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Evaluating the Involvement of Alternative Sigma Factors SigF and SigG in *Clostridium perfringens* Sporulation and Enterotoxin Synthesis▽

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***Clostridium perfringens* type A food poisoning is the second most commonly identified bacterial food-borne illness. Sporulation contributes to this disease in two ways: (i) most food-poisoning strains form exceptionally resistant spores to facilitate their survival of food-associated stresses, and (ii) the enterotoxin (CPE) responsible for the symptoms of this food poisoning is synthesized only during sporulation. In *Bacillus subtilis*, four alternative sigma factors mediate sporulation. The same four sigma factors are encoded by *C. perfringens* genomes, and two (SigE and SigK) have previously been shown to be necessary for sporulation and CPE production by SM101, a transformable derivative of a *C. perfringens* food-poisoning strain (K. H. Harry, R. Zhou, L. Kroos, and S. B. Melville, J. Bacteriol. 2009, 191:2728–2742). However, the importance of SigF and SigG for *C. perfringens* sporulation or CPE production had not yet been assessed. In the current study, after confirming that sporulating wild-type SM101 cultures produce SigF (from a tricistronic operon) and SigG, we prepared isogenic *sigF*- or *sigG*-null mutants. Whereas SM101 formed heat-resistant, phase-refractile spores, spore formation was blocked in the *sigF*- and *sigG*-null mutants. Complementation fully restored sporulation by both mutants. By use of these mutants and complementing strains, CPE production was shown to be SigF dependent but SigG independent. This finding apparently involved regulation of the production of SigE and SigK, which Harry et al. showed to be necessary for CPE synthesis, by SigF. By combining these findings with those previous results, it is now apparent that all four alternative sigma factors are necessary for *C. perfringens* sporulation, but only SigE, SigF, and SigK are needed for CPE synthesis.**

Clostridium perfringens type A isolates producing enterotoxin (CPE) are the second most commonly identified cause of bacterial food-borne disease in the United States, where an estimated 250,000 cases of *C. perfringens* type A food poisoning occur annually (18, 19). This food poisoning begins with the consumption of foods contaminated with vegetative cells of an enterotoxigenic type A strain. Those ingested bacteria then sporulate in the small intestine and produce CPE, which has been shown to cause the diarrheal and abdominal-cramping symptoms that characterize *C. perfringens* type A food poisoning (18, 24). CPE-producing type A strains are also an important cause of human non-food-borne gastrointestinal diseases (2, 24). For example, it has been estimated that these bacteria account for 5 to 15% of all cases of antibiotic-associated diarrhea (2).

Enterotoxigenic type A isolates produce CPE only during sporulation (18). The sporulation-associated nature of CPE expression is controlled at the transcriptional level, since both Northern blotting and reporter construct studies detected enterotoxin gene (*cpe*) transcription in sporulating cultures, but not in vegetative cultures, of CPE-positive *C. perfringens* strains (4, 30). CPE can account for as much as 20% of the total protein present in a sporulating *C. perfringens* cell (5). Three strong promoters controlling *cpe* transcription have been identified upstream of the *cpe* open reading frame (ORF) (30), probably explaining why type A isolates often produce such high levels of CPE during sporulation.

Interestingly, the sequence of one *cpe* promoter (named P1) resembles a *Bacillus subtilis* SigK-dependent promoter, while the sequences of the other two *cpe* promoters (named P2 and P3) show similarity to *B. subtilis* SigE-dependent promoters (30). SigE and SigK are two of the four sporulation-associated sigma factors produced by *Bacillus subtilis*, which has traditionally been the prototype for the study of sporulation in Gram-positive bacteria (6, 11). These four sporulation-associated sigma factors are now well established as major regulators of *B. subtilis* sporulation, where SigF and SigG regulate gene expression in the forespore, while SigE and SigK control gene expression in the mother cell (6, 11). In *B. subtilis*, expression of these sporulation-associated sigma factors occurs in an ordered temporal cascade, with SigF appearing first, followed sequentially by SigE, SigG, and SigK (6, 11).

Sporulation has been much less studied in *C. perfringens* than in *B. subtilis*, but homologues of genes encoding SigE, SigF, SigG, and SigK have been identified in *C. perfringens* genomes (22, 27). In addition, a recent study by Harry et al. (7) used reverse transcription-PCR (RT-PCR) to detect transcripts of all four sigma factors in sporulating cultures of the CPE-positive *C. perfringens* type A isolate SM101, a transformable derivative of a food-poisoning isolate. Furthermore, Western blot analyses performed in that study confirmed SigE and SigK production by SM101 growing in Duncan-Strong (DS) sporulation medium (7). Since putative SigK- and SigE-dependent

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promoters had previously been identified upstream of the *cpe* gene (30), Harry et al. constructed *sigK*- and *sigE*-null mutants of SM101 in order to evaluate the importance of SigK and SigE for sporulation and CPE production by SM101. Consistent with the dependence of *cpe* transcription on these two sporulation-associated sigma factors, both mutants failed to drive β-glucuronidase production when transformed with a plasmid construct carrying the *cpe* promoter region fused to the *Escherichia coli* reporter gene *gusA*. In addition, neither the *sigK*- nor the *sigE*-null mutant of SM101 could sporulate.

The recent study by Harry et al. (7) also suggested differences in some developmental events during the sporulation of *C. perfringens* strain SM101 versus *B. subtilis*. For example, that recent study suggested that the normal accumulation of *sigF* and *sigE* transcripts in sporulating SM101 cells is dependent on SigK, whereas SigK is the last sigma factor expressed during *B. subtilis* sporulation. In addition, *sigF* transcript accumulation was reportedly delayed in a SM101 *sigE*-null mutant, even though *sigF* is the first transcript produced during *B. subtilis* sporulation. Harry et al. also suggested that *C. perfringens* and *B. subtilis* regulate SigG production differently, since (i) *sigG* transcripts were detected in SM101 *sigE* and *sigK* mutants reportedly containing little or no *sigE* or *sigF* transcript, while (ii) *sigG* transcription by *B. subtilis* requires both SigE and SigF. Finally, transcripts of all four sigma factor genes were detected much earlier in SM101 than has been reported for *B. subtilis.*

Given those apparent differences between sporulation in *C. perfringens* and that in *B. subtilis*, the current study constructed isogenic SM101 *sigF*- and *sigG*-null mutants and complementing strains. Those strains were then employed to explore whether these two sigma factors might also be involved in sporulation and CPE production by SM101.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. *C. perfringens* type A isolate SM101, a derivative of food-poisoning isolate NCTC8798 (Hobbs serotype 9), is transformable by electroporation (30). ATCC 3624 is a transformable, CPE-negative type A isolate (5).

Isolates were stored in cooked-meat medium (Oxoid) at −20°C. FTG (fluid thioglycolate; Difco Laboratories) and TGY broth (3% tryptic soy broth [Becton Dickinson and Company], 2% glucose [Fisher Scientific], 1% yeast extract [Difco], and 0.1% sodium thioglycolate [Sigma Chemical]) were used for growing vegetative cultures. Brain heart infusion (BHI) agar (Difco) supplemented with 15 μg/ml of chloramphenicol (Fisher Scientific) was used in screening to identify mutant clones. DS sporulation medium was used to induce the sporulation of *C. perfringens* strains (4). *E. coli* DH5α was grown at 37°C in LB broth, with shaking, or on LB agar containing 30 μg/ml chloramphenicol.

Construction of *sigF*- and *sigG*-null mutants of *C. perfringens* isolate SM101. The *sigF* and *sigG* genes of SM101 were inactivated by insertion of a group II intron using the *Clostridium*-modified TargeTron (Sigma) insertional mutagenesis system (3). Utilizing optimal intron insertion sites identified in the SM101 genome sequence (22) and the Sigma TargeTron website, an intron was targeted, in the sense orientation, between nucleotides 86 and 87 of the *sigF* ORF. The primers used for PCR targeting the intron to the *sigF* ORF were 86/87s-IBS (5′-AAAAAAGCTTATAA TTATCCTTAGGAAACAAGGAAGTGCGCCCAGATAGGGTG-3′), 86/87s-EBS1d (5′-CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAGGAA GCTAACTTACCTTTCTTTGT-3′), and 86/87s-EBS2 (5′-TGAACGCAAGTTTC TAATTTCGATTTTTCCTCGATAGAGGAAAGTGTCT-3′). Another intron was inserted, in the sense orientation, between nucleotides 159 and 160 of the *sigG* ORF. This intron was prepared by PCR using primers 159/160s-IBS (5′-A AAAAAGCTTATAATTATCCTTAAGAAACAAGAAGTGCGCCCAGATA GGGTG-3′), 159/160s-EBS1d (5′-CAGATTGTACAAATGTGGTGATAACA GATAAGTCAAGAAGATTAACTTACCTTTCTTTGT-3′), and 159/160s-EBS2 (5′-TGAACGCAAGTTTCTAATTTCGATTTTTCTTCGATAGAGG AAAGTGTCT-3′). The 350-bp PCR products were inserted into pJIR750ai in order to construct *sigF*- and *sigG*-specific TargeTron plasmids. The resultant plasmids, named pJIR750sigFi and pJIR750sigGi, respectively, were electroporated into wild-type SM101. The transformation efficiency for SM101 was about 5×10^4 transformants/μg plasmid DNA. Transformants were selected on BHI agar plates containing 15 μg/ml of chloramphenicol, and cells carrying an intron insertion were screened by PCR using primers sigFKOF (5′-AACTCTCATAGTCATGGCTAAAG-3′) and sigFKOR (5′-AG TTCTTCCTTATTGTAA-TGAAGC-3′) for *sigF*-null mutants (SM101::*sigF*) or primers sigGKOF (5′-TGTATAGGACTTATGAAATCTATAG-3′) and sigGKOR (5′-ACATATATTGCATCTCCACCATC) for *sigG*-null mutants (SM101::*sigG*).

Each reaction mixture was subjected to the following PCR amplification conditions: cycle 1, 95°C for 2 min; cycles 2 through 35, 95°C for 30 s, 55°C for 40 s, and 68°C for 80 s; and a final extension for 8 min at 68°C. An aliquot (20 μl) of each PCR sample was electrophoresed on a 1.5% agarose gel and was then visualized by staining with ethidium bromide.

A digoxigenin (DIG)-labeled, intron sequence-specific probe was prepared, as described previously (3), using primers IBS and EBS1d and a DIG-labeling kit (Roche). That probe was then employed for Southern blotting to confirm the presence of a single intron insertion in the SM101::*sigF* and SM101::*sigG* mutants. Briefly, DNA from wild-type SM101, the *sigF*-null mutant, or the *sigG*-null mutant was isolated using the MasterPure Gram-positive DNA purification kit (Epicentre, Wisconsin). A 2.5-μg aliquot of each isolated DNA sample was digested overnight with EcoRI according to the manufacturer's (New England Biolabs) instructions. The digested DNA samples were then electrophoresed on a conventional 1% agarose gel. The separated DNA digestion products were transferred onto a nylon membrane (Roche) for hybridization with the intron probe, as described previously (3).

Construction of complementing strains for *sigF*- and *sigG*-null mutants. The SM101 *sigF*-null mutant was complemented by cloning the entire *sigF* operon (see Results), including two upstream ORFs and the *sigF* ORF, along with 500 bp of upstream sequence and 500 bp of downstream sequence, into the pJIR750 *E. coli*/*C. perfringens* shuttle vector (1) and then transforming that plasmid into SM101::*sigF*. Briefly, DNA was isolated from wild-type SM101 as described above. PCR was then performed with that DNA using the Long-Range *Taq* DNA polymerase from New England Biolabs and primers sigFCOMN2F (5′-ttacgaat tcGTGTAAATGGATTGTCTGTTATAG-3′ with an added EcoRI site [lowercase]) and SigFCOMR (5′-atgcctgcagCATTTATAATCAATCCTCTTCTAG-3′ with an added PstI site [lowercase]), designed according to sequencing results from a previous study (22). The PCR was performed in a Techne thermocycler using the following amplification conditions: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 40 s, and 65°C for 3 min; and finally a single extension at 65°C for 10 min. The resultant 2.5-kb PCR product was cloned into the Topo 2.1 vector (Invitrogen). The Topo plasmid was then digested with EcoRI and PstI. The excised insert containing the *sigF* operon was gel purified and ligated into shuttle plasmid pJIR750, which had been digested with EcoRI and PstI, to produce plasmid pJIR750*sigF*comp. The SM101 *sigF*-null mutant was transformed by electroporation with pJIR750*sigF*comp; a complementing strain named SM101*sigF*comp was then selected from those transformants by growth on a BHI agar plate containing 15 μg/ml of chloramphenicol.

The SM101 *sigG*-null mutant was complemented by cloning the *sigG* ORF and 500 bp of upstream sequence into pJIR750 and then transforming that new plasmid into SM101::*sigG*. Briefly, DNA was isolated from wild-type SM101 as described above. PCR was then performed using the Long-Range *Taq* DNA polymerase from New England Biolabs and primers SigGCOMF (5′-ttacgaattc TTGCAACCTATGCATCAAGATG-3′ with an added EcoRI site [lowercase]) and SigGCOMR (5′-atgcctgcagAGAGCTCTATACATACTTCCTC-3′ with an added PstI site [lowercase]), designed according to sequence results from a previous study (22). The PCR was performed in a Techne thermocycler and used the following amplification conditions: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 40 s, and 65°C for 90 s; and finally a single extension at 65°C for 10 min. The resultant 1.2-kb PCR product was cloned into the Topo 2.1 vector (Invitrogen). The Topo plasmid was then digested with EcoRI and PstI. The excised insert containing *sigG* was gel purified and ligated into shuttle plasmid pJIR750, which had been digested with EcoRI and PstI, producing plasmid pJIR750*sigG*comp. The SM101 *sigG*-null mutant was transformed by electroporation with pJIR750*sigG*comp; the complementing strain SM101*sigG*comp was then selected on BHI agar plates containing 15 μg/ml of chloramphenicol.

RNA extraction and RT-PCR. To conduct a time course study of *cpe* gene expression during sporulation, a starter culture of wild-type SM101 was first grown overnight at 37°C in FTG. A 0.1-ml aliquot of that culture was then transferred to 10 ml of DS medium, which was incubated at 37°C for 1 to 8 h, with

TABLE 1. Primers using *sigF* operon overlap PCR

Primer	Sequence	Product size (bp)
sigFCOMN2F	5′-GTGTAAATGGATTGTCTGTTATAG-3′	853
sigFNR	5′-TTCTGCACTATGATGATCTAATTC-3′	
sigFCOMpNF	5′-AATTAGATCATCATAGTGCAGAAG-3′	400
sigFR1	5′-CTTCAATAGTTGGATCTAACTGAG-3′	
sigFCOMpF	5′-CTCAGTTAGATCCAACTATTGAAG-3′	729
sigFKOR	5′-AGTTCTTCCTTATTGTAATGAAGC-3′	
sigFF2	5′-GCTTCATTACAATAAGGAAGAACT-3′	364
sigFR	5′-CTCTATTCTAGAAACTTGAACTTG-3′	
sigFFN	5′-CAAGTTCAAGTTTCTAGAATAGAG-3′	460
sigFR2	5′-CTATTTTATCATATAATCCAAGTCC-3′	

sample aliquots removed each hour. After centrifugation of each removed culture aliquot, total *C. perfringens* RNA was extracted from the pelleted cells by using saturated phenol (Fisher Scientific), as described in a previous study (29). All phenol-extracted samples were then treated with 2 U of DNase I (Ambion) at 37°C for 30 min. To stop this DNase I activity, a DNase I inhibitor (Ambion) was added to each reaction tube. RNA was quantified by absorbance at 260 nm and was stored at −80°C for no more than 1 month.

RT-PCR analysis for *cpe* gene transcription was then performed with these DNase-treated RNA samples, using the AccessQuick RT-PCR kit from Promega. Briefly, each RNA sample (100 ng) was reverse transcribed to cDNA at 45°C for 1 h, and the cDNA was then used as a template for PCR with primers targeting *cpe* sequences or *spo0A* (as a control housekeeping gene). Control RT-PCRs were performed similarly, except for the omission of reverse transcriptase. As an additional control, a PCR amplifying *cpe* or *spo0A* sequences was performed using DNA extracted from the MasterPure Gram-positive DNA purification kit as described above. The PCR primers used to amplify the *cpe* gene were 3F and 4R (21), and the *spo0A* primers were Spo0A-F (5′-AACAA CCAGATTTAGTTGTATTAG-3′) and Spo0A-R (5′-CTCTATTTGTCCTCT-TCCCCAAGC-3′). The RT-PCR conditions used were as follows: 95°C for 2 min; 45°C for 1 h; 35 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 40 s; and finally a single extension at 72°C for 5 min.

Overlapping RT-PCR analyses of *sigF* operon expression were performed using 100 ng of RNA extracted from SM101 grown for 5 h in DS medium as described above. The primers used (Table 1) spanned the *sigF* ORF, three upstream ORFs, and one downstream ORF. Each RT-PCR mixture contained 1 μl of template RNA (100 ng), 10 μl of 2× TAQ Complete mix, 0.5 μl transcriptase, and 1 μl of each primer pair (final concentration, 1 μM). The RT-PCR conditions used were as follows: 95°C for 2 min; 45°C for 1 h; 35 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min 40 s; and finally a single extension at 72°C for 5 min. One control RT-PCR was performed similarly using *spo0A* primers, except for the omission of reverse transcriptase. Another control reaction used these primers and DNA extracted from the MasterPure Gram-positive DNA purification kit as described above.

Northern blot analysis of *sigF* transcription by wild-type SM101, a *sigF*-null mutant, and a complementing strain. Total RNA was phenol extracted from SM101, SM101::*sigF*, and SM101*sigF*comp using pelleted cells from a 5-h DS culture, as described above. Northern blot analysis was then performed using the Northern Max kit (a formaldehyde-based system for Northern blotting) from Ambion. Briefly, gel preparation, electrophoresis, transfer of RNA to a nylon membrane (Roche), prehybridization, and hybridization were each performed according to the manufacturer's instructions. A digoxigenin-labeled, *sigF* internal sequence-specific probe was prepared, as described previously (16, 26), using primers sigFKOF and sigFKOR and the DIG-labeling kit from Roche.

Western blotting. For Western blot analysis of CPE production, a 0.2-ml aliquot of an FTG culture of wild-type SM101, SM101::*sigF*, SM101*sigF*comp, SM101::*sigG*, or SM101*sigG*comp was inoculated into 10 ml of DS sporulation medium. After incubation at 37°C for 8 h, each DS culture was visually examined by phase-contrast microscopy to assess the presence of spores. At that time point, aliquots of each DS culture were sonicated until more than 95% of cells had lysed. The sonicated samples were then centrifuged to remove debris and unlysed cells. The supernatant from each centrifuged DS culture lysate was analyzed for the presence of CPE by using a previously described CPE Western immunoblot procedure (10).

For Western immunoblot analysis of alternative sigma factor production, a 1.5-ml aliquot of a 5-h DS culture was centrifuged. The pellets were then resuspended in Tris-EDTA (TE) buffer, and 1 μl of Ready to Use Lysozyme (Epicentre) was added. After 1 h of incubation at 37°C, SDS loading dye was added, and the sample was boiled for 5 min. Western blot analysis of these samples to detect the presence of SigE, SigK, SigF, and SigG used a 1:1,000 dilution of rabbit antibodies raised specifically against purified recombinant *B. subtilis* sigma factor E, F, G, or K produced by *E. coli*. These antibodies were kindly supplied by Richard Losick and Masaya Fujita.

Quantitative spore counts. Quantitative spore counts were determined as described previously (15). Briefly, an overnight DS culture of wild-type SM101, SM101::*sigF*, SM101::*sigG*, SM101*sigF*comp, or SM101*sigG*comp was heated at 75°C for 20 min to kill the remaining vegetative cells and to promote the germination of mature spores. Each heat-shocked suspension was then serially diluted from 10^{-2} to 10^{-7} with sterile water and was plated onto BHI agar plates, which were incubated anaerobically overnight at 37°C prior to colony counting. Quantitative spore counting was performed three times each for wild-type SM101, isogenic sigma factor mutants, and complementing strains.

Photomicroscopy. Spore formation in 8-h DS cultures was documented using a Zeiss phase-contrast microscope (total magnification, ×1,000).

RESULTS

Construction of SM101 *sigF*- and *sigG*-null mutants. As shown in Fig. 1, inspection of the published *C. perfringens* strain SM101 genomic sequence (22) revealed that the *sigE*, *sigG*, and *sigK* ORFs cluster together, while the *sigF* ORF is separated from those ORFs by ~200 kb. While (i) a previous study had demonstrated that *sigE* and *sigK* are essential for CPE expression and sporulation by SM101 (7) and (ii) both SigF and SigG are necessary for *B. subtilis* sporulation (6), the importance (if any) of *sigF* or *sigG* for either *C. perfringens* sporulation or CPE expression had not yet been experimentally evaluated. Since significant differences between *B. subtilis* and *C. perfringens* sporulation have been reported (7), the current study used *sigF*- and *sigG*-null mutants to directly evaluate whether *sigF* and *sigG* are involved in CPE synthesis by, and sporulation of, SM101.

The SM101 *sigF*- and *sigG*-null mutants were constructed by a TargeTron-mediated insertional mutagenesis approach (3). The identities of the putative mutants obtained from this mutagenesis procedure were first evaluated by PCR using primers corresponding to internal *sigF* or *sigG* ORF sequences (Fig.

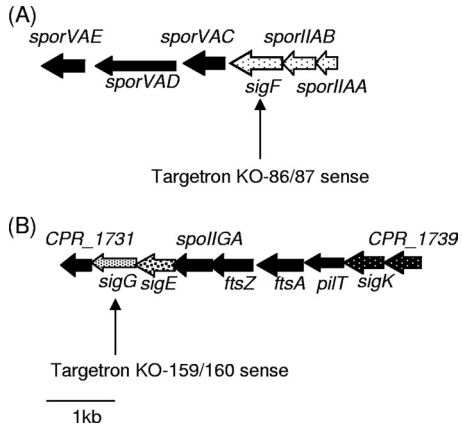

FIG. 1. Arrangement of the sigma factor ORFs in *C. perfringens* SM101 based on previous genome-sequencing results (22). (A) Arrangement of the *sigF* locus in SM101. The results of the current study (see the text) indicate that *sigF*, *sporIIAB*, and *sporIIAA* are transcribed in one operon (dotted arrows). (B) Arrangement of the *sigG*, *sigE*, and *sigK* loci in SM101. As indicated by the black arrows with white dots, *sigK* and *CPR_1739* are also transcribed in one operon, according to reference 7. A 1-kb scale marker is included for size comparisons.

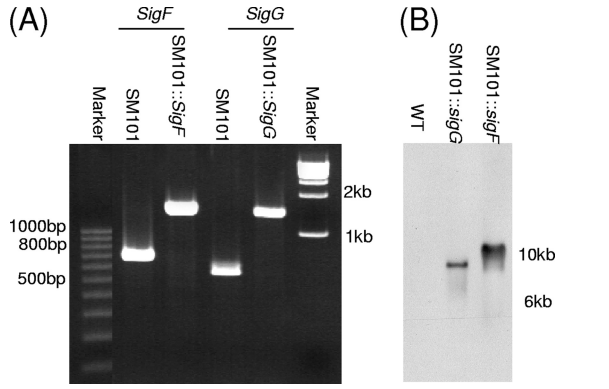

FIG. 2. Intron-based insertional mutagenesis to create SM101 *sigF*- and *sigG*-null mutants. (A) Internal *sigF*- and *sigG*-specific PCR results for wild-type SM101, SM101::*sigF*, and SM101::*sigG*. The migration of 100-bp DNA markers is shown on the left, and the migration of 1-kb DNA markers is shown on the right. (B) Southern blot analysis of wild-type SM101 and SM101::*sigF* and SM101::*sigG* null mutant strains with a DIG-labeled, intron-specific probe. DNA size markers are shown on the right.

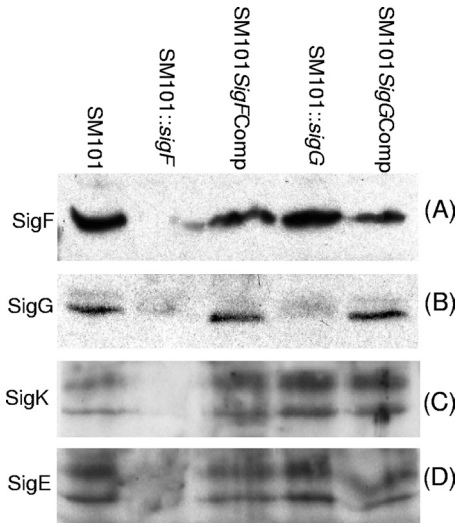

FIG. 3. Western blot analyses of alternative sigma factor production by SM101. Shown are Western blot results for the production of SigF (A), SigG (B), SigK (C), and SigE (D) by DS cultures of wild-type, SM101::*sigF*, SM101*sigF*comp, SM101::*sigG*, and SM101*sigG*comp strains grown for 5 h at 37°C. See Results for discussion of the two SigE or SigK bands present in some lanes.

2A). In wild-type SM101, these internal PCR primers amplified *sigF*- or *sigG*-specific products of ~750 bp or ~550 bp, respectively. However, with DNA from the intron-inactivated mutants, the same primers supported PCR amplification of products that were ~900 bp larger than the products amplified using wild-type SM101 template DNA. The larger sizes of these PCR products amplified from both putative mutants matched the expected size change resulting from an intron insertion into the *sigF* or *sigG* ORF.

To specifically demonstrate the presence of an intron insertion in the *sigF*- and *sigG*-null mutants, and also to show that these mutants carried only a single intron insertion, Southern blot analysis was performed using an intron-specific probe (Fig. 2B). As expected, this experiment detected no hybridization of the intron-specific probe to wild-type SM101 DNA. In contrast, these Southern blot results showed the presence of a single intron insertion in the SM101::*sigF* and SM101::*sigG* mutants.

Disruption of *sigF* or *sigG* expression by the two mutants was then assessed directly using Western blotting (Fig. 3A and B). These analyses first demonstrated that wild-type SM101 produces both SigF and SigG during sporulation. Furthermore, these Western blots also showed the complete loss of SigF or SigG production by sporulating cultures of the *sigF*- or *sigG*-null mutant, respectively.

Complementation of the SM101 *sigF*- and *sigG*-null mutants. The SM101::*sigG* mutant was complemented by PCR cloning, from wild-type SM101, of DNA corresponding to the *sigG* ORF and 500 bp of upstream sequence. This PCR product was cloned into the pJIR750 shuttle plasmid and was then transformed into the *sigG*-null mutant to create SM101::*sigG*comp. The presence of a wild-type *sigG* gene in this complementing strain was confirmed by PCR (data not shown). SigG production by SM101::*sigG*comp was then demonstrated directly by Western blotting (Fig. 3B).

Complementation of the *sigF* mutant proved more complicated. Initial experiments indicated that transformation of a plasmid carrying a PCR product corresponding to the *sigF* ORF and ~500 bp of upstream sequence failed to restore SigF expression to SM101::*sigF* (data not shown). Therefore, it was considered that the *sigF* ORF might be transcribed as part of an operon. To test this hypothesis, overlapping RT-PCR and PCR were first performed. The overlapping PCR primers for these analyses were designed to link *sigF* with three upstream genes, i.e., the ATP-dependent protease gene, *sporIIAA*, and *sporIIAB*, along with one downstream gene, i.e., *sporVAC* (Fig. 4A). Using wild-type SM101 DNA as the template for this overlap PCR assay, each of the five expected products was amplified (Fig. 4B), confirming the validity of the primers. However, using an SM101 RNA template, the RT-PCR amplified products only from reactions R2 to R4, indicating that the *sporIIAA*, *sporIIAB*, and *sigF* ORFs are cotranscribed as a single mRNA.

Northern blot analysis (Fig. 4C) supported this finding by indicating that the *sigF* gene is transcribed as a ~1.5-kb mRNA, matching the expected size of a single mRNA encoding SporIIAA, SporIIAB, and SigF. In addition, these Northern blot analyses also failed to detect the presence of any *sigF* mRNA in the *sigF*-null mutant, consistent with the Western blot results in Fig. 3A.

Since both the overlap RT-PCR and Northern blot results indicated expression of the *sigF* gene as part of a tricistronic operon containing two upstream genes, a PCR product containing those three genes and 500 bp of upstream and downstream sequence was ligated into shuttle vector pJIR750, followed by transformation of the resultant plasmid (pJIR750*sigF*comp) into the *sigF*-null mutant. Both Western blot and Northern blot analyses demonstrated SigF expression by this complementing strain (Fig. 3A and 4C), named SM101::*sigF*comp.

Regulation of sigma factor production by SigF and SigG. The *sigF*- and *sigG*-null mutants were first used to assess whether SigF or SigG might regulate each other's production.

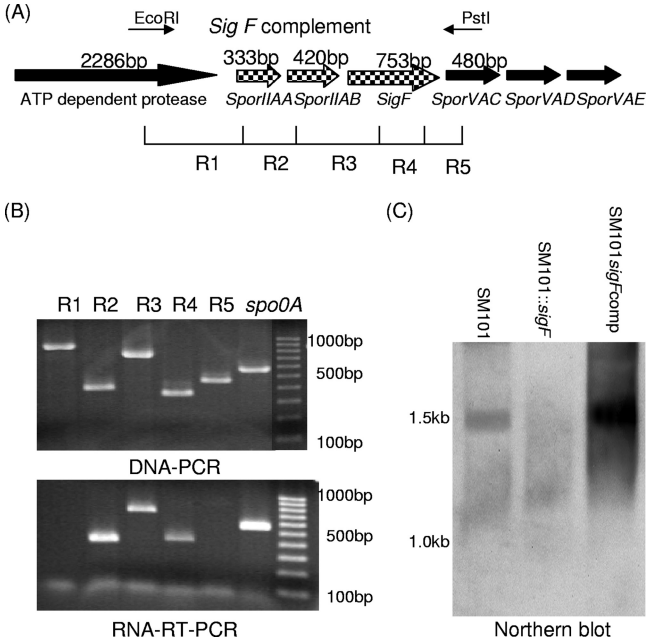

FIG. 4. The *sigF* ORF is transcribed as part of a tricistronic operon. (A) Arrangement of the *sigF* locus in SM101, based on sequencing results (22). The locations of the primers used in panel B for overlap PCR are indicated by R1 to R5. (B) Overlap PCR analysis of the *sigF* locus region in five reactions (R1 to R5). The results of a DNA PCR (top) and an RNA RT-PCR (bottom) using reaction R1 to R5 primers (Table 1) are shown. Size markers are shown on the right. (C) Northern blot analysis using a DIG-labeled *sigF* probe. Results for wild-type, SM101::*sigF*, and SM101*sigF*comp strains are shown. Size markers are shown on the left.

Western blotting (Fig. 3B) showed that the *sigF* mutant was impaired for production of SigG, while the SM101::*sigF*comp complementing strain produced near-wild-type levels of SigG. In contrast, the *sigG* mutant was still able to produce wild-type levels of SigF (Fig. 3A).

The *sigF*- and *sigG*-null mutants were next employed to test whether SigF or SigG might regulate the production of SigE or SigK, which have previously been tied to *C. perfringens* sporulation and the regulation of CPE production (7). Western blot analyses comparing SigE and SigK production by sporulating cultures of wild-type SM101 revealed that, as expected, 5-h DS cultures grown at 37°C produce both SigE and SigK (Fig. 3C and D). The presence of two visible bands for SigE and SigK on Western blots has been observed previously for *C. perfringens* (7), and those bands were suggested to correspond to proprotein or mature, active forms of these two sigma factors.

Compared to the results for wild-type SM101 in Fig. 3, Western blotting detected sharply reduced or no production of SigE or SigK by the SM101 *sigF*-null mutant grown under the same incubation conditions for 5 h at 37°C in DS medium (Fig. 3C and D). Furthermore, complementation of the *sigF*-null mutant fully restored SigE and SigK production after a similar 5 h of incubation at 37°C in DS medium (Fig. 3C and D).

In contrast to these results indicating that SigF regulates the production of SigE and SigK, inactivation of the *sigG* gene had no effect on the production of either SigE or SigK in cultures grown for 5 h in DS medium at 37°C (Fig. 3).

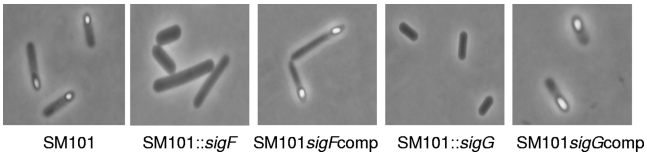

FIG. 5. Formation of phase-refractile spores by wild-type SM101, the *sigF*- and *sigG*-null mutants, and complementing strains. Shown are results for DS cultures of each strain after 8 h of growth at 37°C in DS. Photographs were taken using a phase-contrast microscope (magnification, ×1,000).

Evidence that both SigF and SigG are essential for *C. perfringens* sporulation. To address whether SigF or SigG is required for sporulation by wild-type SM101, the *sigF*- and *sigG*-null mutants (or complementing strains of those mutants) were first examined using phase-contrast microscopy (Fig. 5). This analysis showed that, by 8 h of incubation at 37°C in DS sporulation medium, wild-type SM101 had already formed refractile spores, with an efficiency of >95%. In contrast, the *sigG*- and *sigF*-null mutants failed to form any refractile spores after a similar 8 h of incubation in DS medium at 37°C. Furthermore, both mutants remained unable to form refractile spores, even when incubated for 24 or 48 h at 37°C in DS medium (not shown). However, complementation of either mutant restored sporulation after 8 h of incubation at 37°C in DS medium, by which time >95% of the complementing strain cells had formed refractile spores (Fig. 5).

The sporulating abilities of SM101, the *sigG*- and *sigF*-null mutants, and complementing strains of those mutants were also directly compared by measuring the formation of heat-resistant spores after 24 h of incubation at 37°C in DS medium. Under these conditions, wild-type SM101 formed $\sim 7.7 \times 10^8 \pm 1.7 \times 10^8$ heat-resistant spores/ml. However, under the same incubation conditions, no heat-resistant spores were detected for either the SM101 *sigG*-null mutant or the SM101 *sigF*-null mutant. This inability of the *sigG* and *sigF* mutants to form heat-resistant spores was not due simply to slower spore production, since no heat-resistant spores were detected even when these mutants were incubated at 37°C in DS sporulation medium for 2, 3, or 7 days. Complementation of either sigma factor mutant restored the ability to form heat-resistant spores to near-wild-type levels following overnight incubation in DS medium at 37°C. Specifically, SM101*sigF*comp formed $3.2 \times 10^8 \pm 1.5 \times 10^8$ heat-resistant spores/ml, and SM101*sigG*comp formed $9.2 \times 10^7 \pm 0.4 \times 10^7$ heat-resistant spores/ml. Collectively, these results indicated that SigF and SigG are essential for the formation of heat-resistant mature spores by SM101.

CPE expression during sporulation is SigF dependent but does not require SigG. RT-PCR analyses showed that, for wild-type SM101 growing at 37°C in DS medium, *cpe* gene transcription starts at ~3 to 4 h and then increases over the next several hours (Fig. 6). In addition, Western blot analyses demonstrated that, under the same incubation conditions, CPE production by SM101 became detectable within 5 h in DS cultures incubated at 37°C and then increased markedly by 6 to 8 h (Fig. 6 and 7). In contrast, no *cpe* transcription or CPE production was observed in 8-h cultures of SM101 grown at 37°C in TGY medium, a condition that does not trigger *C. perfringens* sporulation (data not shown). As another control,

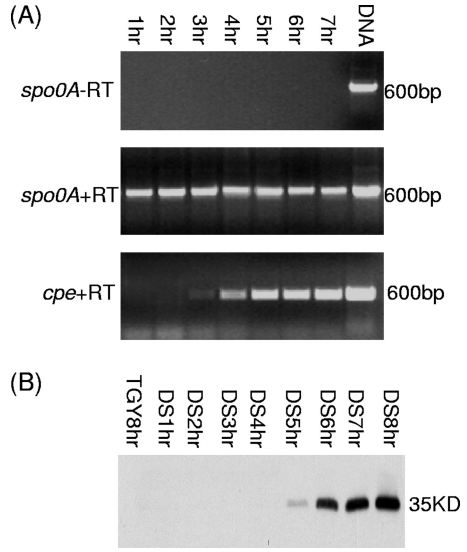

FIG. 6. Kinetics of *cpe* gene expression and CPE production by DS cultures of SM101. (A) RT-PCR analysis of *cpe* gene transcription in cultures growing from 1 to 7 h in DS medium at 37°C. The *spo0A* gene was used as a housekeeping gene, and a sample lacking reverse transcriptase (−RT) was run to show the absence of DNA contamination in these samples. The sizes of products, based on comparison against DNA size markers (not shown), are shown to the right of each gel. (B) Western blot analyses of CPE production after 1 to 7 h of growth in DS medium at 37°C. To show that CPE production is dependent on sporulation, this figure shows the absence of CPE production by an 8-h culture of SM101 grown at 37°C in TGY, a medium that does not induce sporulation. The size of the immunoreactive protein, based on the migration of prestained markers (not shown), is indicated on the right.

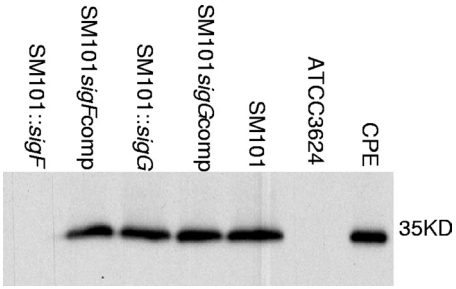

FIG. 7. Comparison of CPE production by wild-type SM101, *sigF*- and *sigG*-null mutants, and complementing strains. Western blot results for CPE production are shown for sporulating 8-h DS cultures incubated at 37°C. As controls, similar analyses are shown for an 8-h DS culture of the CPE-negative strain ATCC 3624 grown at 37°C and for purified CPE. The size of the immunoreactive protein, based on the migration of prestained markers (not shown), is given on the right.

no immunoreactivity with an anti-CPE antibody was observed using 8-h DS cultures of the *cpe*-negative strain ATCC 3624.

Similar Western blot analyses of 8-h DS cultures incubated at 37°C were used to evaluate CPE production by the *sigF*- and *sigG*-null mutants. Those Western blot studies detected no CPE production by the SM101::*sigF* strain (Fig. 7). However, under the same incubation conditions, complementation of the *sigF* mutant to restore SigF expression also restored CPE production to near-wild-type SM101 levels. Collectively, these results demonstrated that SigF is required for CPE expression.

In contrast, similar Western blot experiments revealed that 8-h DS cultures of the *sigG*-null mutant still produced normal levels of CPE, as did similar cultures of the complementing strain for that mutant. Therefore, SigG is necessary for the production of spores by SM101 but is not required for CPE production.

DISCUSSION

The ability to sporulate is an integral contributor to several important *C. perfringens* diseases. For example, contamination of wounds with spores can lead to gas gangrene (28). Spores also contribute to the transmission of *C. perfringens* type A food poisoning, particularly because the survival of food-poisoning strains is often facilitated by the formation of spores that are exceptionally resistant to food environment stresses, such as heat, cold, and preservatives (13–15, 17, 23, 25). Furthermore, the sporulation process itself is essential for *C. perfringens* type A food poisoning, i.e., the enterotoxin (CPE) causing the gastrointestinal symptoms of this food-borne illness is produced only by sporulating cells (5, 18, 19). Therefore, knowledge of *C. perfringens* sporulation is important for full understanding of the pathogenesis of *C. perfringens* type A food poisoning and other *C. perfringens* diseases.

While *C. perfringens* sporulation is still understood only at a rudimentary level, both similarities to and differences from *B. subtilis* sporulation have been reported (7). Regarding sporulation similarities, both bacteria were found to utilize the transcription factor Spo0A (6, 8) and to express four alternative sigma factors that, for *B. subtilis*, have been shown to regulate sporulation-associated gene expression (6, 7). Among sporulation differences, *C. perfringens* lacks the phosphorelay that helps signal the start of *B. subtilis* sporulation (22, 27). Also, it has been suggested that the production of the four alternative sigma factors may be regulated differently in these two bacteria (7). Given these apparent differences between *B. subtilis* versus *C. perfringens* sporulation, it has been important to establish whether all four alternative sigma factors are required for *C. perfringens* sporulation. To begin addressing that question, Harry et al. showed that SigE and SigK are necessary for the sporulation of *C. perfringens* strain SM101 (7).

The current results have demonstrated that SigF and SigG are also required for *C. perfringens* sporulation, i.e., mutants unable to produce SigF or SigG were completely blocked for spore formation, and complementation restored sporulation to near-wild-type levels. This complementation of *sigF* and *sigG* mutants back to near-wild-type sporulation levels contrasts notably with results from the study by Harry et al. (7), where complementation restored only 1 to 2% of wild-type sporulation ability to *sigE*- and *sigK*-null mutants. Harry et al. suggested that their more limited complementation might be attributable to substantial wild-type *sigE* and *sigK* transcription from upstream genes, an effect not encoded by their complementing plasmid. This would be similar to our finding that, as in *B. subtilis* (20), SM101 transcribes *sigF* as part of a *spoIIA* tricistronic operon containing the *sigF* (*spoIIAC*), *spoIIAA*, and *spoIIAB* genes. This genetic similarity could suggest that, as in *B. subtilis* (20), SpoIIAA and SpoIIAB regulate SigF activity in SM101.

The current results add important support for another proposed similarity between the sporulation of SM101 and that of *B. subtilis*, where SigE and SigK, but not SigF or SigG, are initially produced as inactive proproteins that are then proteolytically processed to an active, mature form (6, 7). Specifically, our Western blot analyses showed that antibodies raised against SigF or SigG reacted only with single proteins that corresponded to the appropriate sigma factor based on size, and this immunoreactive band was absent from Western blot lanes containing lysates of *sigF*- or *sigG*-null mutants.

In *B. subtilis*, synthesis of the four alternative sigma factors begins during the initiation of sporulation and follows the temporal pattern of SigF appearing first, followed sequentially by SigE, SigG, and SigK (6). In contrast, Harry et al. (7) found that all four sigma factors accumulated rapidly after SM101 was inoculated into sporulation medium and that early accumulation of *sigF* transcript appeared to be SigK dependent, suggesting that SigK might act prior to SigF in the *C. perfringens* sporulation regulatory network. Therefore, it was interesting that the current study detected little or no or SigK production by the *sigF*-null mutant, although production of that sigma factor was observed after complementation to restore SigF production. As expanded on below, our current results are most consistent with SM101 sporulation involving a cascade of alternative sigma factors reminiscent of the process in *B. subtilis*, where SigF regulates the production of SigG, SigE, and SigK. Notably, the same classical sporulation-associated sigma factor regulatory cascade has also been described for the nonpathogenic organism *Clostridium acetobutylicum* (9).

Harry et al. (7) also concluded that SigG regulation substantially differs between *B. subtilis* and *C. perfringens.* Whereas SigG production in *B. subtilis* is dependent on both SigF and SigE, the study by Harry et al. (7) had reported that a SM101 *sigE*-null mutant still produced SigG, even though it produced little or no SigE or SigF. However, our current study found that a SM101 *sigF*-null mutant no longer produced SigG. Consistent with that finding, bioinformatics analysis identified a consensus *sigF* promoter sequence upstream of *sigG*. These findings would suggest that, as in *B. subtilis*, SigG production by *C. perfringens* requires SigF.

As mentioned in the introduction, the P1 *cpe* promoter possesses sequence similarity to a SigK-dependent promoter, while the P2 and P3 *cpe* promoter sequences resemble sequences found in SigE-dependent promoters (30). Consistent with those homologies, Harry et al. recently used *gusA* fusion assays to conclude that *cpe* transcription is blocked in *sigE*- and *sigK*-null mutants of SM101 (7). The results from our current study now establish that *cpe* transcription and CPE production are also blocked in an SM101 *sigF*-null mutant. However, we also found that, despite the previously reported early appearance of *sigG* transcripts in sporulating SM101 cultures (7), a *sigG*-null mutant still shows normal levels of *cpe* transcription and CPE production, thus documenting that not all sporulation-associated sigma factors are required for *cpe* transcription and CPE production.

At least two possibilities could explain how SigF regulates *cpe* transcription. First, a *cpe* promoter might be SigF dependent. This possibility seems less likely, since promoter-mapping studies identified only SigE- and SigK-dependent *cpe* promoter sequences (30). A second possible explanation, shown in the Fig. 8 model, might be that (as with *B. subtilis* and *C. acetobutylicum*) SM101 uses SigF to regulate the production of other sporulation-associated sigma factors. This model is supported by the current Western blot results showing little or no production of SigE and SigK by the *sigF*-null mutant. This model also indicates that (as with *B. subtilis* and *C. acetobutylicum* [6, 9, 11]) SigG production by SM101 apparently requires SigF, consistent with the Western blot results of our current study.

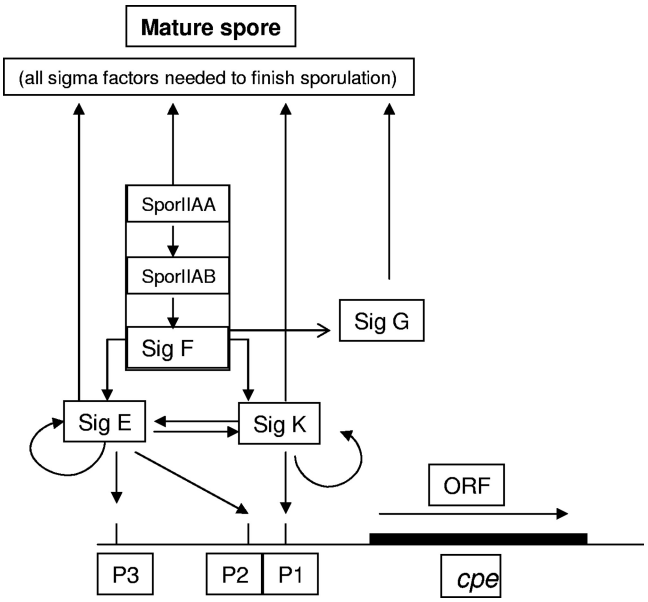

FIG. 8. Model describing sigma factor regulation of *C. perfringens* CPE production and sporulation. The model incorporates findings from the current work and research by Harry et al. (7). Not shown is the proven involvement of Spo0A in *C. perfringens* sporulation (8), since it is not yet clear how that protein leads to sporulation-associated sigma factor production by *C. perfringens*.

Because older studies (12) had suggested that CPE production levels may involve posttranscriptional regulatory effects not modeled by transcriptional fusion assays, the current study directly compared the timing of *cpe* transcription and CPE production in sporulating SM101 cultures. These analyses detected *cpe* transcription beginning ~3 h after inoculation into DS medium. The appearance of CPE protein lagged slightly; it became identifiable by Western blotting at 4 to 5 h postinoculation. These results largely agree with the transcriptional fusion assay results of Harry et al. (7), who noted *cpe* promoter-driven GusA activity ~3 to 4 h after inoculation into a slightly different sporulation medium. However, our results do not necessarily eliminate posttranscriptional contributions to CPE production, a topic worthy of further evaluation.

In summary, there is now increasing evidence for both similarities and differences between the sporulation of *C. perfringens* and that of *B. subtilis*. Sporulation similarities apparently include the involvement of Spo0A and SigF-mediated control of the other sporulation-associated sigma factors, while differences include the absence of a *B. subtilis*-like phosphorelay from *C. perfringens*. Future transcriptomic studies should identify transcripts regulated by each alternative sigma factor in *C.*

perfringens. Another largely unstudied topic for *C. perfringens* sporulation concerns the initial signaling cascade that leads to Spo0A phosphorylation and triggers sporulation. Finally, the results of this study indicate a previously unappreciated level of complexity for the regulation of *cpe* transcription (i.e., the involvement of SigF). However, in combination with the earlier results of Harry et al., the now demonstrated involvement of three sporulation-associated sigma factors in the regulation of *cpe* transcription offers an explanation for the ~40-year-old observation of a strong linkage between CPE production and sporulation.

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