples were collected over an 8 h time period. Western blot analyses using anti-σ^K antibodies generated against *B. subtilis* σ^K were performed on *C. perfringens* strains SM101 (A), KM1 (B), and KM2 (C). *B. subtilis* served as a positive control when analyzing the *C. perfringens* SM101 blot. *C. perfringens* SM101 samples served as a positive control in blots analyzing mutant strains. *C. perfringens* KM1 (*sigK*-) was a negative control when analyzing the *C. perfringens* KM2 (*sigE*-) blot.

The complemented *sigE* and *sigK* mutants indicate that pro-σ^E/σ^E is restored, but not pro-σ^K/σ^K.

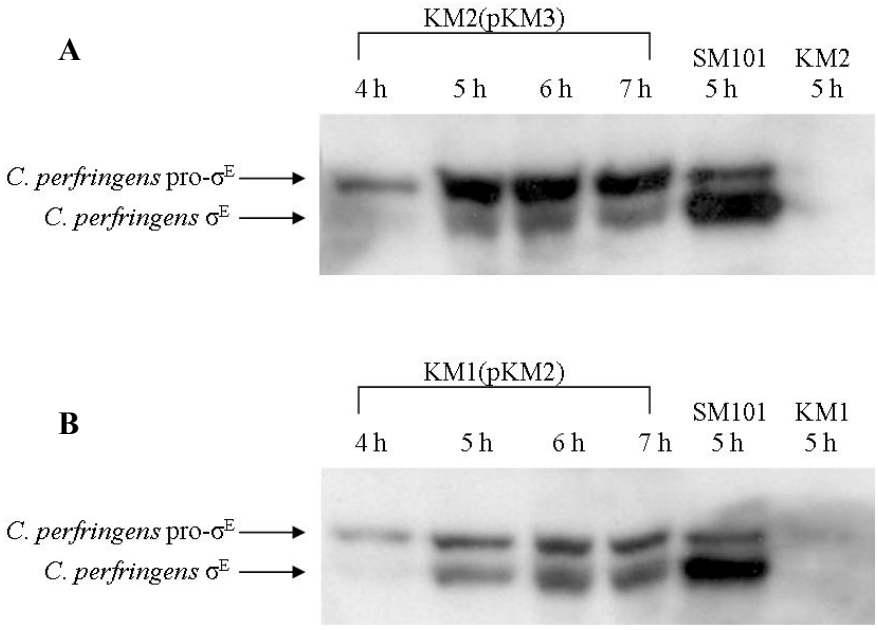
In the complemented *C. perfringens* strains, KM2(pKM3) (complemented *sigE*-) and KM1(pKM2) (complemented *sigK*-) pro-σ^E was present at 4 h with processing of pro-σ^E to active σ^E starting at 5 h after inoculation into sporulation media, but was not processed to active σ^E at the same level as *C. perfringens* SM101 was (Fig. 3-7A). It is possible that the inability of pro-σ^E to be processed completely could account for the lack of complementation observed in *C. perfringens* strains KM1(pKM2) (complemented *sigK*-) and KM2(pKM3) (complemented *sigE*-). There was no pro-σ^K/σ^K detected in *C. perfringens* KM1(pKM2) (complemented *sigK*-) or in *C. perfringens* KM2(pKM3) (complemented *sigE*-) (data not shown).

spoIIID* is not regulated in *C. perfringens* the same way as it is in *B. subtilis.

In the mother cell, in addition to σ^E and σ^K, SpoIIID is essential for gene regulation during sporulation, as it is a DNA-binding protein that activates transcription of the *sigK* gene. In *B. subtilis*, the transcription of *spoIIID* is σ^E-associated RNA polymerase directed (98).

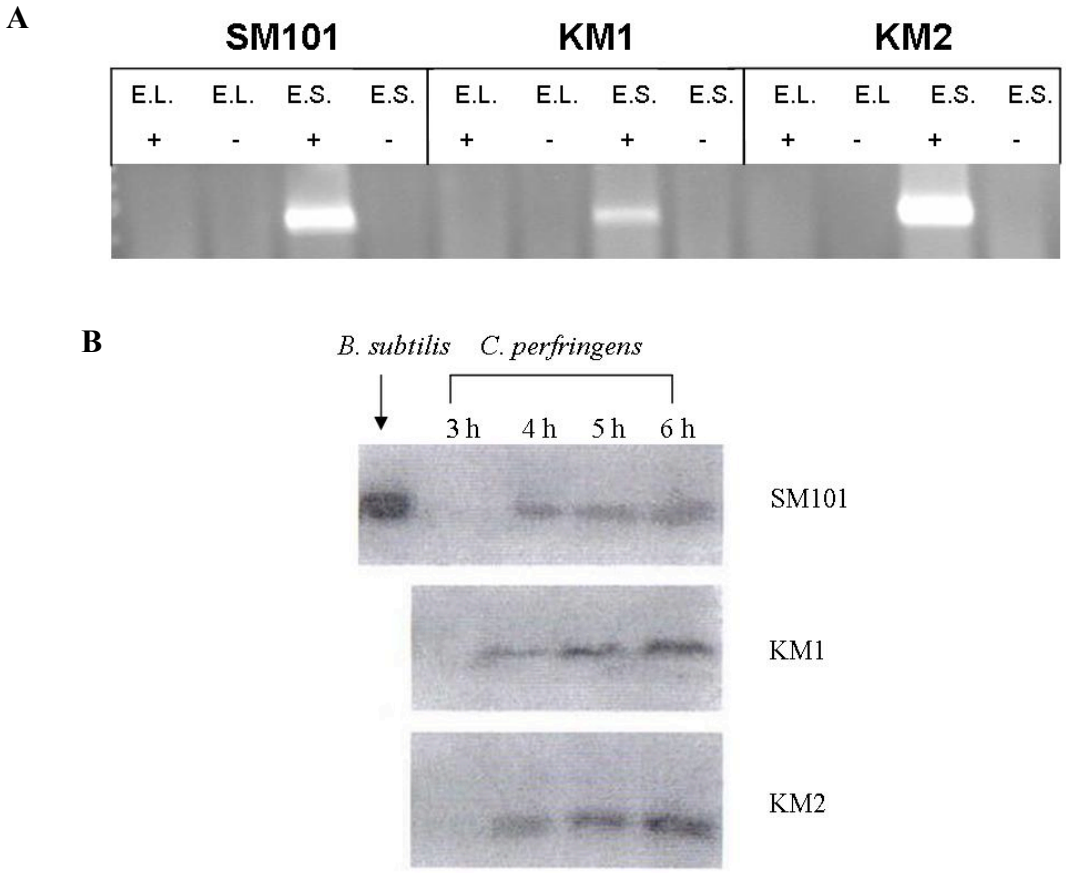
RT-PCR was performed to determine if *spoIIID* is part of an operon with the upstream gene, *CPR_2157*, which codes for a putative peptidase. Primers were designed in *spoIIID* and *CPR_2157*. An amplification product indicated the presence of an RNA transcript. The RT-PCR results indicated that *spoIIID* is transcribed from the promoter of the upstream gene, *CPR_2157*, sometime after early log phase (Fig. 3-8A). *spoIIID* is transcribed in *C. perfringens* KM2 (*sigE*-), and therefore it is not under σ^E-associated RNA polymerase control (Fig. 3-8A).

Figure 3-7. Pro-σ^E and σ^E in the complemented *C. perfringens* KM2 (*sigE*-) and KM1 (*sigK*-) strains.



C. perfringens strains KM2(pKM3) (complemented *sigE*-) and KM1(pKM2) (complemented *sigK*-) were inoculated into sporulation media and samples were collected over an 8 h time period. Western blot analyses using anti-σ^E antibodies generated against *B. subtilis* σ^E were performed on *C. perfringens* strains KM2(pKM3) (complemented *sigE*-) (A) and KM1(pKM2) (complemented *sigK*-) (B). *C. perfringens* SM101 samples served as a positive control. *C. perfringens* KM2 (*sigE*-) served as a negative control when analyzing the *C. perfringens* KM2(pKM3) (complemented *sigE*-) blot while, *C. perfringens* KM1 (*sigK*-) served as a negative control when analyzing the *C. perfringens* KM1(pKM2) (complemented *sigK*-) blot.

Figure 3-8. Transcriptional and translational analysis of SpoIIID.



After inoculation into sporulation media samples of *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) were collected during early log phase (E.L.) and early stationary phase (E.S.) and analyzed for the transcription of *spoIIID* (A). "+" signs indicate reactions in which reverse transcriptase was added to the reaction, "-" signs indicate negative control reactions. Products were viewed on an ethidium-bromide-stained 2% agarose gel. (B) *C. perfringens* strains SM101, KM1 (*sigK*-), and KM2 (*sigE*-) were inoculated into sporulation media and samples were collected over an 8 h time course. Western blot analysis using anti-SpoIIID antibodies generated against *B. subtilis* SpoIIID were performed on *C. perfringens* strains SM101, KM2 (*sigE*-), and KM1 (*sigK*-). *B. subtilis* was used as a positive control.

At the translational level, western blot analysis indicated that SpoIIID in *C. perfringens* is expressed during sporulation of strains SM101, KM2 (*sigE*-), and KM1 (*sigK*-) during late log phase (Fig. 3-8B). This indicated that none of the gene products responsible for synthesis of SpoIIID are affected by the lack of σ^E or σ^K.

Discussion

The regulation of sporulation in *B. subtilis* has been well characterized. The preliminary evidence reported here indicates that transcription is different in *C. perfringens*, both in the timing of sporulation and in the possible regulation of sporulation specific sigma factors.

Sporulation in *B. subtilis* does not occur until the cells have exhausted their nutrient supplies and enter log phase (96). Our results suggest that *C. perfringens* initiates sporulation as soon as early-log phase, as seen by the activation of the *spoIIG* operon promoter. Pro-σ^E is processed almost as soon as it is translated as observed by western blot analysis. Activation of the *sigK* promoter occurs during late log to early stationary phase, about an hour later than that of the *spoIIG* promoter. The majority of the pro-σ^K processing occurs during early stationary phase. These results suggest σ^E is active before σ^K during sporulation; however, analyses of the phenotypes of the *sigE* and *sigK* mutants suggest that σ^K is actually active before σ^E.

In *B. subtilis* σ^A-associated RNA polymerase transcribes the *spoIIG* operon after activation by phosphorylated Spo0A (1, 35). Surprisingly, the β-glucuronidase assay results indicated that in *C. perfringens*, σ^E- and σ^K-associated RNA polymerase are necessary for transcription of the *spoIIG* operon. Western blot analyses showed that pro-σ^E and σ^E were not present in *C. perfringens* KM2 (*sigE*-) or *C. perfringens* KM1 (*sigK*-), which was expected due to the fact that the promoter assays showed no transcription of the *spoIIG* operon.

The β-glucuronidase assays indicated that in *C. perfringens*, σ^E-associated RNA polymerase is necessary for transcription of the *sigK* gene, which is what occurs in *B.*

subtilis (62, 89). We also observed that σ^K-associated RNA polymerase directs a large portion of its own transcription. While it is understood that σ^K-associated RNA polymerase does regulate its own transcription at low levels in *B. subtilis* (89), transcription of *sigK* in *C. perfringens* KM1 (*sigK*-) was 100 fold lower than in *C. perfringens* SM101. Western blot analyses indicated that pro-σ^K and σ^K were not present in *C. perfringens* strains KM1 (*sigK*-) or KM2 (*sigE*-). The fact that no pro-σ^K or σ^K were detected in *C. perfringens* KM2 (*sigE*-) correlates with the observation that there is no transcription from the *sigK* promoter in this mutant strain. Although there was a reduced amount of transcription from the *sigK* promoter in *C. perfringens* KM1 (*sigK*-), the levels were so low that western blotting may not have been able to detect the protein.

Another difference between the regulation of sporulation in *B. subtilis* and *C. perfringens* is the regulation of *spoIIID*. In *B. subtilis, spoIIID* transcription is dependent on σ^E-associated RNA polymerase (61, 123). Therefore, in a *B. subtilis* σ^E mutant, *spoIIID* would not be transcribed. We found that not only is the transcription of *spoIIID* independent of σ^E-associated RNA polymerase, but that it is also co-transcribed with the upstream gene, *CRP_2157*.

In *B. subtilis spoIIAC*, the gene encoding σ^F, is transcribed prior to asymmetric septum formation by σ^H-associated RNA polymerase after activation by phosphorylated Spo0A (133). The phenotype of a *B. subtilis spoIIAC* mutant is a disporic cell (96). *C. perfringens* KM1 (*sigK*-) appears to be mostly blocked in sporulation at stage 0 (Chapter II) indicating an early block at the morphological level and the RT-PCR results show that transcription of *sigF* in *C. perfringens* KM1 (*sigK*-) is practically eliminated, which

indicates an early block at the molecular level. These results suggest that σ^K activation is upstream of σ^F in the regulation of sporulation.

Transcription of both *sigF* and *sigE* appear to be greatly reduced by the lack of σ^K. Both of these genes need phosphorylated Spo0A in order for transcription to occur in *B. subtilis*. It may be possible that σ^K is needed to increase the phosphorylated Spo0A level in *C. perfringens*. If so, this could explain the very early block in sporulation.

Contrary to our β-glucuronidase results, the RT-PCR results indicate that there are high levels of transcription of *sigK* in *C. perfringens* KM1 (*sigK*-) and *sigE* in *C. perfringens* KM2 (*sigE*-). We propose that transcription is not driven by the promoters of *sigE* and *sigK* on the chromosome, but from unidentified promoters located on the plasmids used in mutagenesis. The results suggest that antisense RNA is being transcribed in *C. perfringens* KM2 (*sigE*-) that will bind to the sense strand of *sigE* transcribed from the complementing plasmid pKM3. This antisense-sense duplex structure will result in reduced protein expression. This would explain why *C. perfringens* KM2 (*sigE*-) can only be partially complemented when the wild-type gene is introduced into the mutant on a plasmid.

The results were not as clear when sense and antisense transcription of *sigK* was observed in *C. perfringens* strains SM101 and KM1 (*sigK*-), as both strains showed the transcription of sense and antisense RNA. At this point it is inconclusive as to whether or not antisense RNA is affecting the expression of *sigK* from the complementing plasmid.

Acknowledgements

I would like to acknowledge Stephen Melville for construction of the vectors pSM240 and pSM242. I would also like to thank Runbao Zhou for performing the western blots.

Chapter IV. Overall conclusions

Clostridium perfringens is one of the most common bacterial causes of food poisoning (81). It produces an enterotoxin, CPE, that binds to intestinal epithelial cells, causing diarrheal and abdominal cramping symptoms (20, 29). Sporulation is essential for the production of CPE (19, 70).

Since the regulation of sporulation in *C. perfringens* has not been studied in depth as it has in *Bacillus subtilis*, we use *B. subtilis* as a model. During sporulation, sigma factors regulate the process of cell differentiation from a vegetative cell to a dormant spore. These sigma factors in *B. subtilis* are always activated in a linear form that begins with σ^F, followed by σ^E, then σ^G, and lastly σ^K.

Previous work has demonstrated that there are three promoters that regulate the transcription of *cpe* (138). Based on the upstream regions of the promoters, P1 appears to be σ^K-dependent, while P2 and P3 appear to be σ^E-dependent. In this study, mutations were introduced into the *sigE* and *sigK* genes of *C. perfringens.* We found that there was a loss of *cpe* transcription in the *sigE* and *sigK* mutants.

Electron microscopic evaluation of the *sigE* and *sigK* mutants indicated that σ^K was a very early acting sigma factor during sporulation, which is the opposite of what is observed in *B. subtilis*. Analysis of the transcription of *sigE* and *sigK* indicated that σ^E- and σ^K-associated RNA polymerase may co-regulate each other.

The lack of complementation of the *sigE* and *sigK* mutants has been a problem in this study. We have a few hypotheses for why *C. perfringens* strains KM1 (*sigK*-) and KM2 (*sigE*-) have not been fully complemented. The first hypothesis is based on the dominant negative phenotype exhibited when a mutated *sigK* gene was introduced to wild-type *C. perfringens* SM101. This *sigK* mutant gene upon translation produced a

truncated protein that knocked-out sporulation in *C. perfringens* SM101 to below the limits of detection. When this dominant negative effect is applied to the *sigK* mutant (*C. perfringens* KM1) and the wild-type gene is introduced on a multi-copy number plasmid, it is less surprising that complementation cannot be fully restored. If a *sigE* mutation exhibits the same dominant negative effect, it could be hypothesized that that is why complementation cannot be fully restored in the *sigE* mutant. The second hypothesis for the lack of complementation in the *sigE* and *sigK* mutants is that an uncharacterized promoter located on the multimeric plasmids used in mutagenesis may be producing antisense RNA that is silencing the transcripts from the complementing plasmids and silencing the genes. A third hypothesis is based on the fact that pro-σ^E in the complemented *sigE* and *sigK* mutants is not processed to the same extent as it is in *C. perfringens* SM101. There may not be enough active σ^E available to the cell to complete sporulation efficiently.

Through this project preliminary insights into the regulation of sporulation of *C. perfringens* have been gained. *C. perfringens* forms a spore much faster than does *B. subtilis* once inoculated into sporulation medium. Therefore, it is not surprising that there are differences in the regulation of sporulation between these two organisms.

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