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Sporulation and Enterotoxin (CPE) Synthesis Are Controlled by the Sporulation-Specific Sigma Factors SigE and SigK in *Clostridium perfringens*▽‡

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***Clostridium perfringens* is the third most frequent cause of bacterial food poisoning annually in the United States. Ingested *C. perfringens* vegetative cells sporulate in the intestinal tract and produce an enterotoxin (CPE) that is responsible for the symptoms of acute food poisoning. Studies of *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 σ^F, σ^E, σ^G, and σ^K coordinate much of the developmental process. The *C. perfringens cpe* gene, encoding CPE, is transcribed from three promoters, where P1 was proposed to be σ^K dependent, while P2 and P3 were proposed to be σ^E dependent based on consensus promoter recognition sequences. In this study, mutations were introduced into the *sigE* and *sigK* genes of *C. perfringens*. With the *sigE* and *sigK* mutants, *gusA* fusion assays indicated that there was no expression of *cpe* in either mutant. Results from *gusA* fusion assays and immunoblotting experiments indicate that σ^E-associated RNA polymerase and σ^K-associated RNA polymerase coregulate each other's expression. Transcription and translation of the *spoIIID* gene in *C. perfringens* were not affected by mutations in *sigE* and *sigK*, which differs from *B. subtilis*, in which *spoIIID* transcription requires σ^E-associated RNA polymerase. The results presented here show that the regulation of developmental events in the mother cell compartment of *C. perfringens* is not the same as that in *B. subtilis* and *Clostridium acetobutylicum*.**

Clostridium perfringens is a common cause of food poisoning, responsible for approximately 250,000 cases in the United States each year (47). After the ingestion of vegetative cells in contaminated food, an enterotoxin, CPE, is produced by sporulating cells in the gastrointestinal tract (11, 13). The enterotoxin binds to receptors on the surface of intestinal epithelial cells to form a pore and interacts with tight junction proteins, claudins (46). This triggers the loss of fluids and electrolytes, leading to the symptoms of diarrhea and intestinal cramping (12, 18).

CPE is produced only in the cytoplasm of the mother cell during sporulation of *C. perfringens* (10, 44). After sporulation is completed, the mother cell lyses to release the mature spore and CPE (9). 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 (9). *C. perfringens* strains that do not sporulate do not produce the enterotoxin (48).

Analysis of *C. perfringens* strains containing the *cpe* promoters fused to the *Escherichia 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 (49). It has been shown that only sporulating cells and not vegetative cells produce *cpe* mRNA (8, 49). These results demonstrate that CPE expression is controlled at the transcriptional level during sporulation. Zhao and Melville (73) found that there are three promoters that regulate the transcription of *cpe*. The P1 promoter has sequences similar to σ^K consensus recognition sequences, and the P2 and P3 promoters are similar to σ^E promoters (73).

The molecular regulation of sporulation in *C. 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 (57). It has been observed that in *C. perfringens* the morphological events, which are divided into seven stages (I to VII), during the formation of the spore are similar to those of *B. subtilis* (26). In *B. subtilis* four sporulation-specific sigma factors, σ^F, σ^E, σ^G, and σ^K, are the major regulators of the sporulation process (23, 26, 38, 45). Homologues of these proteins have been found in *C. perfringens* (60).

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 an ordered fashion, with σ^F active first, followed by σ^E, σ^G, and σ^K last (45). In *B. subtilis*, the σ^F-encoding gene, *spoIIAC*, is transcribed by σ^H-associated RNA polymerase during initiation of sporulation (20, 70). The σ^E-encoding gene, *spoIIGB*, is the second gene in an operon with *spoIIGA* (which encodes a protease that activates pro-σ^E) and is also transcribed before asymmetric septation (30, 36), but transcription continues only in the mother cell after septum formation (19). *spoIIGB* is

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transcribed by σ^A-associated RNA polymerase, a sigma factor associated mainly with the transcription of housekeeping genes (3, 23). Transcription of *spoIIIG*, the gene encoding σ^G, involves both σ^F- and σ^G-associated RNA polymerase (64), while *sigK*, the structural gene encoding σ^K, is transcribed by σ^E- and σ^K-associated RNA polymerase (39, 43, 63). While the *sigK* gene in *B. subtilis* is interrupted by a DNA element called *skin* (63) and another *skin* element is found in the *Clostridium difficile sigK* gene (24), these are not present in the *sigK* genes in the *C. perfringens* genomes that have been sequenced thus far (data not shown). In *B. subtilis*, the activation of *sigK* transcription is also regulated by the DNA-binding protein SpoIIID (39, 43). *spoIIID* is transcribed in the mother cell by σ^E-associated RNA polymerase (42, 66).

In *B. subtilis*, each of the four sporulation sigma factors is regulated posttranslationally. σ^F is held inactive by the anti-sigma factor SpoIIAB (14, 50), and inhibition is relieved upon formation of the asymmetrical septum early in sporulation (28, 40). SpoIIAB and another anti-sigma factor, CsfB, can also act as anti-sigma factors to suppress low levels of inappropriate σ^G activity, but the mechanism of σ^G activation to completion of forespore engulfment remains speculative (6, 7, 34, 35). σ^E and σ^K are first translated as inactive proproteins that become active only after proteases cleave amino acid residues from their N-terminal ends, and the pro-σ^E and pro-σ^K cleavage events in the mother cell depend on signals generated by σ^F and σ^G in the forespore, respectively (38, 45).

The genes encoding the four sporulation-specific sigma factors are present in *C. perfringens* and seem to be present in all of the *Clostridium* species for which the complete genome sequence is known (62). The role of clostridial sporulation-specific sigma factors in endospore development has been studied most thoroughly in *Clostridium acetobutylicum*, a solventogenic *Clostridium* species (15, 32). For *C. acetobutylicum*, the pattern of sigma factor expression during sporulation and solventogenesis for *spoIIGA-sigE*, *sigG*, and *sigK* matched that seen with *B. subtilis* (15), although it was spread out over a much longer time (35 h) than that seen with *B. subtilis* expression (~8 h). In the last several years, Papoutsakis and colleagues have used complete genomic microarrays and quantitative reverse transcription (RT)-PCR to characterize the expression levels of sporulation-specific sigma factors and the sporulation transcriptional regulator Spo0A and to identify the transcriptomes specific to each factor in *C. acetobutylicum* (1, 2, 32, 52, 53, 67). They also found that *sigF*, *sigE*, and *sigG* transcription increased ~50- to 200-fold during sporulation, but *sigK* expression was weak and not strongly induced during sporulation (32). Although the transcriptomes associated with each sigma factor in *C. acetobutylicum* are now being elucidated, the underlying mechanisms responsible for regulation of expression of the sigma factors themselves are still unknown for any species of *Clostridium*. Spo0A mutants have been constructed in several clostridia, they are usually blocked in sporulation at an early stage, and sporulation-specific sigma factors are not synthesized (2, 15, 25, 27). Since we have proposed that sporulation-specific sigma factors SigE and SigK play a major role in regulating CPE synthesis (73), we tested this hypothesis by constructing mutants in *sigE* and *sigK* and measuring CPE expression. We also examined expression of key mother cell genes (*sigE*, *spoIIID*, and *sigK*) in the mutants and characterized their sporulation phenotypes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 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 as 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 no. 3, 20 g glucose, 10 g yeast extract, 1 g of sodium thioglycolate per liter) or 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 and OSM173. The sequences for all oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. 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 (68), resulting in pNLDK.

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 and OSM169. OSM168 has a SacI restriction site, and OSM169 has a KpnI restriction site designed into the primer sequence. The resulting PCR product was digested with SacI and KpnI and ligated into pSM300, resulting in the plasmid pNLDE.

pNLDK and pNLDE were transformed into *E. coli* strain JM107 (*recA*$^+$) by electroporation in order to produce a multimeric form of the plasmid. Multimeric plasmids have been found to insert into the chromosome via homologous recombination in *C. perfringens* more efficiently than monomeric forms (69). After passage through JM107, the plasmids were purified via a cesium chloride gradient. Sixteen micrograms of purified pNLDK and 24 μg of purified pNLDE were transformed into *C. perfringens* strain SM101 by electroporation as previously described (73) and then plated on brain heart infusion with 30 μg/ml erythromycin.

Southern blot analysis. To confirm the insertion of the multimeric pNLDE and pNLDK plasmids into the *sigE* or *sigK* gene, Southern blotting was performed on the erythromycin-resistant transformants. Chromosomal DNA of *C. perfringens* strain KM1 (*sigK* mutant) was digested with PstI and EcoRI, and the digested DNA separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose by blotting. Hybridization with a biotinylated 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* mutant). A shift from 2 kb to >10 kb indicated the recombination of pNLDK into the *sigK* gene (data not shown). A similar procedure was performed to confirm the *sigE* mutant, except that chromosomal DNA from the erythromycin-resistant transformant, *C. perfringens* strain KM2 (*sigE* mutant), was digested with XbaI. A shift from a wild-type size of 2.1 kb to >10 kb indicated the recombination of pNLDE into the *sigE* gene (data not shown).

Construction of complementation plasmids for *C. perfringens* KM1 (*sigK* mutant) and *C. perfringens* KM2 (*sigE* mutant). Complementation of *C. perfringens* KM1 (*sigK* mutant) was attempted by first digesting pSM305 with PstI and HincII to produce a fragment spanning the complete *sigK* gene and its promoter. This fragment was then ligated into the *E. coli-C. perfringens* shuttle vector, pJV5, which had been digested with SmaI and PstI, to produce the plasmid pKM2 (Table 1).

Complementation of *C. perfringens* KM2 (*sigE* mutant) was attempted by first PCR amplifying the entire operon, consisting of *spoIIGA*, *sigE*, and 400 bp upstream of *spoIIGA*, from the *C. perfringens* SM101 chromosome using the oligonucleotide primers OKM9, which had a BamHI restriction site designed into the primer, and OKM11, which had an EcoRI restriction site designed into the primer. The PCR product was then digested with BamHI and EcoRI and ligated into pJV5, which had been digested with BamHI and EcoRI, resulting in the plasmid pKM3 (Table 1).

Sporulation assays. *C. perfringens* strains were grown 14 to 16 h in 5 ml fluid thioglycolate medium (FTG) (Difco) with the appropriate antibiotic if needed. Two hundred fifty microliters of cell suspension was subcultured into 5 ml prewarmed sporulation medium, DSSM (15 g proteose peptone no. 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

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS mcrBC*) ϕ80d*lacZ* ΔM15 *lacX74 deoR recA1 araD139* Δ(*ara*, *leu*)*7697 galU galK* Δ-*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*	72
C. perfringens		
SM101	High efficiency of electroporation derivative of NCTC 8798	73
KM1	*sigK* mutant of SM101	This study
KM2	*sigE* mutant of SM101	This study
Plasmids		
pSM240	*E. coli-C. perfringens* shuttle vector containing the *E. coli gusA* gene; chloramphenicol resistance	This study
pKM4	*spoIIG* promoter region fused to pSM240	This study
pSM242	*sigK* promoter region fused to pSM240	This study
pSM300	*E. coli* origin of replication; erythromycin resistance	68
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	69
pSM305	pBluescript SK− 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	69
pKM2	pJV5 with complete *sigK* gene from plasmid pSM305	This study
pKM3	pJV5 with complete *spoIIG* operon	This study
pJIR750	*E. coli-C. perfringens* shuttle vector; chloramphenicol resistance	4
pSM100	pBluescript SK− with the NCTC 10240 *cpe* promoter	49
pSM104	pJIR750 with Φ($cpe_{\text{NCTC 10240}}$-*gusA*) (P1, P2, and P3)	49
pSM127	pSM104 with Φ($cpe_{\text{NCTC 10240}}$-*gusA*) (P1 and P2)	73
pSM170	pSM104 with Φ($cpe_{\text{NCTC 10240}}$-*gusA*) (P3)	73

diluted and plated in duplicate on PGY agar in the absence of antibiotics to determine the number of viable cells per milliliter of culture. Three hundred fifty microliters of samples were heated at 75°C for 15 min, diluted, and plated in duplicate on PGY agar to determine the number of spores per milliliter of culture. All assays were performed in triplicate. The following formula was used to determine the sporulation efficiency: sporulation efficiency (%) = [number of CFU in heated culture/(number of CFU in heated culture + number of CFU in unheated culture)] × 100.

Transmission EM. *C. perfringens* strains were grown 14 to 16 h in 5 ml FTG with the appropriate antibiotic, and then 750 μl was inoculated into 75 ml of DSSM. The optical density at 600 nm (OD_{600}) was measured every hour for 8 h using a Genesys 10 UV scanning spectrophotometer. One-milliliter samples were obtained at 3, 5, and 8 h postinoculation into DSSM, corresponding to mid-log phase, early stationary phase, and stationary phase, respectively. Samples were centrifuged, and the pellet was suspended in 84 μl of 0.5 M $NaPO_4$, pH 7, and 5 μl of 25% electron microscopy (EM)-grade glutaraldehyde by gentle vortexing and then 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 centrifuged at 10,000 × *g* for 3 to 5 min at 4°C. A standard ethanol dehydration was performed, and the samples were embedded in Spurr's resin. Samples were sectioned, collected on grids, and stained with 1% uranyl acetate for 5 min and Reynold's lead for 1 min.

The phenotypes of *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) in mid-log phase, early stationary phase, and stationary phase after inoculation into DSSM, as well as those of *C. perfringens* KM1(pKM2) (complemented *sigK* mutant) and *C. perfringens* KM2(pKM3) (complemented *sigE* mutant) 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 examined for each sample ranged from 17 to 49, except for *C. perfringens* KM2 (*sigE* mutant) at 3 h, in which six cells were counted.

Extraction of RNA, primer extension, and RT-PCR. *C. perfringens* SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) strains were grown 14 to 16 h in FTG, with erythromycin if needed. A 1% inoculum was transferred to prewarmed DSSM, and growth of the culture was measured at an OD_{600}. Fifty milliliters of culture was collected at an OD_{600} of 0.1 and 1.0, representing early log and early stationary phases 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), according to the manufacturer's instructions. RNA was stored in liquid nitrogen until use.

Primer extension for *spoIIGA-sigE* and *sigK* was done using 1 μg of RNA extracted from cells grown to mid-log phase for *spoIIGA-sigE* or early stationary phase for *sigK*. The extension reaction was done according to the manufacturer's instructions (Promega) and included primers OKM19 (*spoIIGA*) and OKM22 (*sigK*), each tagged with 6-carboxy fluorescein on the 5′ end. The primer extension product was separated by capillary electrophoresis and compared to defined size standards to determine the length of the primer extension product.

To determine the transcriptional activity of genes in the *sigK* region, primers were designed to amplify an internal fragment of *pilT* and the intergenic regions between *sigK*, *pilT*, and *ftsA*. A primer was designed 57 bp upstream of the CPR_1739 stop codon (OKM12) and 502 bp downstream of the *sigK* start codon (OJV13) to determine if *sigK* is cotranscribed with CPR_1739, which is annotated as a penicillin binding protein with a transpeptidase domain (http://www.ncbi.nlm.nih.gov/sites/entrez?db = genomeprj&cmd = Retrieve&dopt = Overview&list_uids = 12521). 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) and 626 bp downstream of the *pilT* start codon (OJV22). 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 and OJV22). In order to determine if *pilT* and *ftsA* are cotranscribed and if a mutation in *sigK* blocks this transcription, OJV11 (942 bp upstream of the *pilT* stop codon) was used with a primer designed 53 bp downstream of the *ftsA* start codon (OJV18).

For RT-PCR measurements of the transcriptional activity of sporulation sigma factor-encoding genes, oligonucleotide primers were designed internal to the genes. Oligonucleotide primers OSM166 and OSM167 were used to amplify a 487-bp region of *sigF*, OSM168 and OSM169 to amplify a 433-bp fragment of *sigE*, OSM170 and OSM171 to amplify a 431-bp fragment of *sigG*, and OSM172

and OSM173 to amplify a 382-bp fragment of *sigK*. OSM168 and OSM169 were used in the mutagenesis of the *sigE* gene in *C. perfringens* SM101 to create *C. perfringens* KM2 (*sigE* mutant), and OSM172 and OSM173 were used in the mutagenesis of the *sigK* gene to create *C. perfringens* KM1 (*sigK* mutant).

To determine if *spoIIID* was cotranscribed with the upstream gene CPR_2157, the oligonucleotide primer OKM34 was designed to anneal 248 bp upstream of the stop codon in the CPR_2157 gene and OKM23 was designed to anneal 49 bp downstream of the start codon in *spoIIID*.

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-PCRs set up without reverse transcriptase made it possible to verify the absence of contaminating DNA. All RT-PCRs were performed in duplicate.

Construction of fusions to the *spoIIG* and *sigK* promoter regions. 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 a translational fusion in pKM4 (Table 1). The region was amplified by PCR using the oligonucleotide primers OKM14 and OKM15.

To determine if there was a promoter located upstream of *sigK*, oligonucleotide primers OSM125 and OSM139 were used to amplify a 105-bp region upstream of the *sigK* gene. OSM125 had a PstI restriction site designed into the primer, 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 1).

Western blots. *C. perfringens* strains were grown overnight at 37°C in 5 ml FTG medium. Cells were then subcultured by inoculating 750 μl into 75 ml prewarmed DSSM. Over a time period beginning 1 h after inoculation into DSSM, 1 ml of culture was collected and the OD_{600} was measured. One-milliliter samples were also collected at these time points and centrifuged, and cell pellets were stored at −80°C. Extracts were prepared as described previously (74), except with lysis buffer at pH 8.0. Western blot analysis was performed as described previously (41), except the antibodies used to detect pro-σ^K and σ^K were used at a 1:5,000 dilution. These and other antibodies made against *B. subtilis* proteins were used to detect the corresponding *C. perfringens* proteins. Antibodies used to detect SpoIIID have been described (21) and were used at a 1:10,000 dilution. Monoclonal antibody against σ^E (a gift from W. Haldenwang) was used at a 1:1,000 dilution to detect pro-σ^E and σ^E, with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Promega) at a 1:1,000 dilution serving as the secondary antibodies.

RESULTS

The *C. perfringens sigK* mutant and *sigE* mutant are severely defective in their ability to sporulate. Both *C. perfringens* KM1 (*sigK* mutant) and KM2 (*sigE* mutant) formed less than 10 heat-resistant spores per ml after incubation in DSSM for 24 h, whereas the wild-type strain, SM101, formed an average of 4×10^7 spores/ml. The sporulation efficiencies of KM1 (*sigK* mutant) and KM2 (*sigE* mutant) were 0.0003% and 0.0006%, respectively, while SM101 had a sporulation efficiency of 80% (Fig. 1). These results suggest that σ^E and σ^K are essential for formation of heat-resistant spores.

Complementation of *C. perfringens* KM1 (*sigK* mutant) and KM2 (*sigE* mutant). To complement KM2 (*sigE* mutant) and KM1 (*sigK* mutant), the entire *spoIIGA-sigE* operon and the upstream promoter (plasmid pKM3) and the *sigK* gene and the upstream promoter (plasmid pKM2) were transformed into KM2 (*sigE* mutant) and KM1 (*sigK* mutant), respectively, by electroporation. KM2(pKM3) produced an average of 3.1×10^5 spores/ml, and its sporulation efficiency was 1.7%, which was about 3,000-fold higher than that of its KM2 parent (Fig. 1). Similarly, KM1(pKM2) (the complemented *sigK* mutant strain) produced an average of 2.6×10^5 spores/ml, and its sporulation efficiency was 1.3%, which was about 4,000-fold higher than that of its KM1 parent (Fig. 1). However, neither complemented strain showed a sporulation efficiency equal to that of strain SM101 (80%).

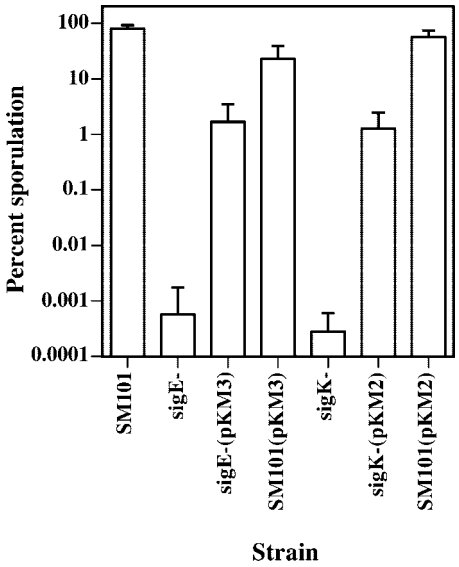


FIG. 1. Sporulation efficiencies of *C. perfringens* SM101 and the sigma factor mutants with and without complementing plasmids. Sporulation efficiencies of wild-type strain SM101 and mutants KM1 (*sigK* mutant) and KM2 (*sigE* mutant) were measured with no plasmid or with complementing plasmid. pKM2, wild-type *sigK* gene; pKM3, wild-type *sigE* gene; bar, standard deviation.

To determine if the increased *spoIIGA-sigE* and *sigK* gene copy numbers would have an effect on sporulation efficiency in the wild-type strain, pKM3 and pKM2 were also transformed into *C. perfringens* SM101. These are multicopy plasmids with an estimated 20 to 25 copies/cell, based on the characteristics of the parent plasmid, pIP404 (58). SM101(pKM3) had a sporulation efficiency of about 23%, producing an average of 6.3×10^6 spores/ml, while SM101(pKM2) had a sporulation efficiency of about 60%, producing an average of 2.3×10^7 spores/ml (Fig. 1). Because the sporulation efficiencies of SM101 with the complementing plasmids were not lowered to 1 or 2%, we determined that the increased copy numbers of the complementing genes contribute to but are not solely responsible for the lack of full complementation in KM1 (*sigK* mutant) and KM2 (*sigE* mutant).

Because we could not achieve full complementation and because *C. perfringens* KM1 (*sigK* mutant) was blocked unexpectedly (based on the *B. subtilis* paradigm) early in sporulation (see below), we remade multiple *sigK* mutant strains using pNLDK and attempted to complement each of them with pKM2. Nine *sigK* mutants were isolated from two separate electroporation experiments, and each mutant produced less than 10 spores/ml (data not shown). When complemented with pKM2, the mutants had an average sporulation efficiency of 2.8% (data not shown), which was similar to that observed in the original *sigK* mutant, KM1. These results indicate that KM1 exhibited typical complementation behavior, so the observed partial complementation is not likely due to a second-site mutation. Possible explanations for the observed partial complementation are presented below and in Discussion.

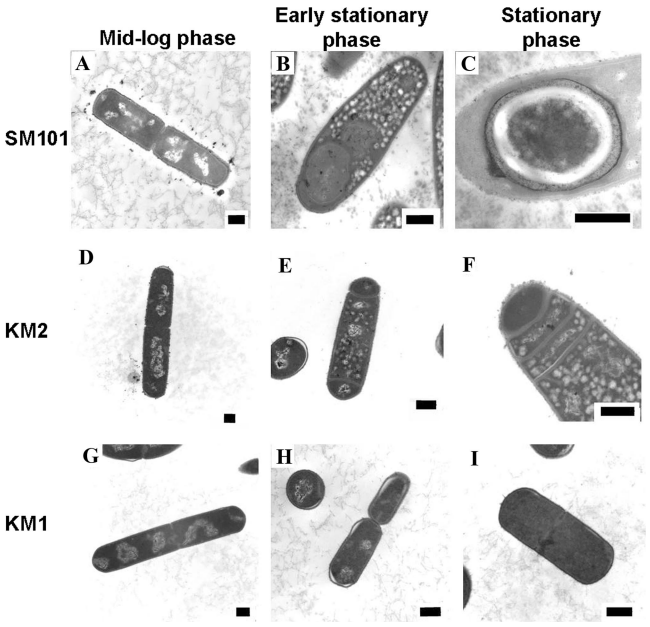


FIG. 2. Transmission electron micrographs comparing sporulation in *C. perfringens* strains SM101, KM2 (*sigE* mutant), and KM1 (*sigK* mutant). Samples of sporulating cultures of strains SM101 (A to C), KM2 (D to F), and KM1 (G to I) were analyzed during mid-log phase (3 h after inoculation into DSSM) (A, D, and G), early stationary phase (5 h after inoculation into DSSM) (B, E, and H), and stationary phase (8 h after inoculation into DSSM) (C, F, and I) of growth. Bars in bottom right corners of electron micrographs represent 500 nm.

***C. perfringens* KM1 (*sigK* mutant) is blocked earlier in sporulation than KM2 (*sigE* mutant).** Samples of *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) were analyzed by transmission EM to determine how mutations in the *sigK* and *sigE* genes affected the development of spores. Representative images of cells observed at mid-log phase (3 h postinoculation), early stationary phase (5 h postinoculation), and middle stationary phase (8 h postinoculation) are shown in Fig. 2. The cell morphology phenotypes observed in electron micrographs were quantified, and the results are shown in Table 2.

For wild-type strain SM101 in mid-log phase, the large majority of cells were vegetative rods (Fig. 2A), although a few cells had begun the sporulation process by forming an asymmetric septum (stage II) or initiating engulfment (stage III) (Table 2). In early stationary phase, most cells were undergoing the process of engulfment (Fig. 2B); however, some remained in the vegetative state. By middle stationary phase, the majority of cells had formed mature endospores (stage VI) (Fig. 2C) or existed as free spores. In mid-log and especially in early stationary phase, granules, which we believe are either starch granules or polyhydroxybutyrate inclusions, were present in the mother cell (Fig. 2B). Similar granules have been reported in other sporulating organisms and serve as an energy source for the cell during sporulation (16, 37, 61).

C. perfringens KM2 (*sigE* mutant) cells never developed beyond the stage of asymmetric septum formation. During mid-log phase, all of the cells viewed were vegetative rods (Fig. 2D and Table 2). In the early stationary phase, cells were forming asymmetric septa and some had a disporic phenotype (Fig. 2E) characteristic of *sigE* mutants in *B. subtilis* (29). By middle stationary phase, many cells had formed multiple asymmetric septa (Fig. 2F), which is another characteristic of mutants lacking σ^E in *B. subtilis*. Cells in early stationary and middle stationary phases also had granules (Fig. 2E and F), in contrast to the wild-type strain, in which granules were present only in early-stationary-phase cells.

In *B. subtilis*, a mutation in *sigK* blocks sporulation at stage IV, when phase-gray forespores have formed (17). Surprisingly, *C. perfringens* KM1 (*sigK* mutant) appeared to be blocked early in development. During mid-log phase and early stationary phase, all of the cells were in the vegetative state (Fig. 2G and H and Table 2). Most of the cells were still vegetative at middle stationary phase (Fig. 2I); however, a low number of cells had formed an asymmetric septum (stage II), were disporic, or had completed engulfment (stage III) (Table 2). None of the cells that were characterized had granules (Table 2). Considering the absence of granules and the low number of cells with asymmetric septa, we propose that the *sigK* mutant is blocked at an earlier stage of development than the *sigE* mutant, which is different than what occurs in *B. subtilis*.

Transcription of sporulation sigma factor-encoding genes during early log and early stationary phases in *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant). RT-PCR was utilized to determine if and when *sigF*, *sigE*, *sigG*,

TABLE 2. Comparison of cell morphologies of *C. perfringens* wild-type SM101 and the mutant strains KM2 (*sigE* mutant) and KM1 (*sigK* mutant)[a]

Strain	Hours after inoculation	Granules (%)	Vegetative (%)	Asymmetric septum (%)	Engulfment (%)	Disporic (%)	Multiseptate at one pole (%)	Stage IV/free spores (%)
SM101 (wild type)	3	15	90	5	5	0	0	0
	5	80	37	0	63	0	0	0
	8	0	11	0	16	0	0	74
KM2 (*sigE* mutant)	3	0	100	0	0	0	0	0
	5	32	71	15	0	12	3	0
	8	35	41	0	0	12	35	0
KM1 (*sigK* mutant)	3	0	100	0	0	0	0	0
	5	0	100	0	0	0	0	0
	8	0	76	11	9	4	0	0

[a] The phenotypes noted were quantified by observing only cells in which complete longitudinal sections were present.

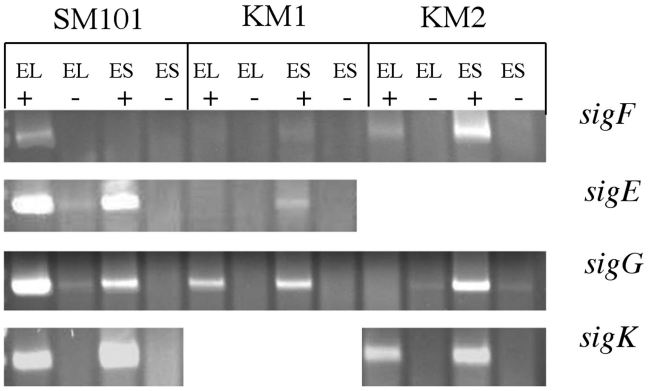


FIG. 3. Transcription of sporulation sigma factor genes in *C. perfringens* SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) strains. RT-PCR of the four sporulation sigma factor genes in strains SM101, KM1, and KM2. Samples were collected during early log phase (EL) (OD_{600} of 0.1) and early stationary phase (ES) (OD_{600} of 1.0). + indicates reactions in which reverse transcriptase was added to the reaction. − indicates 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.

and *sigK* transcripts accumulate in *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant). RT-PCR amplification of *sigE*, *sigG*, and *sigK* in SM101 determined that transcripts from these three genes accumulate during early log (OD_{600} of 0.1) and early stationary phases (OD_{600} of 1.0), while *sigF* transcripts accumulated only during the early log phase (Fig. 3). The early-log-phase transcription of all the sporulation sigma factor-encoding genes was unexpected, because the orthologous genes in *B. subtilis* and *C. acetobutylicum* are not highly expressed during logarithmic growth but are induced during transition into sporulation (15, 32).

RT-PCR analysis of *C. perfringens* KM1 (*sigK* mutant) indicated that σ^K-associated RNA polymerase is necessary for normal accumulation of *sigF* and *sigE* transcripts, but not *sigG* transcripts, although there appeared to be a reduced level of *sigG* transcripts in early log phase in strain KM1 compared to SM101 (Fig. 3). While there was no accumulation of *sigF* or *sigE* transcripts in early log phase, there appeared to be very low levels of these transcripts in early stationary phase. The dependence of *sigF* and *sigE* transcript accumulation on σ^K-associated RNA polymerase was unexpected, on the basis of the *B. subtilis* model (σ^K is last in the cascade) but could in part account for the early developmental block observed for KM1 (*sigK* mutant) (Fig. 2G to I and Table 2).

For the sporulation sigma factor encoding-genes in *C. perfringens* KM2 (*sigE* mutant), accumulation of all *sig* transcripts was affected (Fig. 3). The *sigF* transcript accumulated in early stationary phase (unlike in SM101). There was no accumulation of the *sigG* transcript during early log phase (unlike SM101), but this transcript did accumulate in early stationary phase (comparable to SM101). The *sigK* transcript was present during early log and early stationary phase, but in slightly reduced amounts compared to SM101. In *B. subtilis*, *sigK* is not transcribed in the absence of σ^E-associated RNA polymerase, so the presence of the *sigK* transcript in KM2 (*sigE* mutant) was surprising and was investigated further as described below.

Expression from the *spoIIGA* and *sigK* promoter regions is eliminated in *C. perfringens* KM2 (*sigE* mutant) and KM1 (*sigK* mutant). Since the results shown in Fig. 3 indicated that mutations in the *sigE* and *sigK* genes have effects on *sigK* and *sigE* transcript levels, respectively, we decided to obtain a more quantitative analysis of expression by using translational fusions of the *spoIIGA* (the *spoIIGA* promoter drives transcription of the *spoIIGA-sigE* operon in *B. subtilis*) and *sigK* promoter regions (i.e., the promoter immediately upstream of the *sigK* gene) to a promoterless version of the *E. coli gusA* gene, which encodes the enzyme β-glucuronidase. However, we first needed to identify potential promoters upstream of the *spoIIGA* and *sigK* genes by primer extension experiments (see Fig. S1 and S2 in the supplemental material). For *spoIIGA*, a 5′ end at −15 relative to the ATG initiator codon was seen, and for *sigK*, 5′ ends at −68 and −117 were seen relative to the ATG initiator codon (see Fig. S1 and S2 in the supplemental material). Using this information, we constructed the fusion to contain sequences that include ~200 bp upstream of the open reading frame for *spoIIGA* and for *sigK* to include the promoter located at −68 and upstream sequences (see Materials and Methods).

Strains were induced to sporulate by inoculation into DSSM, and samples were removed to measure β-glucuronidase activity. The activities of the *spoIIGA-gusA* fusion in *C. perfringens* strains SM101, KM2 (*sigE* mutant), and KM1 (*sigK* mutant) are shown in Fig. 4A. In SM101, the specific activity increased considerably between 2 and 3 h, which correlates with early or mid-log growth phase (Fig. 4C). This result is consistent with early-log *sigE* transcript accumulation (Fig. 3). The activity of the *spoIIGA-gusA* fusion was not above background levels of pSM240 (vector control with no promoter upstream of *gusA*) in KM2 (*sigE* mutant) and KM1 (*sigK* mutant) (Fig. 4B), indicating that both σ^E- and σ^K-associated RNA polymerase are directly or indirectly necessary for expression from the *spoIIGA-gusA* fusion. The dependence of expression on σ^K-associated RNA polymerase is consistent with the greatly reduced *sigE* transcript level that we observed with KM1 (*sigK* mutant) (Fig. 3). Although we cannot be certain that the *spoIIGA* promoter is the source of the small amount of *sigE* transcript detected by RT-PCR in KM1 (*sigK* mutant) (Fig. 3) or that the *spoIIGA* promoter fragment fused to *gusA* functions completely normally (Fig. 4), the small difference in the results could indicate that RT-PCR is more sensitive than the *gusA* fusion. The dependence of *spoIIGA-gusA* activity on σ^E-associated RNA polymerase suggests direct or indirect autoregulation, a feature not found with *B. subtilis* (phosphorylated Spo0A stimulates transcription from the *spoIIGA* promoter by σ^A-associated RNA polymerase) (23).

The activity of the *sigK-gusA* fusion in *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) was also examined. SM101 showed induction of activity between 3 and 4 h after inoculation into sporulation media (Fig. 4D), corresponding to the late log or early stationary phase of growth (Fig. 4F). The absence of activity in the early log phase suggested that a different promoter was responsible for the *sigK* transcript detected by RT-PCR of RNA from early-log-phase cells (Fig. 3) (see below). Expression from the *sigK-gusA* fusion was significantly reduced during sporulation, but above the background level of activity seen with plasmid pSM240, in

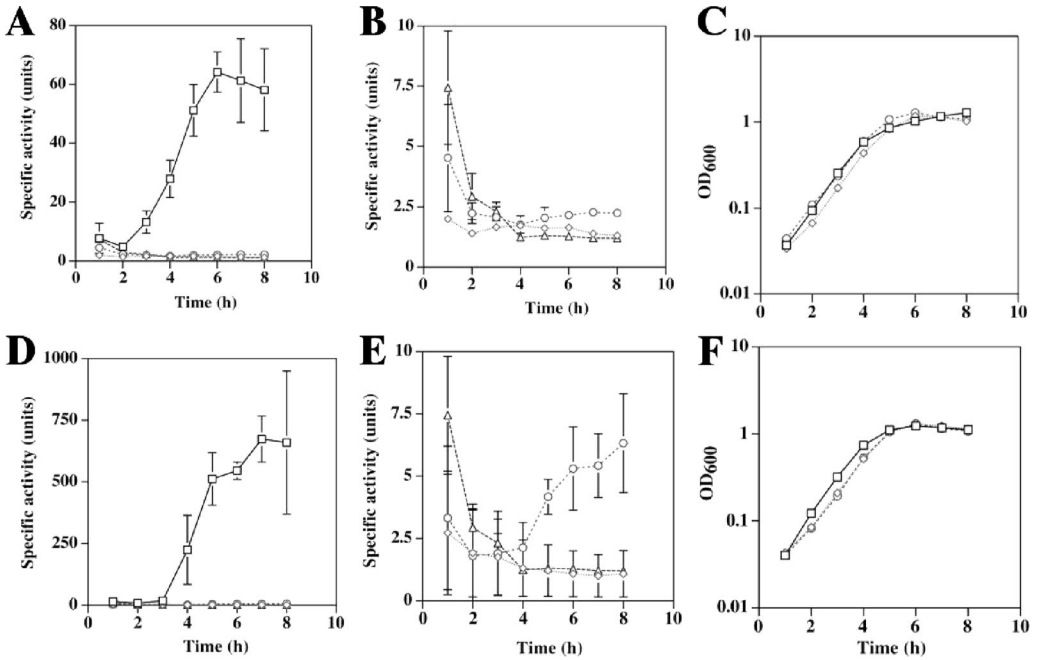


FIG. 4. Expression of the *spoIIGA-gusA* and *sigK-gusA* translational fusions in *C. perfringens* wild-type SM101 and mutants KM1 (*sigK* mutant) and KM2 (*sigE* mutant). (A) β-Glucuronidase activities of the *spoIIGA-gusA* fusion in *C. perfringens* strains SM101 (squares), KM2 (diamonds), KM1 (circles), and SM101 with the plasmid backbone pSM240 (triangles). (B) β-Glucuronidase activities shown in panel A are shown on a different scale. (C) Representative growth curves of strains SM101 (squares), KM2 (diamonds), and KM1 (circles) containing the *spoIIGA-gusA* fusion. (D) β-Glucuronidase activities of the *sigK-gusA* fusion in strains SM101 (squares), KM2 (diamonds), KM1 (circles), and the SM101(pSM240) control (triangles). (E) β-Glucuronidase activities shown in panel D are shown on a different scale. (F) Representative growth curves of strains SM101 (squares), KM2 (diamonds), and KM1 (circles) containing the *sigK-gusA* fusion.

KM1 (*sigK* mutant) (Fig. 4E), indicating that σ^K-associated RNA polymerase is needed for most, but not all, of the expression. However, *sigK-gusA* fusion activity was not above background levels in KM2 (*sigE* mutant) during sporulation (Fig. 4E), indicating that σ^E-associated RNA polymerase is absolutely necessary for expression. Absolute dependence of the *sigK* promoter on σ^E-associated RNA polymerase and partial dependence on σ^K-associated RNA polymerase (i.e., autoregulation) are features of the *sigK* promoter in *B. subtilis* (43).

***sigK* is transcribed by readthrough from an upstream gene, and the *sigK* mutation does not prevent transcription of downstream genes.** The results shown in Fig. 4E, in which there was no transcription from the *sigK* promoter in the *sigE* mutant (KM2), seemed to contradict the results that we obtained by RT-PCR analysis (Fig. 3), in which there was appreciable *sigK* transcription in the *sigE* mutant. One possible explanation that could account for these results was that a transcript originating from the gene (CPR_1739) upstream of the *sigK* gene was reading through into the *sigK* gene. To determine if this was occurring and also to determine if the mutation we constructed in *sigK* affects transcription of downstream genes, we characterized transcripts from the region surrounding *sigK* using RT-PCR (Fig. 5A). RNA was extracted from *C. perfringens* SM101 and KM1 cultures during early log phase and early stationary phase in sporulation medium. We found that in SM101 there is a transcript that extends from CPR_1739 into *sigK* in both early-log and early-stationary-phase cells (Fig. 5B). As shown in Fig. 5A, the upstream primer, OKM12, lies in the coding region of CPR_1739. It hybridizes to DNA upstream of the region that was amplified during RT-PCR analysis of the *sigK* transcript (Fig. 3). This result provided an explanation for the RT-PCR product seen with the *sigE* mutant: it was actually derived from readthrough transcription from CPR_1739 into *sigK* and did not originate from the *sigK* promoter.

There was no transcript detected by RT-PCR between the *sigK* and *pilT* genes (Fig. 5B), indicating that *pilT* is under the control of its own promoter. However, *pilT*, whose gene product is an ATPase necessary for type IV pilus-dependent motility in *C. perfringens* (69), is transcribed in early-log and stationary-phase cells of SM101 (Fig. 5B). It also seems that *pilT* and the downstream gene, *ftsA*, are cotranscribed in SM101, especially in early-log-phase cells. *ftsA* is upstream of *ftsZ* in strain SM101, and the two genes are located in an operon in *B. subtilis* (5).

RNA was extracted from cultures of *C. perfringens* KM1 (*sigK* mutant) grown in sporulation medium during early log phase and early stationary phase, and transcripts of genes in the *sigK* region were analyzed (Fig. 5C). There was a very low level of a transcript spanning from CPR_1739 to *sigK* in early log phase and a somewhat higher level in early stationary phase, but less than in that of wild-type cells (Fig. 5B). As in *C. perfringens* SM101, no transcript was observed between *sigK* and *pilT*. Also, as in SM101, transcripts from *pilT* and spanning from *pilT* to *ftsA* were detected in early-log and stationary-phase cells, indicating that the mutation in the *sigK* gene does not prevent transcription of downstream genes and that σ^K-associated RNA polymerase is not required for transcription of *pilT* and *ftsA*.

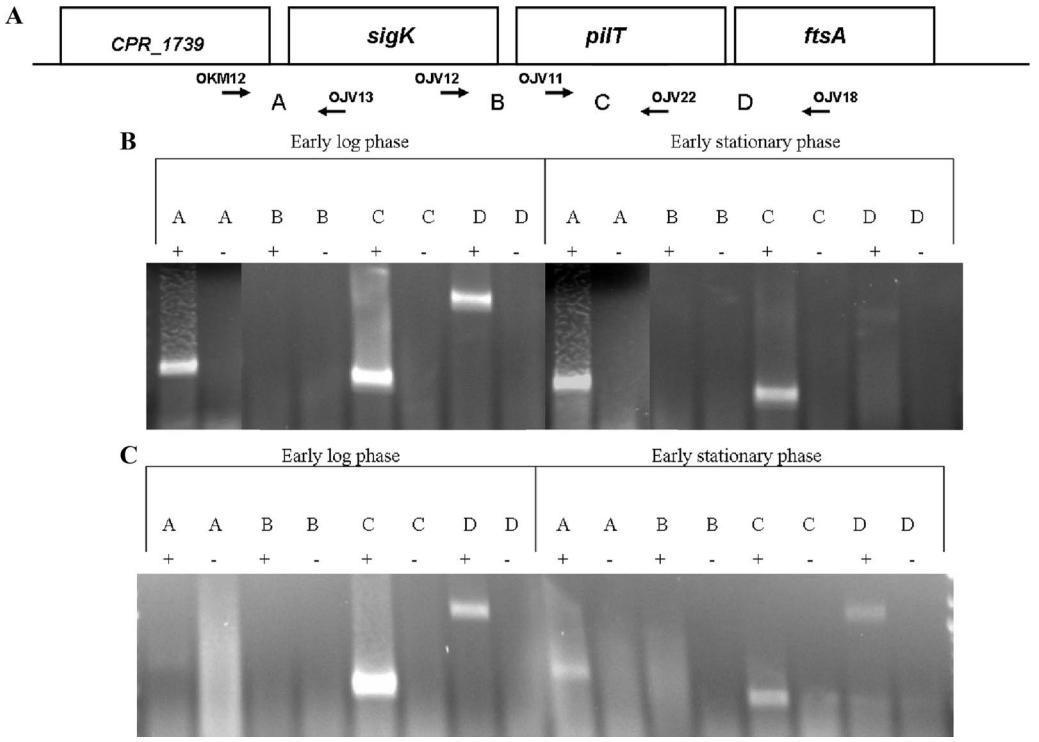


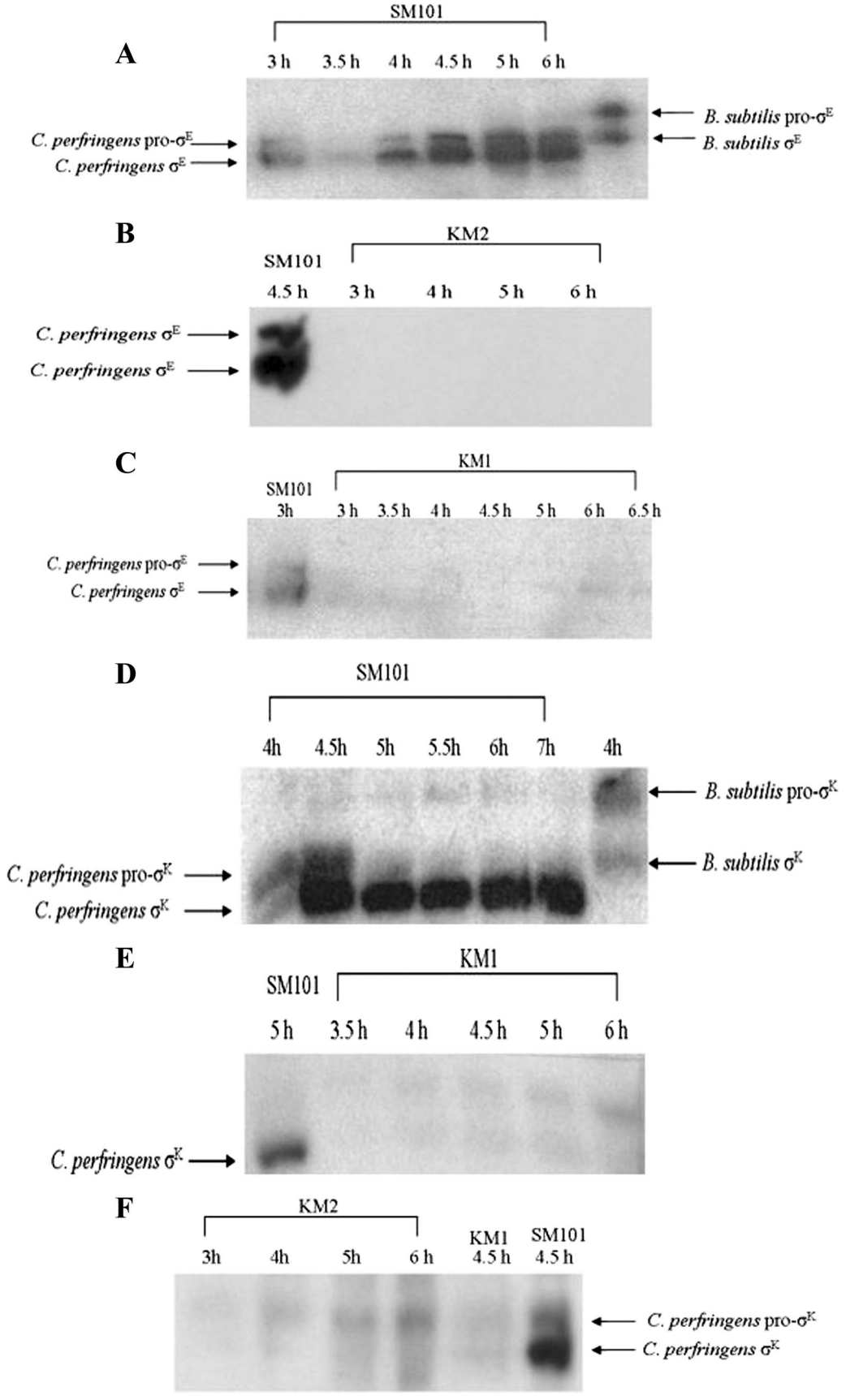
FIG. 5. Transcription of genes in the *sigK* region in *C. perfringens* SM101 and KM1 (*sigK* mutant). (A) The location of genes surrounding the *sigK* gene. The letter "A" indicates the region between primers OKM12 and OJV13, "B" between OJV12 and OJV22, "C" between OJV11 and OJV22, and "D" between OJV11 and OJV18. Arrows indicate the locations where primers anneal in relation to the respective genes. RNA from *C. perfringens* SM101 (B) and KM1 (*sigK* mutant) (C) was harvested during early log (OD_{600} of 0.1) and early stationary phase (OD_{600} of 1.0), after inoculation into DSSM sporulation medium. Letters indicate which primer pairs were used in the reaction. + indicates reactions in which reverse transcriptase was added to the reaction, while − indicates negative controls in which reverse transcriptase was omitted. Products were viewed on an ethidium bromide-stained 2% agarose gel.

Pro-σ^E/σ^E and pro-σ^K/σ^K were not detected in *C. perfringens* KM1 (*sigK* mutant), and only a low level of pro-σ^K was observed with KM2 (*sigE* mutant). In order to determine if mutations in the *sigE* and *sigK* genes abolished production of these proteins, samples were taken from cultures after inoculation into DSSM sporulation medium, and Western blot analyses were performed. Antibodies generated against *B. subtilis* pro-σ^E/σ^E were found to detect *C. perfringens* pro-σ^E/σ^E in strain SM101 at 3 h postinoculation (Fig. 6A), correlating with the mid-log phase of growth (data not shown). There was no pro-σ^E/σ^E detected in KM2 (*sigE* mutant) (Fig. 6B) or KM1 (*sigK* mutant) (Fig. 6C). These results were expected, since no activity was detected from the *spoIIGA* promoter in KM2 (*sigE* mutant) or KM1 (*sigK* mutant) (Fig. 4B) and very little *sigE* transcript was detected by RT-PCR (Fig. 3), which might be more sensitive than the *gusA* fusion or the immunoblotting.

Antibodies generated against *B. subtilis* pro-σ^K/σ^K were found to detect *C. perfringens* SM101 pro-σ^K/σ^K at 4 h postinoculation (Fig. 6D), correlating with the late log phase of growth (data not shown). By 5 h, almost all of the pro-σ^K had been processed to σ^K. There was no pro-σ^K/σ^K detected in KM1 (*sigK* mutant) (Fig. 6E), but a very low level of pro-σ^K was observed in KM2 (*sigE* mutant) (Fig. 6F), consistent with our finding that the *sigK* transcript is present, albeit at a reduced level compared with that in strain SM101 (Fig. 3), presumably due to readthrough transcription from CPR_1739 (Fig. 5B). In agreement with the suggestion above that RT-PCR is more sensitive than immunoblotting, we note that the *sigK* transcript was readily detected in the *sigE* mutant strain (Fig. 3), but pro-σ^K was barely detectable in this strain (Fig. 6F).

Pro-σ^E/σ^E is partially restored, but pro-σ^K/σ^K is not detectable in the complemented *sigE* mutant and *sigK* mutant strains. In the complemented *C. perfringens* strains, KM2(pKM3) (complemented *sigE* mutant) and KM1(pKM2) (complemented *sigK* mutant), 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 processing to σ^E and total σ^E levels were reduced in comparison to that seen in *C. perfringens* SM101 (Fig. 7). There was no pro-σ^K/σ^K detected in KM1(pKM2) or KM2(pKM3) (data not shown). Although σ^K was not restored to a detectable level in KM1(pKM2) or KM2(pKM3), presumably both strains make a very low level of σ^K, which allows the partial complementation of sporulation that we observed (Fig. 1). The failure to fully restore σ^E and σ^K synthesis may explain the observed partial complementation (see Discussion).

SpoIIID is not regulated in *C. perfringens* in the same manner as it is in *B. subtilis*. In the *B. subtilis* 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 (39). In *B. subtilis*, the


A
SM101
3 h
3.5 h
4 h
4.5 h
5 h
6 h
C. perfringens pro-σE
C. perfringens σE
B. subtilis pro-σE
B. subtilis σE
B
KM2
SM101
4.5 h
3 h
4 h
5 h
6 h
C. perfringens σE
C. perfringens σE
C
KM1
SM101
3h
3 h
3.5 h
4 h
4.5 h
5 h
6 h
6.5 h
C. perfringens pro-σE
C. perfringens σE
D
SM101
4h
4.5h
5h
5.5h
6h
7h
4h
B. subtilis pro-σK
B. subtilis σK
C. perfringens pro-σK
C. perfringens σK
E
KM1
SM101
5 h
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5 h
6 h
C. perfringens σK
F
KM2
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C. perfringens pro-σK
C. perfringens σK

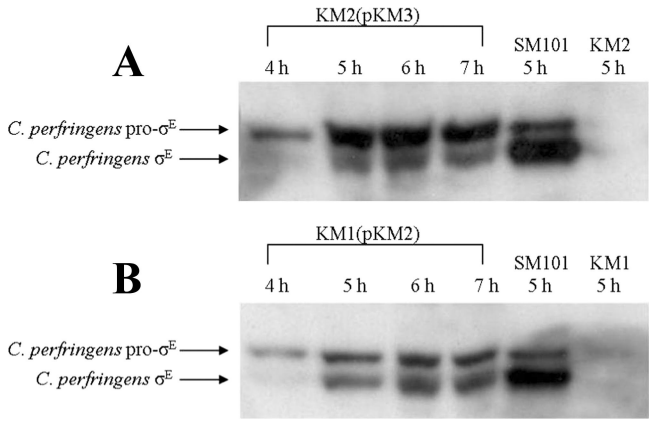

FIG. 7. Western blots of pro-σ^E and σ^E in the complemented *C. perfringens* KM2 (*sigE* mutant) and KM1 (*sigK* mutant) strains. *C. perfringens* strains KM2(pKM3) (complemented *sigE* mutant) and KM1(pKM2) (complemented *sigK* mutant) were inoculated into sporulation medium, and samples were collected over an 8-h time period. (A and B) Western blot analyses using anti-σ^E antibodies generated against *B. subtilis* σ^E were performed on the indicated strains. A *C. perfringens* SM101 sample served as a positive control. A *C. perfringens* KM2 (*sigE* mutant) sample served as a negative control when analyzing the KM2(pKM3) (complemented *sigE* mutant) blot, while *C. perfringens* KM1 (*sigK* mutant) served as a negative control on the KM1(pKM2) (complemented *sigK* mutant) blot.

transcription of *spoIIID* is directed by σ^E-associated RNA polymerase (31).

A SpoIIID ortholog, the CPR_2156 protein, exhibiting 62% identity to the SpoIIID protein of *B. subtilis*, was detected in the genomic sequence of *C. perfringens* strain SM101 (http://www.ncbi.nlm.nih.gov/sites/entrez?db = genomeprj&cmd = Retrieve&dopt = Overview&list_uids = 12521). RT-PCR was performed to determine if the *C. perfringens spoIIID* gene is part of an operon with the upstream gene, CPR_2157, which codes for a putative peptidase. Primers were designed to hybridize to sequences in *spoIIID* and CPR_2157. An amplification product indicated that an RNA transcript was present, suggesting that *spoIIID* is transcribed from a promoter upstream of CPR_2157, sometime after early log phase (Fig. 8A). The *spoIIID* transcript was also detected in *C. perfringens* KM2 (*sigE* mutant) and therefore is not under σ^E-associated RNA polymerase control (Fig. 8A), as it is in *B. subtilis* (31). The *spoIIID* transcript was also observed in the *sigK* mutant strain KM1 (Fig. 8A).

Western blot analysis using antibodies directed against the *B. subtilis* SpoIIID protein indicated that SpoIIID in *C. perfringens* is expressed during sporulation of strains SM101, KM2 (*sigE* mutant), and KM1 (*sigK* mutant) during late log phase (Fig. 8B). We conclude that in *C. perfringens* SpoIIID is not under σ^E control, as it is in *B. subtilis*.

Both σ^E and σ^K are necessary for expression of the *cpe* gene. In previous studies, Zhao and Melville (73) 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*, they are possibly σ^E- and σ^K-dependent in *C. perfringens*. Plasmids containing different segments spanning the *cpe* promoter region fused to the *E. coli* reporter gene *gusA* were described previously (Fig. 9A) (49, 73). These plasmids were transformed into *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) by electroporation, sporulation was induced by growing the cells in DSSM, and samples were removed to measure β-glucuronidase activity. The construct containing all three *cpe* promoters (pSM104) and the construct containing P1 and P2 (pSM127) produced similar levels of β-glucuronidase activity in SM101 (Fig. 9B and C). The construct containing only P3 (pSM170) exhibited 19% as much activity as the other two constructs (Fig. 9D). For all three *cpe-gusA* constructs, induction of β-glucuronidase activity occurred between 3 h and 4 h after inoculation into sporulation medium, when cells were between the mid-log and early stationary phases of growth (Fig. 9E). These results are similar to those reported previously (73) and suggest that most *cpe* transcription is derived from P1 and P2. However, there was no β-glucuronidase activity detected for pSM104, pSM127, or pSM170 in *C. perfringens* KM1 (*sigK* mutant) or KM2 (*sigE* mutant) (Fig. 9B to D), indicating that σ^E and σ^K are needed for expression from all three *cpe* promoters.

DISCUSSION

By making mutations in the *C. perfringens sigE* and *sigK* genes, we not only confirmed the hypothesis that *cpe* expression would depend on these sigma factors (73), we discovered that expression and function of these sigma factors are quite different than in *B. subtilis*. First, a *sigK* mutant is blocked earlier during the sporulation process than a *sigE* mutant in *C. perfringens* (Fig. 2), whereas the opposite is observed for *B. subtilis* (57). Consistent with the early block in the *C. perfringens sigK* mutant, *sigF* and *sigE* transcripts fail to accumulate normally (Fig. 3). Also, consistent with an early role of σ^K, we discovered that *sigK* is cotranscribed with an upstream gene during the early log phase of growth (Fig. 5). Moreover, *sigF*, *sigE*, and *sigG* transcripts also accumulate in the early log phase in *C. perfringens*, a second major difference from *B. subtilis*. A third important difference between the regulatory networks is that expression of a key mother cell transcription factor, SpoIIID, depends on σ^E-associated RNA polymerase in *B. subtilis* (31), but not in *C. perfringens* (Fig. 8). We speculate that these differences reflect different signals for initiation of sporulation by the

FIG. 6. Western blots of σ^E and σ^K in *C. perfringens* wild-type and mutant strains. Wild-type strain SM101 and mutant strains KM2 (*sigE* mutant) and KM1 (*sigK* mutant) were inoculated into sporulation medium, and samples were collected over an 8-h time course. (A to C) Western blot analyses using anti-σ^E antibodies generated against *B. subtilis* σ^E were performed on the indicated strains. A *B. subtilis* sample served as a positive control when analyzing the *C. perfringens* SM101 blot. *C. perfringens* SM101 samples served as positive controls in blots analyzing σ^E in the mutant strains. (D to F) Western blot analyses using anti-σ^K antibodies generated against *B. subtilis* pro-σ^K were performed on the indicated strains. A *B. subtilis* sample served as a positive control when analyzing the *C. perfringens* SM101 blot. *C. perfringens* SM101 samples served as positive controls in blots when analyzing mutant strains. *C. perfringens* KM1 (*sigK* mutant) was a negative control on the *C. perfringens* KM2 (*sigE* mutant) blot.

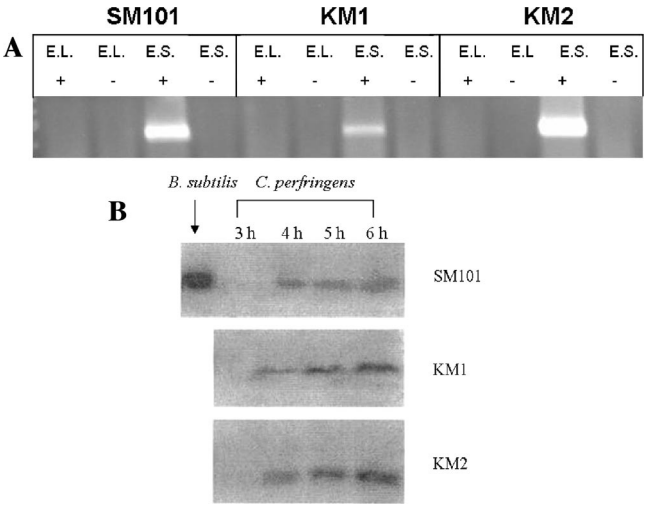

FIG. 8. Analysis of *spoIIID* transcript and SpoIIID protein levels in *C. perfringens* wild-type and mutant strains. (A) After inoculation into sporulation medium, samples of the wild-type strain SM101 and mutant strains KM1 (*sigK* mutant) and KM2 (*sigE* mutant) were collected during early log phase (E.L.) (OD_{600} of 0.1) and early stationary phase (E.S.) (OD_{600} of 1.0) and analyzed for the *spoIIID* transcript by RT-PCR. + indicated a reaction in which reverse transcriptase was added to the reaction, − indicates a negative control reaction in which reverse transcriptase was omitted. Products were viewed on an ethidium bromide-stained 2% agarose gel. (B) *C. perfringens* strains SM101, KM1 (*sigK* mutant), and KM2 (*sigE* mutant) were inoculated into sporulation medium, and samples were collected over an 8-h time course. Western blot analysis using anti-SpoIIID antibodies generated against *B. subtilis* SpoIIID was performed on the samples. *B. subtilis* SpoIIID was used as a positive control.

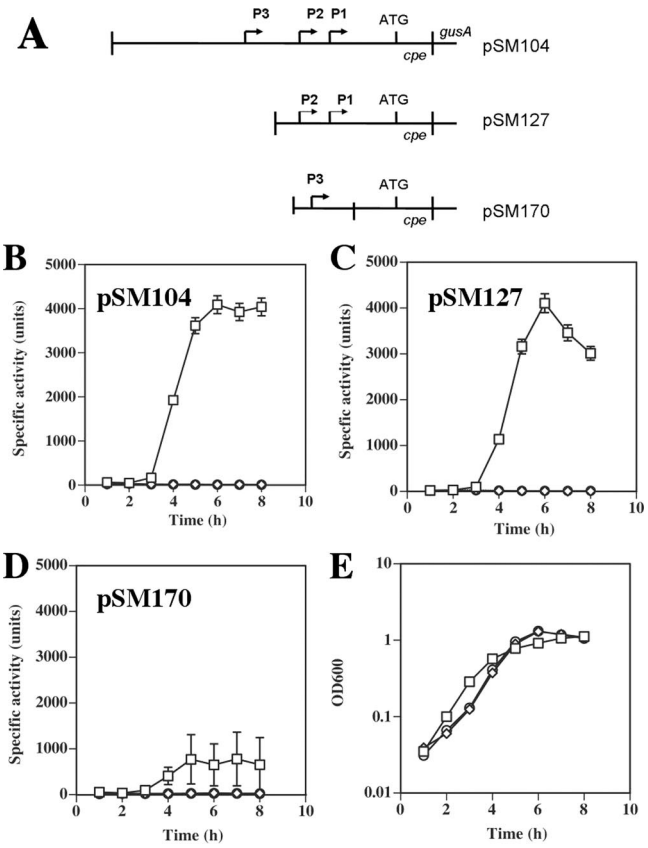

FIG. 9. Expression of *cpe-gusA* fusions in *C. perfringens* wild-type and mutant strains. (A) Schematic diagram of the *C. perfringens cpe* translational fusions to *E. coli gusA*. (B to D) Expression from the *cpe* promoters in the indicated plasmids was measured by the specific activity of β-glucuronidase in samples collected during an 8-h time period after inoculation of wild-type SM101 (squares) and mutants KM1 (*sigK* mutant) (diamonds) and KM2 (*sigE* mutant) (circles) into sporulation medium. (E) Representative growth curves of strains SM101 (squares), KM1 (*sigK* mutant) (diamonds), and KM2 (*sigE* mutant) (circles) containing pSM104 in sporulation medium (growth patterns with strains containing the other plasmids were very similar).

two bacteria and the need for *C. perfringens* to rapidly produce spores in its ecological niches.

The *sigE* and *sigK* genes were mutagenized by insertion of a multimeric plasmid into the chromosomal copy of each gene. We found that KM2 (*sigE* mutant) and KM1 (*sigK* mutant) strains of *C. perfringens* produce less than 10 spores/ml. When complemented with wild-type copies of the *spoIIGA-sigE* and *sigK* genes, sporulation increased dramatically, 3,000- to 4,000-fold, but was not fully restored (1 to 2% efficiency compared with 80% efficiency for the wild-type strain) (Fig. 1). Repeated attempts were made to mutagenize the *sigE* and *sigK* genes by using transformation with monomeric forms of the plasmids and also transforming with large quantities (~20 μg) of linearized DNA to enhance the frequency of allele replacement, but none of these experiments resulted in antibiotic-resistant colonies (data not shown). In the past, our group has constructed mutations in strain SM101 using allele replacement by homologous recombination methods (68), so the lack of transformants with anything except the multimeric form of the plasmids indicates that the *sigE* and *sigK* genes are relatively resistant to recombination events.

The similar complementation efficiencies of the *C. perfringens sigE* and *sigK* mutants (Fig. 1) were accompanied by partial rescue of σ^E production in both mutants (Fig. 7), but neither pro-σ^K nor σ^K was detected (data not shown). In the case of *C. perfringens* KM2(pKM3) (the complemented *sigE* mutant), the *spoIIGA-sigE* genes in pKM3 restored production of pro-σ^E, but processing of pro-σ^E to σ^E was delayed and reduced (Fig. 7A) compared with that seen with the wild-type strain (Fig. 6A). This may have been due to aberrant levels of expression of the putative protease, SpoIIGA, and its substrate, pro-σ^E, and the decreased level of σ^E may be insufficient for detectable production of pro-σ^K/σ^K, resulting in a lack of full complementation. For *C. perfringens* KM1(pKM2) (the complemented *sigK* mutant), pKM2 did not include the upstream gene CPR_1739, from which transcription reads through into *sigK* during the early log and early stationary phases (Fig. 5B). We attempted to clone both the CPR_1739 gene and *sigK* together in the vector used for complementation, but for unknown reasons, we were unable to obtain any such plasmids in *E. coli*. We hypothesize that the lack of *sigK* transcription from the upstream promoter accounts for the observed partial complementation of KM1 by pKM2 (Fig. 1). Specifically, production of σ^E was compromised (Fig. 7B). The decreased level of σ^E may be insufficient for detectable production of pro-σ^K/σ^K from pKM2, just as we propose it is insufficient for production of detectable pro-σ^K/σ^K from the

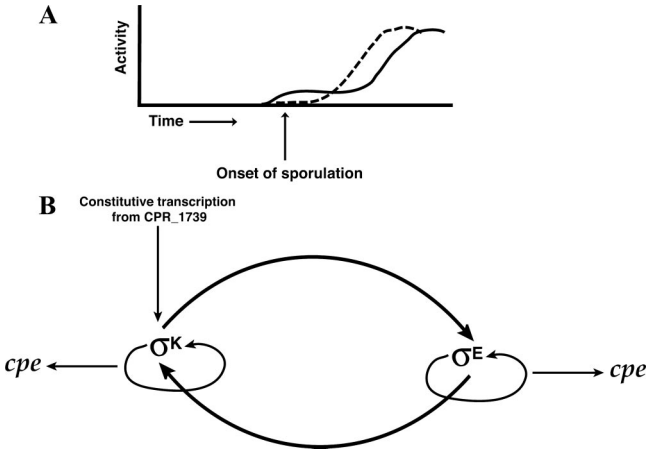

FIG. 10. Activity and regulation of σ^E and σ^K in *C. perfringens*. (A) Diagram of the proposed activity profile of σ^E (dashed line) and σ^K (solid line) during growth and sporulation of *C. perfringens*. (B) Model illustrating proposed regulation of σ^E, σ^K, and *cpe* in the mother cell compartment of *C. perfringens*. Arrows indicate positive regulation, which may be direct or indirect.

chromosome in KM2(pKM3), resulting in a similar lack of full complementation (Fig. 1). Presumably, although σ^K was undetectable by immunoblotting, a small amount was made in these strains, allowing sporulation at low efficiency. While we favor this explanation and note that incomplete complementation of mutants made by plasmid insertion has been reported in several *C. perfringens* studies (54, 55, 59, 68), we cannot rule out the possibility that our results are due to an unknown secondary mutation.

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 (29). Our results demonstrate that a *C. perfringens sigE* mutant is blocked at a similar stage of development as a *B. subtilis sigE* mutant, stage II, as revealed by EM (Fig. 2 and Table 2). A mutation in the *B. subtilis* σ^K-encoding gene is characterized by the completion of engulfment, but spore development stops before spore cortex and spore coat can be added (57). Unexpectedly, a *C. perfringens sigK* mutant appeared to be blocked in sporulation at an earlier stage of development (stage 0) than the *sigE* mutant (Fig. 2 and Table 2). The block in sporulation appears to be earlier because of the lack of asymmetric septa and granules in the *sigK* mutant strain. How could σ^K be active before σ^E if the *sigK* promoter is dependent on a functional *sigE* gene (Fig. 4)? A model that can explain our results is shown in Fig. 10. We hypothesize that *sigK* expression is biphasic, with transcription from a promoter within or upstream of CPR_1739 (Fig. 5), providing in log and early stationary phases a low level of σ^K that is needed at an unknown early step in the sporulation process (Fig. 10A). After this early σ^K-dependent step, *sigE* is expressed in a σ^K-dependent step, and σ^E is then responsible for transcription of the *sigK* gene from the *sigK* promoter (Fig. 4), resulting in a high level of σ^K (Fig. 10A). However, because σ^E synthesis is dependent on σ^K activity and σ^E then directs a high level of both σ^K and σ^E synthesis later in sporulation, σ^E production and σ^K production are depicted as being codependent on each other, as shown in Fig. 10B.

It is likely that in its early phase of activity at the onset of sporulation, σ^K is needed for functions other than just positively regulating transcription of the *spoIIGA-sigE* operon, since the *sigK* mutant has a different phenotype than does the *sigE* mutant (Fig. 2). The effect of σ^K on transcription of the *spoIIGA-sigE* operon may be indirect since our evidence suggests this operon is transcribed by σ^A-associated RNA polymerase with activation by (presumably phosphorylated) Spo0A.

Additional evidence in support of a role for σ^K early in sporulation comes from analysis of the transcript levels for *sigF* in *C. perfringens*. In *B. subtilis*, *sigF* is transcribed prior to asymmetric septum formation by σ^H-associated RNA polymerase, with activation by phosphorylated Spo0A (71). The phenotype of a *B. subtilis sigF* mutant is a disporic cell (57). *C. perfringens* KM1 (*sigK* mutant) appears to be blocked in sporulation mostly at stage 0 (Fig. 2 and Table 2), indicating an early block at the morphological level, and the RT-PCR results show that accumulation of *sigF* transcripts is nearly eliminated in KM1 (Fig. 3), suggesting that σ^K acts prior to σ^F in the regulatory network that governs sporulation.

Interestingly, *sigG* transcripts accumulated in *C. perfringens* KM1 (*sigK* mutant) and KM2 (*sigE* mutant), albeit accumulation was reduced and delayed, respectively (Fig. 3). The finding that *sigG* transcripts accumulated at all is somewhat surprising, since in *B. subtilis* the transcription of *sigG* depends on σ^F (33, 65) and σ^E (56), and we expect very little σ^F to be present in *C. perfringens* KM1 (given the low *sigF* transcript level), and no σ^E was detected in KM1 or KM2 (Fig. 6). The lack of stronger dependence of *sigG* transcription on σ^E in *C. perfringens* is clearly different from the *B. subtilis* paradigm, and another difference may be that *sigG* transcription does not depend on σ^F, although we cannot rule out the possibility that a small amount of σ^F in KM1 accounts for initial *sigG* transcription, which is followed by autoregulation (i.e., σ^G-directed transcription of *sigG*), which is observed in *B. subtilis* (64).

In *B. subtilis*, σ^A-associated RNA polymerase transcribes the *spoIIGA-sigE* operon, with activation by phosphorylated Spo0A (3, 20). Surprisingly, our *gusA* fusion results indicated that in *C. perfringens*, σ^E- and σ^K-associated RNA polymerase are both necessary for expression of the *spoIIGA-sigE* operon (Fig. 4). As additional evidence for this effect, Western blot analyses showed that pro-σ^E and σ^E were not detected in *C. perfringens* KM2 (*sigE* mutant) or KM1 (*sigK* mutant) (Fig. 6B and C). A very low level of *sigE* transcript was detected in KM1 (Fig. 3) but apparently is insufficient to produce a detectable level of pro-σ^E or σ^E (Fig. 6C). As depicted in our model (Fig. 10B), σ^E production depends on both σ^K and σ^E, but we do not know whether these directly recognize the *spoIIGA-sigE* promoter or indirectly affect σ^A-associated RNA polymerase or Spo0A.

The β-glucuronidase assays indicated that in *C. perfringens*, σ^E-associated RNA polymerase is necessary for expression from the *sigK* promoter (Fig. 4D and E), which is consistent with genetic analysis in *B. subtilis* (43, 51). The *B. subtilis* σ^E-associated RNA polymerase has been shown to transcribe from the *sigK* promoter in vitro, with activation by SpoIIID (22). We also observed that σ^K-associated RNA polymerase is necessary for the vast majority of transcription from the *sigK* promoter (Fig. 4D and E). While it appears from genetic

studies that σ^K-associated RNA polymerase is responsible for about two-thirds of the *sigK* transcription in *B. subtilis* (43) and while in vitro studies demonstrate that σ^K-associated RNA polymerase can recognize the *sigK* promoter (39), expression of *sigK* in *C. perfringens* KM1 (*sigK* mutant) was 100-fold lower than that in SM101 (Fig. 4D and E). Western blot analyses indicated that pro-σ^K and σ^K were not detectable in *C. perfringens* strains KM1 (*sigK* mutant) and KM2 (*sigE* mutant) (Fig. 6E and F). The fact that pro-σ^K and σ^K were not detected in KM2 indicates that readthrough transcription from the CPR_1739 gene (Fig. 3 and 5) is insufficient to produce detectable levels of these proteins. Like production of σ^E, the production of a detectable level of σ^K depends on both σ^K and σ^E (Fig. 10B), and we do not know whether these directly recognize the *sigK* promoter, but readthrough transcription from the CPR_1739 gene presumably produces a small amount of σ^K that catalyzes codependent production of σ^E and σ^K at higher levels. Processing of pro-σ^K to σ^K presumably relies on the *C. perfringens* ortholog of *B. subtilis spoIVFB*, which in *C. perfringens* would need to be expressed during growth, unless another mechanism exists to generate a small amount of σ^K from the pro-σ^K produced by readthrough transcription from the CPR_1739 gene.

Another difference between the regulation of sporulation in *B. subtilis* and *C. perfringens* is the regulation of *spoIIID*. In *B. subtilis*, *spoIIID* transcription is absolutely dependent on σ^E-associated RNA polymerase (31, 42, 66). We found that in *C. perfringens*, the transcription of *spoIIID* is independent of σ^E-associated RNA polymerase and that *spoIIID* is cotranscribed with the upstream gene CPR_2157 (Fig. 8A). In *B. subtilis*, SpoIIID functions as an activator of *sigK* transcription (39, 43). Since we did not mutagenize the *spoIIID* gene in *C. perfringens*, we do not know if it regulates *sigK* transcription, but the lack of regulation of *spoIIID* by σ^E in *C. perfringens* suggests that SpoIIID plays a somewhat different role in this bacterium.

Results from the *cpe* promoter fusions to *gusA* in pSM240 indicate that expression of *cpe* is dependent on σ^E and σ^K (Fig. 9). This coincides with the fact that there was no sporulation detected in *C. perfringens* KM1 (*sigK* mutant) and KM2 (*sigE* mutant) and that enterotoxin production is always associated with sporulation (48). Although we have not provided direct biochemical evidence that σ^E- and σ^K-associated RNA polymerases are responsible for transcription of the three *cpe* promoters, our results support the hypothesis that this toxin gene is under direct control of these sigma factors.

The regulation of sporulation in *B. subtilis* has been well characterized. The evidence reported here indicates that the regulatory network is different in *C. perfringens*, both in the timing of sporulation and in the regulation of sporulation-specific sigma factors. Sporulation in *B. subtilis* does not occur until the cells have exhausted their nutrient supplies and entered stationary phase (57). Our results suggest that *C. perfringens* initiates sporulation as soon as early log phase, as seen by the presence of *sigF*, *sigE*, *sigG*, and *sigK* transcripts (Fig. 3) and by activation of the *spoIIGA-sigE* operon promoter (Fig. 4). Pro-σ^E is processed almost as soon as it is translated (Fig. 6A). Activation of the *sigK* promoter occurs during late log or early stationary phase (Fig. 4), about an hour later than that of the *spoIIGA-sigE* promoter, and the majority of pro-σ^K processing occurs during early stationary phase (Fig. 6D). These results show that σ^E becomes abundant before σ^K during sporulation; however, as discussed above, analyses of the phenotypes of the *sigE* and *sigK* mutants indicate that σ^K is actually active before σ^E, and this early σ^K activity is likely due to transcription originating from the upstream gene CPR_1739 (Fig. 5). Our findings support a model of codependent σ^E and σ^K production (Fig. 10B), and we hypothesize that *C. perfringens* sporulation is not regulated by levels of nutrients as much as it is by the environmental conditions that the bacterium finds itself in while still in exponential growth. It has been proposed that *C. acetobutylicum* initiates sporulation to survive the acidifying effects of its own fermentative metabolism (15), but this does not appear to be the case in *C. perfringens*. Clearly, *C. perfringens* has evolved a sporulation cycle that is characterized by the ability to rapidly produce a heat-resistant endospore, which likely plays an important role in the ability of the bacterium to be one of the most ubiquitous bacteria in all of nature (58).

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