Global Analysis of the Sporulation Pathway of *Clostridium difficile*

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**Abstract**

The Gram-positive, spore-forming pathogen *Clostridium difficile* is the leading definable cause of healthcare-associated diarrhea worldwide. *C. difficile* infections are difficult to treat because of their frequent recurrence, which can cause life-threatening complications such as pseudomembranous colitis. The spores of *C. difficile* are responsible for these high rates of recurrence, since they are the major transmissive form of the organism and resistant to antibiotics and many disinfectants. Despite the importance of spores to the pathogenesis of *C. difficile*, little is known about their composition or formation. Based on studies in *Bacillus subtilis* and other *Clostridium* spp., the sigma factors $\sigma^E$, $\sigma^F$, $\sigma^G$, and $\sigma^K$ are predicted to control the transcription of genes required for sporulation, although their specific functions vary depending on the organism. In order to determine the roles of $\sigma^E$, $\sigma^F$, $\sigma^G$, and $\sigma^K$ in regulating *C. difficile* sporulation, we generated loss-of-function mutations in genes encoding these sporulation sigma factors and performed RNA-Sequencing to identify specific sigma factor-dependent genes. This analysis identified 224 genes whose expression was collectively activated by sporulation sigma factors: 183 were $\sigma^E$-dependent, 169 were $\sigma^F$-dependent, 34 were $\sigma^G$-dependent, and 31 were $\sigma^K$-dependent. In contrast with *B. subtilis*, *C. difficile* $\sigma^E$ was dispensable for $\sigma^G$ activation, $\sigma^G$ was dispensable for $\sigma^K$ activation, and $\sigma^F$ was required for post-translationally activating $\sigma^K$. Collectively, these results provide the first genome-wide transcriptional analysis of genes induced by specific sporulation sigma factors in the Clostridia and highlight that diverse mechanisms regulate sporulation sigma factor activity in the Firmicutes.

**Introduction**

*Clostridium difficile* is a Gram-positive, spore-forming, obligate anaerobe that causes gastrointestinal diseases including diarrhea, pseudomembranous colitis, and toxic megacolon [1–3]. *C. difficile* infections and *C. difficile*-related deaths have risen dramatically in the past decade, increasing the financial burden on health care systems [4–7]. While *C. difficile* is best known for causing hospital-acquired antibiotic-associated infections, recent epidemiologic studies indicate that community-acquired *C. difficile* infections are increasingly more common and associated with significant morbidity [6,7]. A key element to the success of *C. difficile* as a pathogen is its ability to produce spores. Spores are resistant to most disinfectants and antibiotics, making them difficult to eliminate both from infected humans and the environment [1,2,9]. As a result, *C. difficile* spores disseminate readily from person to person and cause high rates of recurrent infections, which can lead to serious illness or even death [1–3,9].

Although spores are critical to the pathogenesis of *C. difficile*, their composition and formation remain poorly characterized. Less than 25% of the spore coat proteins identified in the well-characterized spore-former *Bacillus subtilis* have homologs in *C. difficile* [10]. In contrast, the regulatory proteins that control spore coat gene expression and other sporulation events in *B. subtilis* are conserved in *C. difficile* and all other spore-forming Firmicutes [10–13]. These include the master sporulation transcriptional regulator, Spo0A, and the sporulation sigma factors $\sigma^F$, $\sigma^K$, $\sigma^E$, and $\sigma^G$.

In *B. subtilis* the sporulation sigma factors function at discrete stages during spore development to couple changes in gene expression with specific morphological changes in the cell [14–16]. The morphological changes begin with the formation of a polar septum, which creates two compartments, the mother cell and the forespore. The mother cell engulfs the forespore and guides the assembly of the spore until it lyses once spore maturation is complete. By coupling these developmental changes to the sequential activation of compartment-specific sporulation sigma factors, the mother cell and forespore produce divergent transcriptional profiles that coordinate to lead to the formation of a dormant spore [16].

Sporulation gene transcription in *B. subtilis* begins with the activation of the transcription factor Spo0A, which in turn
Author Summary

_C. difficile_ is the leading cause of healthcare-associated infectious diarrhea in the United States in large part because of its ability to form spores. Since spores are resistant to most disinfectants and antibiotics, _C. difficile_ infections frequently recur and are easily spread. Despite the importance of spores to _C. difficile_ transmission, little is known about how spores are made. We set out to address this question by generating _C. difficile_ mutants lacking regulatory factors required for sporulation and identifying genes that are regulated by these factors during spore formation using whole-genome RNA-sequencing. We determined that the regulatory pathway controlling sporulation in _C. difficile_ differs from related Clostridium species and the non-pathogenic model spore-former *Bacillus subtilis* and identified 314 genes that are induced during _C. difficile_ spore development. Collectively, our study provides a framework for identifying _C. difficile_ gene products that are essential for spore formation. Further characterization of these gene products may lead to the identification of diagnostic biomarkers and the development of new therapeutics.

activates early sporulation gene transcription, such as the genes encoding the early sigma factors σ^E_ and σ^K_. σ^F_ is initially held inactive by an anti-σ factor and only undergoes activation after septum formation is complete; this mode of regulation couples σ^F_ activation in the forespore to a morphological event [17,18]. Active σ^F_ induces the transcription of genes whose products mediate cleavage of an inhibitory pro-peptide from σ^K_ in the mother cell via trans-septum signaling [19]. Active σ^K_ induces the transcription of genes whose products lead to the activation of the late sporulation sigma factor σ^G_ in the forespore, which occurs during or after engulfment [20,21]. Activated σ^G_ in the forespore subsequently induces the expression of genes whose products proteolytically activate σ^K_ in the mother cell via trans-septum signaling [22]. Notably, the activity of each sigma factor relies on the activation of the preceding sigma factor [11,14–16,23]. As a result, the sigma factors operate in a sequential, “criss-cross” manner and collectively control the expression of hundreds of genes during sporulation [24–26].

The regulatory pathway controlling sporulation sigma factor activation in _B. subtilis_ is thought to be conserved across endospore-forming bacteria, since all four sigma factors are conserved [11,12]. However, a growing body of work in the Clostridia suggests that diverse pathways regulate sporulation sigma factor activity in the Firmicutes. In _C. perfringens_, a sigG^-mutant still produces cleaved σ^K_, suggesting that σ^K_ does not control the proteolytic activation of σ^K_ as it does in _B. subtilis_ [27]. Furthermore, a _C. perfringens_ sigK^-mutant exhibits a phenotype more severe than a _B. subtilis_ sigE^-mutant in that it fails to initiate asymmetric division or produce σ^K_ [28], suggesting that in _C. perfringens_ σ^K_ functions upstream of σ^K_. Indeed, _C. perfringens_ σ^K_ and σ^K_ have been suggested to be dependent on each other for full activity, in contrast with _B. subtilis_ [28]. A similar early sporulation defect has been observed in a sigK^-mutant of _C. botulinum_, which also exhibits reduced expression of early sporulation genes spo0A and sigF_ [29]. In contrast with _B. subtilis_ and _C. perfringens_, however, a _C. acetobutylicum_ sigF^-mutant does not initiate asymmetric division [30], and a sigE^-mutant fails to complete asymmetric division [31]. In addition, a _C. acetobutylicum_ sigE^-mutant produces wildtype levels of σ^K_ [31] in contrast with _B. subtilis_, and a sigG^-mutant exhibits elongated forespores and pleiotropic defects in coat and cortex formation [31].

To determine how these sporulation sigma factors regulate sporulation in _C. difficile_, we constructed mutations in the genes encoding the sporulation transcription factor Spo0A and the sigma factors σ^F_ and σ^K_. σ^F_ and σ^K_ and determined the transcriptional profiles of these mutants using RNA-sequencing (RNA-Seq). The transcriptional analyses, combined with cytological characterization of the sigma factor mutants, suggest that divergent mechanisms regulate the activity of σ^K_ and σ^G_ in _C. difficile_ relative to _B. subtilis_ and other Clostridium spp. In addition, these analyses have identified a set of 314 genes that are upregulated during sporulation in a _spo0A_, σ^K_-, σ^K_-, σ^K_-, and/or σ^K^-dependent manner. These sporulation-induced genes provide a framework for identifying and characterizing _C. difficile_ spore proteins that may have diagnostic or therapeutic utility.

Results

_C. difficile_ sporulation sigma factors are essential for mature spore formation

In order to identify genes that are regulated by the sporulation-specific sigma factors, we used a modified TargeTron gene knockout system to disrupt the genes encoding σ^F_, σ^K_, and σ^K_ in _C. difficile_ [32]. This system uses a group II intron to insert an erythromycin resistance cassette into the target gene (Figure S1A). JIR8094- [33], an erythromycin-sensitive derivative of the sequenced _C. difficile_ strain 630 [34], was used as the parental strain. As a control, we also constructed a targeted disruption in _spo0A_ [35,36]. Colony PCR of the intron-disrupted mutants confirmed the expected size change resulting from the intron insertion into the _spo0A_, sigF_, sigE_, sigG_, and sigK_ genes (Figure S1B).

To determine the effect of blocking sigma factor production on sporulation, the mutants were induced to sporulate on solid sporulation media and visualized by phase contrast microscopy [37]. It should be noted that sporulation is asynchronous in this assay, and the extent and timing of sporulation exhibits variability even between biological replicates (Figure S2). Nevertheless, after 18 hrs of growth, sufficient numbers of cells have initiated sporulation to detect the production of immature phase-dark forespores and mature phase-bright spores in the wildtype strain (Figure 1 and S2). In contrast, _spo0A_-, _sigF_-, _sigE_-, _sigG_-, and _sigK_ cultures failed to produce phase-bright spores (Figure 1). No phase-dark or phase-bright forespores were observed in the _spo0A_-, _sigF_-, or _sigE_^-strains, suggesting a block early in sporulation.

Analysis of live, sporulating cultures with the lipophilic dye FM4-64 (to stain mother cell and forespore membranes) and Hoechst 33342 (to stain cell nucleoids) revealed polar septum formation in wild type and the sigma factor mutants but not in the _spo0A^-mutant (Figure 1). This result was consistent with the observation that Spo0A is necessary to induce the sporulation pathway in _C. difficile_ [35,36]. Overall, the proportion of sporulating cells detected by membrane and DNA staining in the culture was 23%, 41%, 24%, 26%, and 18% for wildtype, _sigF_-, _sigE_-, _sigG_-, and _sigK_-, respectively, as indicated by the presence of a polar septum, immature forespore compartment, or mature forespore (Table S1). Wildtype cultures contained a heterogeneous population of sporulating cells at discrete stages of sporulation: 28% of sporulating cells exhibited intense DNA staining of an FM4-64-labeled forespore compartment (yellow arrows, Figure 1, Table S1); 28% showed phase-dark forespores that stained with both FM4-64 and Hoechst (Table S1); 28%
exhibited phase-dark forespores that stained intensely with FM4-64 but not Hoechst (green arrows, Figure 1, Table S1), and 16% contained a phase-bright forespore that failed to stain with either FM4-64 or Hoechst (pink arrows, Figure 1, Table S1). In contrast, sigF and sigE sporulating cells were arrested at the asymmetric division stage, with 95% and 92% of sporulating cells, respectively, exhibiting intense DNA staining of an FM4-64-labeled forespore compartment (yellow arrows, Figure 1, Table S1). The sigF mutant strain was arrested at the phase-dark forespore stage, with 69% of sporulating cells exhibiting intense forespore membrane and nucleoid staining (yellow arrows, Figure 1, Table S1). While only 4% of the sigG cells were observed to produce forespores that stained only with FM4-64, 44% of sporulating sigK cells were captured at this stage of sporulation, a phenotype that was also observed in wildtype (green arrows, Figure 1, Table S1). Taken together, these results indicate that all four sporulation sigma factors are required to complete sporulation and suggest that $\sigma^G$ is necessary to complete the stage of sporulation development required to exclude the Hoechst dye from staining the forespore chromosome. The results are also consistent with studies investigating B. subtilis forespore development, which indicate that nucleic acid stains are excluded earlier than membrane stains during spore development [38-40].

To confirm that the gene disruptions prevented sigma factor production in each of the respective sigma factor mutants, we performed Western blot analyses using antibodies raised against C. difficile sigma factors. Similar to B. subtilis, Spo0A was required for the production of all the factors, and $\sigma^F$ was observed in the sigE, sigG, and sigK strains at wildtype levels (Figure 2, [41]). $\sigma^K$ was detected in both its pro- and cleaved form in wildtype, sigG- and sigK strains, whereas the majority of $\sigma^K$ was unprocessed in the sigF strain (Figure 2). This result slightly deviates from the B. subtilis model, where pro-$\sigma^K$ processing is completely abrogated in a B. subtilis sigF strain [42]. In contrast, a C. perfringens sigF mutant fails to produce pro-$\sigma^K$ altogether [27], and $\sigma^K$ processing has not been demonstrated in C. acetobutylicum [31]. $\sigma^K$ was present in wildtype and sigG- mutant strains but absent in the sigF and sigE strains (Figure 2), analogous to observations in B. subtilis where $\sigma^K$ is required for sigK expression. A C. perfringens sigF strain in contrast produces low amounts of $\sigma^K$ [28]. Consistent with the observation that C. difficile $\sigma^K$ lacks an N-terminal propeptide [43], no processing of $\sigma^K$ was observed in wildtype C. difficile (Figure 2), even though $\sigma^K$ undergoes proteolytic activation in B. subtilis and C. perfringens [27]. $\sigma^K$ was detected in the C. difficile sigF, sigE and sigK mutants (Figure 2) in contrast with studies of other endospore-forming bacteria, where $\sigma^K$ activity and autoactivation of sigK transcription is partially dependent on $\sigma^K$ in B. subtilis [44-46], and $\sigma^K$ production depends on $\sigma^K$ in C. perfringens and C. acetobutylicum [27,30].

We next performed transmission electron microscopy (TEM) to identify the precise developmental stage at which each sigma factor mutant was stalled. Cortex and coat layers were present on forespores in wildtype sporulating cells, while the spo0A mutant exhibited no signs of spore formation (Figure 3). The sigF mutant failed to progress beyond asymmetric division (Figure 3), similar to a B. subtilis sigF mutant [47] but in contrast with a C. acetobutylicum sigF mutant which does not initiate asymmetric division [50]. Nevertheless, unlike B. subtilis, a more electron-translucent region in the mother cell cytosol surrounded by electron dense layers was observed in some sigF mutant cells; this region resembled mislocalized spore coat ([37], Figure 3). The C. difficile sigK strain was arrested at the asymmetric division stage similar to the sigF mutant, although electron-translucent regions surrounded by coat-like layers were not observed in any sigE cell analyzed. The C. difficile sigE mutant phenotype resembled the phenotype of sigE mutants of B. subtilis [47] and C. perfringens [28], with frequent observations of disporic cells or cells with multiple septa at one pole (Figures 1 and 3). This observation was in contrast with a C. acetobutylicum sigE mutant, which does not complete asymmetric division [31]. The C. difficile sigG mutant produced forespores lacking an apparent cortex layer, similar to B. subtilis [21,44]; however, unlike B. subtilis, the forespores were surrounded by thin layers that resembled the spore coat layers visible in wildtype cells (Figure 3). In addition, the C. difficile sigG mutant exhibited pleiotropic defects including forespore ruffling, incomplete membrane fission during engulfment, and a septated forespore compartment (Figures 3 and S3). Quantitation of the prevalence of each phenotype revealed that forespore ruffling, incomplete engulfment, and a septated forespore compartment were observed in 98, 87 and 21% of sigG cells, respectively (Figure S3). Lastly, the C. difficile sigK mutant produced forespores surrounded by a layer that resembled the cortex layer of wildtype, but no coat layers were apparent (Figure 3). This phenotype was more similar to a B. subtilis sigK mutant, which lacks both cortex and coat [22], than C. perfringens, which fails to initiate polar septum formation [28].

Plasmid complementation rescues the sporulation defects of sigF, sigE, sigG, and sigK mutants

To validate that the observed mutant phenotypes were due to the targeted insertions, we complemented the mutant strains by expressing a wildtype copy of the gene encoding the corresponding sigma factor from a plasmid. We used either the pMTL83151 or pMTL84151 multicopy plasmids [48] to express the complementing genes or operons from their native promoters. The complementation constructs all restored production of phase-bright forespores when expressed in their respective mutant backgrounds (Figure S4A), although phase-bright spore formation by the sigK complementation strain was delayed relative to wildtype. Western blot analysis further confirmed that the complementation constructs restored production of the respective sigma factor to wildtype levels (Figure S4B). TEM analysis revealed that all four complementation constructs restored heat and cortex formation to their respective mutant strains (Figure S5). Heat resistance assays to measure complementation strain sporulation efficiency revealed that the sigF and sigE constructs fully complemented heat resistance relative to wildtype and that the sigG and sigK constructs partially complemented heat resistance (70 and 23%, respectively, Figure S4C).
RNA-Seq analysis reveals the regulatory relationships between C. difficile sporulation sigma factors

While these analyses showed that σK, σG, σF, and Spo0A were all required for mature spore formation, they did not reveal which genes were being misregulated in the sporulation sigma factor mutants to produce their respective sporulation defects. To identify these genes and gain insight into the regulatory network controlling sporulation sigma factor activity, we used RNA-Sequencing (RNA-Seq) to transcriptionally profile our sporulation mutants and wild type during sporulation. Three biological replicates of wildtype, spo0A-, and sporulation sigma factor mutant strains were grown on sporulation media (Figure S2), and RNA was isolated. Following DNase-treatment, ribosomal RNA depletion and reverse transcription, Illumina-based RNA-Seq was used to determine the complete transcriptome of wildtype C. difficile and the sporulation mutants. Genome coverage and sequencing counts for each strain and replicate can be found in Table S2.

The DeSeq variance analysis package [49] was used to identify genes that were downregulated by ≥4-fold with an adjusted p-value of ≤0.05 in the spo0A- strain relative to wild type. This pairwise analysis identified 276 genes as being Spo0A-dependent (Table S3). Consistent with the role of Spo0A as the master regulator of sporulation, 65 of these genes were predicted to be involved in sporulation (Table S4) [11,50–52]. Six of these Spo0A-dependent genes were recently identified as encoding components of the C. difficile spore coat [50,53], and 36 sporulation-related genes (Table S4) were shown to depend on σH, the stationary phase sigma factor that induces spo0A transcription in C. difficile [51] and B. subtilis [54]. σK-, σF-, σG-, and σE-dependent genes were identified by comparing the transcriptional profiles of the σK-, σF-, σG-, and σE strains to wild type, respectively, using the same parameters as above. This analysis identified 185 genes as being dependent on σH for their expression (Table S5). One hundred eighteen of these σH-dependent genes were also σK-dependent (Table S6), indicating that σK has some activity in a σF- mutant consistent with the reduced levels of cleaved σK being detected by Western blot (Figure 2); 29 of the σK-dependent genes formed a separate subset of genes that were also σG-, dependent but σF-independent. Indeed, the majority of the 34 σG-dependent genes identified in this analysis were not dependent on σK for their expression (Table S7), since only four of the σG-regulated genes were also σK-regulated. Notably, none of the genes identified as being σG-dependent required σK for their expression (Table S8), suggesting that the σK produced in the sigF- and sigK- mutants is active (Figure 2). This result differs from the B. subtilis model where σK is needed to fully activate σG function [20,21,46,55,56].

Of the 169 genes that depended on σK for their expression (Table S6), 85% and 78% of these genes were dependent on Spo0A and σK, respectively (Figure 4). The expression of 29 of these genes was also σE-dependent (Table S5). Indeed the majority of the 31 σE-dependent genes were σK-dependent (Table S8; Figure 4), consistent with σE being required for σK production (Figure 2). In contrast, as described earlier, no overlap was observed between σK- and σF-dependent genes (Figure 4). Taken together, the RNA-Seq analyses suggested that (1) a small subset of σF-dependent genes are neither σK- nor σE-dependent; (2) σK activity depends on Spo0A and σE but not σG or σF; (3) σK activity depends on Spo0A, σE, and σK but not σG; and (4) σG activity depends on Spo0A and σF but not σK or σE. The latter two findings differ from the B. subtilis model, where the σK-dependent genes are also σG-dependent because σK activity depends on σG [11,15,22], and σG-dependent genes are σE-dependent because full activation of σG requires σK [20,21,46,55,56].

To visually represent the differences in gene expression profiles between the sigma factor mutants and wild type, we generated a heat map for genes downregulated by ≥4-fold with an adjusted p-value of ≤10−5 in the spo0A- strain relative to wild type. The expression levels of wild type and the sigma factor mutants relative to spo0A- strain were centered, scaled, and mapped to a red-green color scale. The heat map revealed a cluster of genes that was poorly expressed in the sigE- mutant relative to the wildtype, sigG-, and sigK- strains; these genes were also expressed at reduced levels in the sigF- mutant (Figure 5) and were primarily σK-dependent (Table S4). A separate cluster of genes was downregulated in both the sigK- and sigE- mutants relative to the wildtype and sigG- strains (Figure 5); these genes were all identified as σK-dependent genes (Table S5). Another discrete cluster of genes was downregulated in the sigG- and sigF- strains relative to the wildtype, sigE-, and sigK- strains (Figure 5); again, most of these genes were identified as σF-dependent genes, although two genes were σF-dependent but not σG-dependent (Tables S5 and S7). Thus, identification of variably expressed genes between the strains confirmed the findings of our earlier pairwise analyses: σK-dependent genes were largely Spo0A-dependent, σF-dependent genes were largely σK-dependent, and σG-dependent genes were σE-dependent and σK-dependent but not σF- or σG-dependent. These results support a model where (1) σK controls the activation of both σF and σG, (2) σE induces the production and activation of σK, and (3) σF and σK are dispensable for σG activation. Alternative statistical models were also employed to validate these findings (see Text S1 and Figures S6 and S7).

Quantitative RT-PCR validates the RNA-Seq Data

To validate the RNA-Seq data, we isolated RNA from three separately prepared biological replicates of wildtype, spo0A-, sigF-, sigE-, sigG-, and sigK- strains grown on sporulation media for 18 hrs. RNA was reverse transcribed and quantitative RT-PCR (qRT-PCR) was performed using primers specific for three
genes within each of the sigma factor-dependent transcriptomes. Gene expression levels in the wildtype and the sigma factor mutant strains relative to spo0A were determined by comparative CT analysis normalized to the housekeeping gene rpoB. These analyses confirmed that the transcript levels of the \( \sigma^F \)-dependent gene gpr was reduced by 50-fold (\( p = 0.0001 \)) in the \( \sigma^F \) mutant relative to wild type, and reduced in the \( \sigma^G \) mutant by 4 fold (\( p = 0.01 \)); gpr expression was not affected in the \( \sigma^E \) and \( \sigma^K \) mutants. cd0125 (spoIIQ, [13]) transcription was reduced by 10-fold in the \( \sigma^F \) mutant relative to wild type (\( p = 0.01 \)), but no reduction in transcript levels was observed in \( \sigma^G \) and \( \sigma^K \) mutants (Figure 6A). Transcription of cd2376 was reduced by 3-fold in the \( \sigma^F \) mutant relative to wild type (Figure 6A). Although this correlation was not statistically significant, it approached statistical significance (\( p = 0.065 \)) (Figure 6A); this result is likely due to the low number of overall cd2376 transcripts present in the samples. Transcript levels of the \( \sigma^G \)-dependent genes spoVT, spoB, and daeF showed significant reductions in the \( \sigma^G \) (\( p < 0.0004 \), <0.0002 and <0.0001, respectively) and \( \sigma^F \) mutants (\( p < 0.0001 \)) compared to wild type but no significant reduction in the \( \sigma^E \) and \( \sigma^K \) mutants relative to wild type (Figure 6B). This observation was consistent with the RNA-Seq data indicating that \( \sigma^G \) activity depends on \( \sigma^F \), although it is likely that \( \sigma^F \) directly induces the transcription of some \( \sigma^G \)-dependent genes given the predicted overlap in their promoter specificities [11]. Nevertheless, given that \( \sigma^G \) is present at wildtype levels in a \( \sigma^F \) strain, these observations suggest that \( \sigma^F \) regulates \( \sigma^G \) activity through a post-translational mechanism.

\( \sigma^E \)-dependent genes cd3522 and spoIVA were reduced by >100-fold, and cd1511 by >50-fold, in \( \sigma^F \) relative to wild type, (\( p < 0.0001 \), <0.0001, and <0.006, respectively), but not in \( \sigma^G \) and \( \sigma^K \) mutants (Figure 6C). Transcript levels of these \( \sigma^E \)-
dependent genes were reduced by ~5 to 6-fold (p<0.01) in the \( \sigma^E \) mutant relative to wildtype, indicating that, in the absence of \( \sigma^F \), \( \sigma^E \) activity is reduced but detectable. Transcript levels of the \( \sigma^K \)-dependent genes \( cd1433 \), \( cd1067 \), and \( sleC \) were significantly reduced by \( \leq 100 \)-fold in the \( \sigma^E \) (p<0.0001 for each gene) and \( \sigma^K \) (p<0.0001 for each gene) strains compared to wild type (Figure 6D). \( \sigma^K \)-dependent gene expression was reduced in the \( \sigma^F \) mutant by 8 to 10-fold (p<0.01), suggesting that \( \sigma^K \) has reduced but detectable activity in the \( \sigma^F \) strain. Importantly, no statistically significant change for any of these \( \sigma^K \)-dependent genes was observed in the \( \sigma^G \) mutant relative to wild type, consistent with the RNA-Seq results indicating that \( \sigma^K \) activity does not depend on \( \sigma^G \) (Figures 4 and 5). Altogether, the qRT-PCR data validated the RNA-Seq data identifying \( \sigma^F \), \( \sigma^E \), \( \sigma^G \), and \( \sigma^K \)-dependent genes and confirmed that (1) \( \sigma^K \), \( \sigma^G \), and \( \sigma^K \) activity depend on \( \sigma^F \), (2) full \( \sigma^G \) activity requires \( \sigma^F \) but not \( \sigma^G \), and (3) \( \sigma^K \) activity requires \( \sigma^F \) but not \( \sigma^G \). It should be noted however that, although \( \sigma^F \) is required for full \( \sigma^E \) and \( \sigma^K \) activity, some degree of \( \sigma^G \)- and \( \sigma^K \)-dependent gene expression is observed even in the absence of \( \sigma^F \).

Western blot analyses confirm that \( \sigma^K \) activity depends on \( \sigma^E \) but not \( \sigma^G \)

Having validated the RNA-Seq data at the transcript level, we next investigated whether changes in transcript levels correlated with changes in protein levels for \( \sigma^F \), \( \sigma^E \), \( \sigma^G \), and \( \sigma^K \)-regulated genes. To this end, we raised antibodies against proteins encoded by genes identified by RNA-Seq as being \( \sigma^F \)-, \( \sigma^E \)-, \( \sigma^G \)-, and \( \sigma^K \)-regulated genes. Western blot analyses of the germination protease Gpr confirmed that only \( \sigma^F \) is required for gpr expression, while production of the regulatory protein SpoVT and the small acid-soluble protein SspA depended on both \( \sigma^E \) and \( \sigma^G \). These results...
Figure 5. Comparison of Spo0A-dependent gene expression in wildtype and sporulation sigma factor mutants. Heat map representation of the genes that were downregulated by ≥4-fold with an adjusted p-value of ≤10^{-5} in the spo0A^- strain relative to wild type. Expression levels of these genes in wildtype (WT), sigF^-, sigE^-, sigG^-, and sigK^- strains relative to spo0A^- were centered, scaled, and mapped to a red-green color scale, with green indicating that the gene was upregulated in the strain relative to the other strains, and red indicating that the gene was downregulated relative to the centered expression level. σF-regulated genes (blue), σK-regulated genes (purple), σE-regulated genes (orange), σG-dependent genes (green) are colored as indicated, and clusters of coordinately regulated genes are bracketed. Genes colored in black were identified as depending only on Spo0A for expression.

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Figure 6. qRT-PCR validation of RNA-Seq transcriptional profiling. Transcript levels for three genes that were determined to be dependent on σF, σE, σG, and/or σK for expression as measured by qRT-PCR on three biological replicates. Samples were distinct from those used for RNA-Seq. (A) σF-dependent genes included gpr, CD0125 (spoIIQ [13]) and CD2376. (B) σE-dependent genes included spoVT sspB, and dacF. (C) σG-dependent genes included CD3522, spoIVA, and CD1511. (D) σK-dependent genes included CD1433, CD1067 and sleC. cDNA was produced from RNA samples harvested from wildtype (WT), spo0A^-, sigF^-, sigE^-, sigG^- and sigK^- strains grown on sporulation media for 18 hrs. Data represent the averages of three biological replicates and at least two technical replicates. Transcripts were calculated relative to spo0A^- and normalized to rpoB (housekeeping gene). Error bars indicate the standard error of the mean. Statistically significant changes in transcript levels were determined relative to WT and represented by adjusted p-values determined by a Dunnett's one-way ANOVA. ****p<0.0001, ***p<0.001, **p<0.01. CD2376 transcript levels were ~3-fold reduced in the sigF^- mutant relative to wild type (p=0.065).

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indicate that σ^G can directly activate the expression of spoVT and sigF (Figure 7). Western blot analyses for CD3522, SpoIVA, and CD1511 demonstrated that their production depends on σ^F but not σ^G or σ^K; these proteins were detected, albeit at greatly reduced levels, in the sigF- mutant (Figure 7). These results were consistent with the observation that active, processed σ^G is present in both sigG- and sigF- strains, while only trace amounts of processed σ^K could be detected in the sigF- strain (Figure 2).

Analysis of σ^K-dependent protein production using antibodies specific for CD1433, CD1067 and SleC confirmed that these proteins were absent in the sigE- and sigK- mutants and present in wild type and the sigG- mutant (Figure 7). Only SleC was reliably detected in the sigF- mutant, even though cd1433 and cd1067 transcripts could be detected in the sigF- strain (Figure 6D). Nevertheless, taken together these observations confirm that (1) σ^K does not require σ^G, σ^K, or σ^K for activation, (2) full σ^K activation requires σ^K, (3) full σ^K activation requires σ^K but not σ^K or σ^K, and (4) σ^K activation requires σ^K but not σ^K.

Discussion

The regulation of sporulation in the Clostridia has remained poorly characterized relative to the model spore-forming bacterium B. subtilis because the function and activity of all four sporulation sigma factors has not been simultaneously interrogated in a given Clostridium sp. to date. By constructing mutations in genes encoding for individual sporulation sigma factors in the nosocomial pathogen C. difficile and performing whole genome transcriptional profiling on these mutants, we identified 314 genes whose expression is activated during sporulation (Table S9) in a Spo0A, σ^K, σ^K, σ^K, and/or σ^K-dependent manner (Tables S3, S5, S6, S7, S8). These experiments reveal that the sporulation pathway of C. difficile exhibits numerous differences relative to B. subtilis and other Clostridium spp., highlighting the diversity of mechanisms that regulate sporulation sigma factor activity in the Firmicutes.

Diverse mechanisms regulate sporulation sigma factor activity in the Firmicutes

While mutation of all four sporulation sigma factors in C. difficile abrogated functional spore formation as expected [11], the regulation and function of these sigma factors in C. difficile differed from the regulatory pathways determined for B. subtilis and other Clostridium spp. The differences between C. difficile, C. perfringens, C. acetobutylicum, and B. subtilis sporulation pathways are summarized in Figure 8, as are the similarities.

Similar to B. subtilis, our transcriptional and cytological analyses reveal that C. difficile σ^K functions downstream of σ^K to regulate late-stage sporulation events, and σ^K functions downstream of σ^K to regulate forespore maturation (Figures 2 and 6). In contrast with B. subtilis, C. difficile σ^K is fully active in the absence of σ^K, and σ^K is fully active in the absence of σ^K (Figures 6 and 7). The latter observation could have been anticipated given that C. difficile σ^K lacks an N-terminal pro-peptide, in contrast to all other spore formers [43]. However, the former observation was unexpected because σ^K-regulated gene products function to activate σ^K in the forespore of B. subtilis, initiating a positive feedback pathway that increases σ^K levels through auto-activation of the sigG promoter [44,46,57]. In particular, B. subtilis σ^K activation requires the formation of a σ^K-dependent "feeding tube" [20,21,55,58,59], which maintains forespore integrity by transporting small molecules from the mother cell into the forespore [20,21,55]. This mode of regulation couples the activation of the forespore-specific σ^K to σ^K-controlled events in the mother cell. In contrast, our results indicate that C. difficile σ^K is active in the absence of σ^K-dependent feeding tube gene expression (Figures 5 and 6, Tables S6 and S7). Nevertheless, even though σ^K was active at wildtype levels in the sigK- mutant (Figures 6 and 7), it remains possible that σ^K activity may be mislocalized in the mother cell cytosol, similar to the premature activation of σ^K in Lon- and anti-σ^K sigma factor CdB- cells [57,60,61].

Even though C. difficile σ^K can be fully activated in the absence of σ^K, our results further show that σ^K is post-translationally activated in a σ^K-dependent manner (Figures 2 and 6). These results raise the intriguing question as to how σ^K activates σ^K independent of σ^K in C. difficile. In B. subtilis, multiple post-translational mechanisms control σ^K activity; however, aside from the feeding tube, these mechanisms are inhibitory rather than activating. In B. subtilis the Lon protease reduces σ^K activity in the forespore until engulfment is complete. Whether these factors inhibit σ^K activity in C. difficile is unknown, although C. difficile does not appear to encode a CsdB homolog. In future studies, it will be interesting to determine whether σ^K functions to activate σ^K directly or alleviate its inhibition, and whether C. difficile sporulation sigma factors exhibit compartment-specific activity similar to B. subtilis.

Interestingly, the morphology of the C. difficile sigK- mutant differed considerably from a B. subtilis sigK- mutant. While B. subtilis sigK- mutant forespores are normal in appearance despite lacking both a coat and cortex [44], C. difficile sigK- mutant forespores produced layers resembling spore coat around the forespore and exhibited defects in engulfment and structural integrity (Figures 3 and S3). The forespore membrane ruffling phenotype of C. difficile sigK- mutants was reminiscent of B. subtilis feeding tube mutant phenotypes [21], suggesting that σ^K may encode proteins required to "nurture" the C. difficile forespore. Alternatively, σ^K could regulate a cytoskeletal or cortex component that confers structural integrity to the forespore. Such proteins could be represented in the σ^K-regulated genes identified in this study (Table S7).

The phenotype of the C. difficile sigF- mutant also differed from its cognate mutant in B. subtilis, since the sigF- mutant produced low levels of σ^K and σ^K-induced gene products (Figure 7) and regions that resembled mislocalized coat in the mother cell cytosol (Figure 3) [47]. In B. subtilis, σ^K is required to activate the expression of spoIIA, which encodes an intercellular signaling protein that activates SpoIIGA, the protease responsible for activating pro-σ^K [65,66]. Whether the trace amounts of σ^K processing observed in the C. difficile sigF- mutant results from low-level expression of spoIIA or spoIIA, or whether an unknown protease activates σ^K, remains to be examined.

Comparison of the sporulation pathway of C. perfringens relative to C. difficile indicates that both organisms proteolytically activate σ^K in a σ^K-dependent manner (Figure 2), although it should be noted that a C. perfringens sigF- mutant does not make σ^K, σ^K, or σ^K[27] in contrast with C. difficile (Figure 2). Since the phenotypes of C. perfringens sigF- and sigK- mutants have not been examined by electron microscopy, the precise stage at which they are arrested remains unclear. Nevertheless, unlike C. perfringens (and C. botulinum) where σ^K is essential for both early and late stage sporulation events (Figure 8) [28,29], C. difficile σ^K is needed only at late stages of sporulation. Furthermore, C. perfringens σ^K is produced at low levels in an unprocessed form in a sigK- mutant; σ^K is made at low levels in a C. perfringens sigK- mutant; and sigE and sigK expression appear to be auto-activated [28]. In contrast, no sigK expression was observed in the absence of σ^K in C. difficile.
The sporulation pathway of *C. difficile* appears to be most similar to the *C. acetobutylicum* pathway. Both *C. difficile* and *C. acetobutylicum* sigma factors $\sigma^F$, $\sigma^E$, and $\sigma^G$ appear to function at similar stages of sporulation, although *C. acetobutylicum* sigF and sigE mutants exhibit more severe phenotypes than in *C. difficile* in that they fail to initiate and complete asymmetric division, respectively [30,31], and $\sigma^F$ is required to activate sigG transcription in *C. acetobutylicum* [30] unlike *C. difficile*. Similar to *C. difficile*, however, *C. acetobutylicum* $\sigma^F$ does not require $\sigma^E$ for auto-activation of sigG expression, although it is unclear whether *C. acetobutylicum* $\sigma^G$ is active in the absence of $\sigma^E$ [31]. Lastly, loss of sigG expression in *C. acetobutylicum* results in pleiotropic defects in coat and cortex formation and forespore integrity similar to *C. difficile* (Figure 3, [31]). Since a sigK mutant in *C. acetobutylicum* has not been described, it will be interesting to determine whether *C. acetobutylicum* $\sigma^K$ function is more similar to *C. difficile* than to *C. perfringens* and whether these differences correlate to the presence of the skin element, an ~15 kb prophage-like element that interrupts the sigK gene in *C. difficile* but not other *Clostridium* spp. [43]. Nevertheless, our collective transcriptional and cytological analyses of sporulation sigma factor function in *C. difficile* suggest that novel mechanisms regulate $\sigma^G$ and $\sigma^K$ activation relative to other spore-forming organisms (Figure 8). Further studies are needed to determine the regulatory interplay between *C. difficile* sporulation sigma factors and their downstream auxiliary regulators SpoVT and SpoIIIID, which modulate the expression of $\sigma^G$- and $\sigma^K$-regulated genes, respectively, in *B. subtilis* [24,26,67,68] and are conserved in *Clostridium* spp.

**Figure 7. Western blot analyses of proteins encoded by genes induced by specific sigma factors during sporulation.** Western blot analyses of proteins encoded by genes identified as being upregulated during sporulation by specific sigma factors. Wildtype (WT), spo0A, sigF, sigE, sigG, and sigK strains were grown on sporulation media for 18 hrs. SleC undergoes multiple processing steps [76,85], but only the pro-SleC form is shown. Spo0A was used as a loading control. doi:10.1371/journal.pgen.1003660.g007

Transcriptional profiling of sporulation in *C. difficile* identifies new sporulation genes

By performing whole genome transcriptional profiling on the different sporulation sigma factor mutants, we have identified
distinct subsets of genes that are $\sigma^F$, $\sigma^E$, $\sigma^G$, and $\sigma^K$-dependent. The number of genes determined to be $\sigma^G$-dependent in $C. difficile$ was relatively small (34 genes) relative to $B. subtilis$, where the $\sigma^G$ regulon comprises ~100 genes [11,25,26]. Similarly, the $\sigma^K$ and $\sigma^K$-dependent genes (169 and 31 genes, respectively) identified by our study were smaller than their cognate regulons in $B. subtilis$ (270 and 150 genes, respectively, [24]). While the parameters we used to define genes as being $\sigma^F$, $\sigma^E$, $\sigma^G$, and $\sigma^K$-dependent were relatively stringent, relaxing these parameters did not result in large

Figure 8. Comparison of sporulation regulatory network architecture in the Firmicutes. The temporal progression of sporulation is shown from top to bottom as determined in $B. subtilis$, $C. difficile$, $C. perfringens$, and $C. acetobutylicum$. Transcription factors and sigma factors are shown in bold, and proteins enclosed in boxes directly participate in trans-septum signaling. Dashed boxes indicate that the function of the proteins in trans-septum signaling has not been tested yet. Text color denotes whether the factor has been detected at both the transcript and protein level (black), at either the transcript or protein level (purple), or has not been tested yet at the transcript or protein level (blue), indicating the need for further experimentation. SpoIIQ* denotes the predicted clostridial homolog to $B. subtilis$ SpoIIQ based on bioinformatics analyses [13]. Pro-$\sigma^K$ in $C. acetobutylicum$ is shown in parentheses to indicate that the pro-form has not been detected by Western blot [31]. Black arrows indicate transcriptional control of gene expression, red arrows indicate signaling pathways, dashed arrows indicate that the regulatory relationship between the factors has not been tested, and thick arrows demarcate notable points of divergence from the pathway defined in $B. subtilis$. AND gates are indicated. Unique features of the sporulation pathway in $C. difficile$ include the post-translational activation of $\sigma^G$ by $\sigma^F$ and the absence of proteolytic activation of $\sigma^K$; the $\sigma^K$-dependent SpoIIA-H feeding tube appears to be dispensable for $\sigma^K$ activation.

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increases in gene numbers. One explanation for the smaller size is that \textit{C. difficile} activates fewer genes during sporulation than \textit{B. subtilis}. A more likely explanation is that the asynchronous population of sporulating cells (Figures 1 \& 82, [37]) limits the detection of genes that are transiently expressed during discrete stages of sporulation or genes that are expressed at low levels during sporulation [24–26,69]. Since the RNA samples used in the RNA-Seq analysis were harvested from a sporulation timepoint in which phase-bright forespores were produced by wildtype cells (Figure S2), fewer cells in the population are likely to be at early stages sporulation. As a result, early sporulation genes may be under-represented in our data set; for example, \(\sigma^G\)-dependent early sporulation gene transcripts from \textit{spoIIR} and \textit{spoIIP} were almost undetectable by RNA-Seq (Table S4). In addition, genes that are regulated by more complex mechanisms beyond upregulation by a specific sigma factor are likely to be under-represented in our data set. Sporulation genes that are subject to incoherent feed forward loop regulation, in which their expression is induced by a given sigma factor and repressed by a downstream regulator such as SpoVT-mediated inhibition of \(\sigma G\) transcription in \textit{B. subtilis} [26], may not be detected in our data set. Unraveling the complexities of sporulation gene regulation in \textit{C. difficile} will require further characterization of the kinetics of sporulation and the analysis of mutants defective in auxiliary sporulation regulators.

Of the 51 genes proposed to comprise the core set of sporulation genes in spore-forming Firmicutes by bioinformatics analyses that are conserved in \textit{C. difficile} [13], 34 were identified in our RNA-Seq analyses, leaving 17 that were not identified in our transcriptional analyses (Table S4). Seven of these genes do not have detectable homologs in the 630 \textit{C. difficile} genome, and 6 were expressed at low levels with a base mean of expression less than 15 (Table S4). Although some sporulation-induced genes were likely not detected in our analyses due to low levels of expression, the transcriptional profiling data presented here identify a promising set of genes that are likely to encode proteins with important roles in spore formation. Of the six spore coat proteins recently identified in a proteomic analysis of \textit{C. difficile} [50,53], all were identified in our RNA-Seq experiments. Three of these spore coat genes were determined to be \(\sigma^K\)-dependent, consistent with their predicted role as components of the outer coat (Table S4). Notably, \(\sigma^K\)-regulated genes were among the most abundantly expressed genes induced during sporulation, comprising 6 of the 10 most highly expressed sporulation genes (Table S9). The \(\sigma^K\)-regulated \textit{CD1067} gene was the most highly expressed gene induced during sporulation in \textit{C. difficile}. Cysteine-rich \textit{CD1067} was also one of the most abundant spore proteins identified in proteomic analyses of purified spores and is encoded in a 7.5 kB region enriched in genes encoding spore proteins [70]. Western blot analyses of cysteine-rich \textit{CD1067} revealed that it forms higher order multimers that are highly resistant to denaturing conditions (data not shown), consistent with the proposal that \textit{CD1067} may form a rigid, disulphide-bonded structure around the spore coat upon exposure to atmospheric oxygen, for example during excretion from the host [70]. Intriguingly, \textit{CD1067} is encoded in a region enriched in highly expressed, \(\sigma^K\)-regulated genes encoding hypothetical proteins unique to \textit{C. difficile}, with 8 of the 9 genes in this region being induced during sporulation and 6 of the 9 being \(\sigma^K\)-regulated. These genes may encode coat proteins that confer structural integrity and/or resistance to the \textit{C. difficile} spore coat and thus may play important roles in disease transmission and/or represent good candidates for developing diagnostic reagents.

Although the number of \(\sigma^G\)-dependent genes identified by our study was small, a number of these genes encode proteins with important functions in the forespore of \textit{B. subtilis}, specifically \textit{sofA}, \textit{sofB}, \textit{dacF}, \textit{spoVT}, and \textit{spoVAD} [26,67,71–73]. Since \textit{B. subtilis} \(\sigma^G\) induces the expression of genes encoding the germinant receptors (of which there are no homologs in \textit{C. difficile} [12,34]), it seems likely that some of the \(\sigma^G\)-dependent genes identified in our study encode proteins that transduce the germinant signal into the spore core. It will be interesting to determine whether any of the \(\sigma^G\)-regulated genes identified in our study play important roles in regulating germination and thus disease transmission.

Genes encoding hypothetical proteins were the most abundant class of genes identified in our study (82 in total, Table S10). Twenty of these hypothetical proteins were detected in proteomic analyses of \textit{C. difficile} spores [70]. Indeed, two of the hypothetical proteins were previously shown to be part of the spore coat [50], and we have validated three additional proteins as localizing to the spore coat (data not shown). BLAST searches with the hypothetical proteins identified by RNA-Seq indicate that 16 have no known homologs. These \textit{C. difficile}-specific proteins could comprise part of the spore coat, since coat proteins are often poorly conserved, species-specific, and categorized as hypothetical proteins [10,11].

Taken together, by examining the regulatory interplay between sporulation sigma factors in \textit{C. difficile}, our study highlights that diverse pathways regulate sporulation in the Firmicutes and that considerable work is needed to map these pathways in the Clostridia. By using whole genome transcriptional profiling to define a large set of genes that are activated by Spo0A, \(\sigma^K\), \(\sigma^G\), and/or \(\sigma^F\), our study also provides a framework for identifying new proteins that are necessary for sporulation and determining the role of these proteins in forming a functional, infectious spore. Studies of this nature may lead to the identification of biomarkers for \textit{C. difficile} spores and candidates for vaccine development.

\textbf{Materials and Methods}

\textbf{Bacterial strains and growth conditions}

All \textit{C. difficile} strains are listed in Table 1 and derive from the parent strain JIR8094 [33], an erythromycin-sensitive derivative of the sequenced clinical isolate 630 [34]. \textit{C. difficile} strains were grown on solid brain heart infusion media supplemented with yeast extract (BHIS: 37 g brain heart infusion, 5 g yeast extract, 0.1% (w/v) \textit{L}-cysteine, 15 g agar per liter) [74]. Taurocholate (TA; 0.1% w/v), thiamphenicol (5–10 \(\mu\)g/mL), kanamycin (50 \(\mu\)g/mL), cefoxitin (16 \(\mu\)g/mL), FeSO\(_4\) (50 \(\mu\)M), and/or erythromycin (10 \(\mu\)g/mL) were used to supplement the BHIS media as indicated. Cultures were grown at 37°C, under anaerobic conditions using a gas mixture containing 85% \textit{N}_2, 5% \textit{CO}_2, and 10% \textit{H}_2.

Sporulation was induced on media containing BHIS and SMC (90 g BactoPeptone, 5 g protease peptone, 1 g KH\(_2\)PO\(_4\), 1.5 g Tris base, 13 g agar per liter) [50], at 70% SMC and 30% BHIS (70:30 media, 63 g BactoPeptone, 3.5 g Protease Peptone, 11.1 g BH1, 1.5 g yeast extract, 1.06 g Tris base, 0.7 g KH\(_2\)PO\(_4\), 15 g agar per liter) [37], 70:30 agar (supplemented as appropriate with thiamphenicol at 5–10 \(\mu\)g/mL) was inoculated from a starter culture grown on solid media.

\textit{HB101/pK242} strains were used for conjugations and \textit{BL21(DE3)} strains were used for protein expression. \textit{E. coli} strains were routinely grown at 37°C, shaking at 225 rpm in Luria-Bertani broth (LB). Media was supplemented with chlorampheni-
E. coli strain construction

All strains are listed in Table S11; all plasmids are listed in Table S12; and all primers are listed in Table S13. For disruption of spo0A, sigE, sigG, sigK, and sigF, a modified plasmid containing the retargeting group II intron, pCE245 (a gift from C. Ellermeier, University of Iowa), was used as the template. Primers containing the retargeting group II intron, pCE245 (a gift from C. Ellermeier, University of Iowa), was used as the template. Primers used to amplify the targeting sequence from the template carried flanking regions specific for each gene target and are listed as follows: spo0A (#539, 540, 541 and 532), the EBS Universal primer as specified by the manufacturer (Sigma Aldrich), sigE (#563, 564, 635 and 532), sigG (#728, 729, 780, and 532), sigK (#681, 682, 683, and 532) and sigF (#775, 776, 777, and 532). The spo0A disruption mutant was constructed using the same primers as Underwood et al. [36]. The resulting retargeting sequences were digested with BsrGI and HindIII and cloned into pJS107 (a gift from J. Sorg, University of Texas A&M), a derivative of pJIR750ai (Sigma Aldrich) [32]. The ligations were transformed into DH5α and confirmed by sequencing. The resulting plasmids were used to transform HB101/pK124.

To construct the sigE complementation construct, primers #725 and #726 were used to amplify a fragment containing 252 bp upstream and 156 bp downstream of the two gene spoIIGA-sigE operon using 630 genomic DNA as the template. To construct the sigG complementation construct, primers #883 and 884 were used to amplify 288 bp upstream and 16 bp downstream of sigG using 630 genomic DNA as the template. The sigK complementation construct was made using PCR splicing by overlap extension (SOE) [75]. Primer pair #734 and 736 was used to amplify the 5′ SOE product, while primer pair #735 and 737 was used to amplify the 3′ SOE product. The resulting fragments were mixed together, and the flanking primers #734 and #737 were used to amplify an 898 bp fragment corresponding to the sigK gene including 256 bp region of upstream sequence. This strategy was used to clone an intact sigK gene with the skin element excised [43]. To construct the sigF complementation construct, primers #954 and #956 were used to amplify 88 bp upstream and 19 bp downstream of spoIIBA-spoIAB-sigF operon, using 630 genomic DNA as the template. All complementation constructs were digested with NotI and XhoI and ligated into pMTL83151 [40] digested with the same enzymes, with the exception of the sigF complementation construct, which was cloned into pMTL84151 digested with the same enzymes [48].

To construct strains producing recombinant CD3522, σE, σG, σF, Gpr, SpoVT, and SspA for antibiotic production, primer pairs #490 and #499; #596 and #597; #727 and #724; #790 and #791; #883 and #884; #975 and #976; and #885 and #886 were used to amplify the cd3522, sigE, sigG, sigF, gpr, spoVT, and sspA genes lacking stop codons, respectively, using 630 genomic DNA as the template. The sigE expression construct deletes the sequence encoding the first 23 amino acids of σE, which removes its membrane-anchoring domain and improves the solubility of the protein in E. coli. The resulting PCR products were digested with NdeI and XhoI, (or NheI and XhoI for gpr) ligated to pET22b (or pET21a for gpr and sspA), and used to transform DH5α. To construct a strain producing recombinant σK, PCR SOE was used to amplify the sigK gene lacking the skin element. Primer pair #629 and 630 was used to amplify the 5′ SOE product, while primer pair #689 and 736 was used to amplify the 3′ SOE product. The resulting fragments were mixed together, and the flanking primers #689 and #737 were used to amplify the 3′ SOE product. The resulting fragments were ligated together, digested with NotI and HindIII, and cloned into pMTL83151 digested with the same enzymes, and used to transform DH5α.

Table 1. C. difficile strains used in this study.

| Strain | C. difficile strain | Relevant genotype or features |
|--------|---------------------|------------------------------|
| 11     | JIR8094             | erm-sensitive derivative of 630 [33] |
| 13     | 630                 | Clinical isolate 630 [34] |
| 35     | spo0A               | JIR8094/spo0A:ermB |
| 50     | sigG                | JIR8094 sigG:ermB |
| 67     | sigK                | JIR8094 sigK:ermB |
| 71     | JIR8094/pMTL84151   | JIR8094/pMTL84151 |
| 99     | sigE                | JIR8094 sigE:ermB |
| 106    | sigF                | JIR8094 sigF:ermB |
| 110    | JIR8094/pMTL83151   | JIR8094/pMTL83151 |
| 127    | sigE/pMTL83151-spoIAB-sigE | JIR8094 sigE:ermB/pMTL83151-spoIAB-sigE |
| 143    | sigG/pMTL83151-sigG | JIR8094 sigG:ermB/pMTL83151-sigG |
| 145    | sigK/pMTL83151-sigK | JIR8094 sigK:ermB/pMTL83151-sigK |
| 159    | sigG                 | JIR8094 sigG:ermB/pMTL83151 |
| 164    | sigF                 | JIR8094 sigF:ermB/pMTL83151 |
| 167    | sigE                 | JIR8094 sigE:ermB/pMTL83151 |
| 214    | sigF/pMTL84151      | JIR8094 sigF:ermB/pMTL84151 |
| 218    | spo0A/pMTL83151     | JIR8094 spo0A:ermB/pMTL83151 |
| 222    | sigF/pMTL84151      | JIR8094 sigF:ermB/pMTL84151 |
| 289    | spo0A/pMTL84151     | JIR8094 spo0A:ermB/pMTL84151 |

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pET21a-ght, pET22b-spoVT, and pET21a-spoA plasmids were used to transform BL21(DE3) for protein expression.

C. difficile strain construction

C. difficile strains were constructed using TargeTron-based gene disruption as described previously (Figure S1, [32,37,76]). TargeTron constructs in pJS107 were conjugated into C. difficile using an E. coli HB101/pK424 donor strain. HB101/pK424 strains containing the appropriate pJS107 construct were grown aerobically to exponential phase in 2 mL of LB supplemented with ampicillin (50 µg/mL) and chloramphenicol (10 µg/mL). Cultures were pelleted, transferred into the anaerobic chamber, and resuspended in 1.5 mL of low-exponential phase C. difficile JIR0994 cultures (grown aerobically in BHIS broth). The resulting cell mixture was plated as seven 100 µL spots onto pre-dried, pre-reduced BHIS agar plates. After overnight incubation, all growth was harvested from the BHIS plates, resuspended in 2.5 mL pre-reduced BHIS, and twenty-one 100 µL spots per strain were plated onto BHIS agar supplemented with thiamphenicol (10 µg/mL), kanamycin (50 µg/mL), and cefoxitin (16 µg/mL) to select for C. difficile containing the pJS107 plasmid. After 24–48 hrs of anaerobic growth, single colonies were patched onto BHIS agar supplemented with thiamphenicol (10 µg/mL), kanamycin (50 µg/mL), and FeSO₄ (50 µM) to induce expression of the ferredoxin promoter of the group II intron system. After overnight growth, patches were transferred to BHIS agar plates supplemented with erythromycin (10 µg/mL) for 24–72 hrs to select for cells with activated group II intron systems. Erythromycin-resistant patches were struck out for isolation onto the same media and individual colonies were screened by colony PCR for a 2 kb increase in the size of spoOA (primer pair #556 and 557), sigE (primer pair #687 and 688), sigG (primer pair #725 and 724), sigK (primer pair #689 and 690), and sigF (primer pair #790 and 791) (Figure S1). A minimum of two independent clones from each mutant strain was phenotypically characterized.

C. difficile complementation

HB101/pK424 donor strains carrying the appropriate complementation construct were grown in LB containing ampicillin (50 µg/mL) and chloramphenicol (20 µg/mL) at 37°C, 225 rpm, under aerobic conditions, for 6 hrs. C. difficile recipient strains spoOA⁻, sigE⁻, sigF⁻, sigG⁻, sigK⁻, and sigF⁺, containing group II intron disruptions, were grown aerobically in BHIS broth at 37°C with gentle shaking for 6 hrs. HB101/pK424 cultures were pelleted at 2500 rpm for 5 min and the supernatant was removed. Pellets were transferred to the anaerobic chamber and gently resuspended in 1.5 mL of the appropriate C. difficile culture. The resulting mixture was inoculated onto pre-dried, pre-reduced BHIS agar plates, as seven 100 µL spots for 12 hrs. All spots were collected anaerobically and resuspended in 1 mL PBS. The resulting suspension was spread onto pre-dried, pre-reduced BHIS agar plates supplemented with thiamphenicol (10 µg/mL), kanamycin (50 µg/mL), and cefoxitin (10 µg/mL) at 100 µL per plate, five plates per conjugation. Plates were monitored for colony growth for 24–72 hrs. Individual colonies were struck out for isolation and analyzed for complementation by phase contrast microscopy, Western blot analysis and transmission electron microscopy. A minimum of two independent clones from each complementation strain was phenotypically characterized.

For the sigF complementation, a pMTL84151 plasmid backbone was used. The complementation protocol was followed as described except that after spots were collected from overnight growth on BHIS plates, the resulting PBS suspension was spotted onto three BHIS agar plates supplemented with thiamphenicol (10 µg/mL), kanamycin (50 µg/mL), and cefoxitin (16 µg/mL) with 7–100 µL spots per plate.

Sporulation assay

C. difficile strains were grown from glycerol stocks on BHIS plates supplemented with thiamphenicol (5–10 µg/mL) and cefoxitin (16 µg/mL) for 24–72 hrs to select for cells with activated group II intron systems. Erythromycin-resistant cells were serially diluted and plated on pre-reduced BHIS-TA plates. After 20 hrs on BHIS-TA, colonies were counted, and cell counts were determined. The percent of heat-resistant spores was determined based on the ratio of heat-resistant cells to total cells, and sporulation efficiencies were determined based on the ratio of heat-resistant cells for a strain compared to wild type. Results are based on a minimum of three biological replicates. spoOA⁻ containing empty vector was included as a control for all assays [77].

Fluorescence and light microscopy

For fluorescence microscopy studies, C. difficile strains were harvested in PBS after 18 hours of growth on 70:30 media, pelleted, and resuspended in 1.0 mL PBS containing 1 µg/mL FMI-64 (Molecular Probes) and 15 µg/mL Hoechst 33342 (Molecular Probes). The bacterial suspension (4 µL) was added to a freshly prepared 1% agarose pad on a microscope slide, covered with a 22 x 22 mm #1 coverslip and sealed with VALAB (1:1:1 of vaseline, lanolin, and beeswax) as previously described [78]. Phase and fluorescence microscopy were performed using a Nikon PlanApo 100 x Ph3 oil immersion objective (1.4 NA) on a Nikon Eclipse TE300 epifluorescence microscope. Five fields for each sample were acquired with an iXon3 885 EMCCD camera (Andor) cooled to −70°C with frame averaging set to 4 and an EM gain setting of 3, and driven by NIS-Elements software (Nikon). Images were subsequently imported into Adobe Photoshop CS6 for minimal adjustments in brightness/contrast levels and pseudocoloring.

Phase-contrast microscopy for imaging the samples used for RNA-Seq was performed as previously described [37]. Quantification of total cells undergoing sporulation was determined by analyzing multiple fields for each strain at random. Greater than 200 cells were enumerated for each strain. For cultures analyzed by fluorescence microscopy, sporulating cells were identified as either having a polar septum with or without DNA staining in the forespore, a phase-dark forespore with or without DNA staining in the forespore compartment, a phase-bright forespore without DNA staining, or a free spore (no mother cell compartment).
Electron microscopy

One hundred microliters of bacterial cell suspension samples from sporulation assays were prepared as previously described [37].

Western blot analyses

Sporulation assay C. difficile cells (50 μL of PBS suspension) were freeze-thawed three times, diluted in 100 μL EBB buffer (8 M urea, 2 M thiourea, 4% (v/v) SDS, 2% (v/v) β-mercaptoethanol), and incubated at 95°C for 20 min with vortexing every 5 min. Samples were centrifuged for 5 min at 15,000 rpm and 7 μL of 4× sample buffer (40% (v/v) glycerol, 1 M Tris pH 6.8, 20% (v/v) β-mercaptoethanol, 8% (w/v) SDS, and 0.04% (w/v) bromophenol blue), was added. Protein samples were incubated again at 95°C for 15 minutes with vortexing followed by centrifugation for 5 min at 15,000 rpm. SDS-PAGE gels (12%-15%) were loaded with 5 μL of protein prep. Gels were transferred to Bio-Rad PVDF membrane and blocked in 50% PBS:50% Odyssey Blocking Buffer with 0.1% (v/v) Tween for 30 min at RT. Polyclonal rabbit anti-σE, anti-σD, anti-σS, anti-SpoIVA [37], and anti-CD1433 [76], anti-CD1067, anti-Gpr, anti-SpoVT, and anti-Sspa antibodies were used in this study were raised in rabbits by Cocalico Biologicals (Reamstown, PA). The antigens A230aa-σE-His6, σD-His6, σS-His6, σK-His6, CD3522-His6, Gpr-His6, SpoVT-His6, and Sspa-His6 were purified on Ni²⁺-affinity resin from E. coli strains #755, 743, 756, 921, 853, 881, and #Sspa respectively, as described above. Cultures were grown and protein expression was induced for 30 min, supernatant was harvested, pelleted, and resuspended in 25 mL of low imidazole buffer (HIB; 500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 15 mM imidazole, 10% (v/v) glycerol). Cells were flash frozen in liquid nitrogen, thawed, and lysed by sonication (45 sec burst followed by 3 cycles). Read pairs were mapped to libraries (demultiplexed) and converted to Fastq format using Illumina CASAVA 1.8.2 with default parameters. Adapters were clipped and reads were trimmed to remove the first 12 and last 11 cycles using Trimmomatic [79], dropping read pairs for which at least one read was less than 50 bp. The C. difficile 630 genome (NC_009089) sequence was modified by removing the sigK intervening (skin) element. The C. difficile 630 genome annotation was modified by the addition of sigK. Read pairs were aligned to the modified C. difficile 630 genome (NC_009089) using BWA 0.6.1 with default parameters with one exception (q 20). Read pairs were mapped to NC_009089 gene annotation using the countOverlaps procedure of the R/Bioconductor IRanges package [80-82]. Counts associated with rRNA were removed. Counts associated with the same library were pooled. Reads were of high quality (median Phred score of 39 and a first quartile of 35) as were alignments (median mapping quality score, MAPQ of 60). Median fragment lengths were between 180 and 250. The vast majority of unmapped sequences failed to align to sequences in the NCBI non-redundant database using a blastn and blastx search. There was no indication of highly represented reads among unmapped sequences. Since the majority of reads failed to map to known natural sequences, and since sequences can arise during library preparation particularly when the input sample is small, sequences that failed to map to the C. difficile genome likely represent spurious sequences produced during library construction.

RNA processing

RNA for RNA-Seq was extracted from WT, spo0A Δ, sigF Δ, sigG Δ, sigF Δ, sigG Δ, and sigK Δ C. difficile cell suspensions, from an 18 hr sporulation assay (described earlier), using a FastRNA Pro Blue Kit (MP Biomedical) and a FastPrep-24 automated homogenizer (MP Biomedical, setting 6.0, 45 seconds for 3 cycles). Contaminating genomic DNA was depleted using a column-bound DNase treatment with an RNase Kit (Qiagen) followed by two suspension DNase treatments (New England Biolabs), according to manufacturer’s recommendations. Samples were tested for genomic DNA contamination using quantitative PCR for 16S rRNA and the sleC gene. DNase-treated RNA (5 μg) was mRNA enriched using a Rho-Zero Magnetic Kit (Epicentre).

RNA isolated for qRT-PCR was processed identically except that mRNA enrichment was done using an Ambion MICROExpress Bacterial mRNA Enrichment Kit (Invitrogen). Reverse transcription of enriched RNA was done using the Super Script First Strand cDNA Synthesis Kit (Invitrogen) with random hexamer primers.

RNA-Seq library construction and sequencing

Sequence calls and quality scores were produced in BCL format from images using Illumina RTA v1.13 with default parameters. Read pairs were mapped to libraries (demultiplexed) and converted to Fastq format using Illumina CASAVA 1.8.2 with default parameters. Adapters were clipped and reads were trimmed to remove the first 12 and last 11 cycles using Trimmomatic [79], dropping read pairs for which at least one read was less than 50 bp. The C. difficile 630 genome (NC_009089) sequence was modified by removing the sigK intervening (skin) element. The C. difficile 630 genome annotation was modified by the addition of sigK. Read pairs were aligned to the modified C. difficile 630 genome (NC_009089) using BWA 0.6.1 with default parameters with one exception (q 20). Read pairs were mapped to NC_009089 gene annotation using the countOverlaps procedure of the R/Bioconductor IRanges package [80-82]. Counts associated with rRNA were removed. Counts associated with the same library were pooled. Reads were of high quality (median Phred score of 39 and a first quartile of 35) as were alignments (median mapping quality score, MAPQ of 60). Median fragment lengths were between 180 and 250.

The vast majority of unmapped sequences failed to align to sequences in the NCBI non-redundant database using a blastn and blastx search. There was no indication of highly represented reads among unmapped sequences. Since the majority of reads failed to map to known natural sequences, and since sequences can arise during library preparation particularly when the input sample is small, sequences that failed to map to the C. difficile genome likely represent spurious sequences produced during library construction.
Differential expression statistics reflecting both effect size (fold-change) and statistical significance (p-value adjusted based on the method of Benjamini and Hochberg [93]) were calculated using DESeq [49]. Duplicate reads were excluded from these analyses. Differentially expressed genes were identified based on a minimum fold-change (higher in the reference sample than the query) and maximum p-value. Tables showing genes whose expression was downregulated by ≥4-fold with an adjusted p-value of ≤0.05 during sporulation are provided in the Supplementary Information (Tables S3, S4, S5, S6, S7, S8, S9, S10). A table showing genes whose expression was upregulated by ≥4-fold with an adjusted p-value of ≤0.05 in a spo0A-dependent manner are shown in Table S14.

The log2-transformed expression of genes that were downregulated by ≥4-fold with an adjusted p-value of ≤10^-5 in the spo0A strain relative to wild type expression were represented in a heat map using the heatmap2 procedure of the R/Bioconductor gplots package with default options [84]. Expression levels in spo0A were not shown because the differential expression between spo0A and wild type was biased by the method used to select genes. Expression levels in the other four strains relative to spo0A were centered, scaled, and mapped to a red-green color scale.

Quantitative RT-PCR
For the RNA-Seq validation, expression levels of gtr, cd1025 (spolQ), cd2376, cd1511, cd3522, spoIVA, cd1433, cd1006, sloC, sspR, sspV, dafC, and spoB (housekeeping gene) were run on WT, spo0A-, sigF-, sigE-, sigG-, and sigK- cDNA templates in three replicate reactions using gene-specific primer pairs #1187 and 1188; #1213 and 1214; #1191 and 1192; #796 and 797; #989 and 990; #796 and 799; #792 and 793; #1030 and 1031; #575 and 576; #810 and 811; #995 and 996; #993 and 994; #1002 and 1003, respectively. Quantitative real-time PCR was performed using SYBR Green JumpStart Taq Ready Mix (Sigma), 50 nM of gene specific primers (Table S12), and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Mean Ct values were normalized to the spolA- (negative control) sample and further normalized to rpoB. Relative expression values reported are representative of three biological replicates.

Supporting Information
Figure S1 Construction of spo0A-, sigE-, sigG-, sigK- and sigF- mutants in C. difficile. (A) Schematic of the group II intron targeted gene disruption system. (B) Colony PCR analysis of spo0A-, sigE-, sigG-, sigK-, and sigF- strains compared to wild type (WT) using primers that flank the gene of interest. The group II intron insertion is ~2 kb.

Figure S2 Phase contrast microscopy of strains used for RNA-Seq analyses. Phase-contrast microscopy of WT, spo0A-, sigF-, sigE-, sigG-, and sigK- strains grown on sporulation media for 18 hrs. White triangles mark mature phase-bright spores, and black triangles indicate immature phase-dark forespores. Phase-bright spores were not observed in the sigma factor mutants. The percentage of sporulating cells, defined as containing phase-dark forespores, phase-bright forespores, or free spores, is shown for each of the biological replicates. Scale bar represents 5 μm.

Figure S3 Prevalence of sigG- phenotypes. TEM of sigG- mutants during growth on sporulation media. sigG- mutant cells (n = 80) containing a forespore with a putative coat layer (black triangle) were scored for the presence of a ruffled membranes (98%), a double forespore compartment (21%), and incomplete engulfment (87%). A black arrow indicates incomplete membrane fission during engulfment, and a white arrow indicates septum-like structures in the forespore. No cortex was detected in any of the sigG- mutant cells analyzed. All wildtype forespores surrounded by a coat layer had completed engulfment (n = 80 cells, data not shown). Scale bar represents 500 nm.

Figure S4 Plasmid complementation rescues spore formation in C. difficile sigma factor mutants. (A) Phase-contrast microscopy of sigF-, sigE-, and sigG- strains grown on sporulation media for 30 hrs and the sigK- strain for 42 hrs. The strains carry either empty pMTL83151 or pMTL84151 vector [48] or pMTL8151-sigE, sigG, or sigK genes, respectively, or pMTL84151-sigF, expressed from their native promoters. White triangles mark mature phase-bright spores, and black triangles indicate immature phase-dark forespores. Phase-bright spores were not observed in the sigma factor mutants. Scale bar represents 5 μm. (B) Western blot analyses of wildtype (WT), spo0A-, sigF-, sigG-, and sigK- carrying either empty pMTL83151 vector (EV) or a complementation construct using antibodies raised against σF, σE, σG, and σK. SpolA levels were also measured to compare the induction of sporulation between strains [37,86]. The asterisk demarcates a non-specific band observed in all strains tested. (C) Sporulation efficiencies determined by heat resistance assays of complementation strains sigF+pMTL84151-sigF+, sigE+/pMTL83151-sigE+, and sigK+/pMTL83151-sigK+ relative to wildtype. No heat-resistant spores were detected in mutant strains carrying empty vector.

Figure S5 Plasmid complementation rescues coat and cortex formation in sigma factor mutants. The sigF-, sigE-, and sigG- strains were grown on sporulation media for 28 hrs, while the sigK- strains were grown for 40 hrs. The strains carry either empty pMTL83151 or pMTL84151 vector for sigF- [48] or sigE, sigG, or sigK genes, respectively, expressed from their native promoters. White triangles indicate cortex and black triangles indicate coat. Scale bar represents 250 μm.

Figure S6 Analysis of sigma factor regulation network topology in C. difficile. Circles represent genes and arrows indicate activation of expression (see Text S1). s0 = spo0A, ε = sigE, g = sigG, and k = sigK. (A) Network topology proposed for B. subtilis. (B) Expression profile of a query sporulation gene, q, among the spo0A-, sigE-, sigG-, and sigK- mutants, illustrated in the context of the B. subtilis network topology. The circles of the network topology (A) represent genes whereas the columns in the heat map (Figure 5) represent strains; by coloring the circles of the topology using the expression level of q in the associated knockout strain (red = low; green = high), the consistency of the expression profile (B) with the network topology can be readily evaluated. More precisely, a network is consistent with the expression profile of q if and only if the red circles form the path between s0 (spo0A-) and q. The example is inconsistent with the B. subtilis topology because there is no way to attach q that will result in a consistent topology. (C and D) Expression profile for σE- and σG-dependent genes, respectively, in the proposed topology for C. difficile. Red coloring of a gene indicates that q is downregulated when the former is knocked out. For example, in (D) q is downregulated in s0 (spo0A-) and ε (sigE) mutants but upregulated in the g (sigG) and k (sigK) mutants.
Figure S7 Statistical analysis rejects the B. subtilis network topology for sporulation sigma factor regulation. Each gene was fit to models associated with the null, $\sigma^E$, and $\sigma^K$-dependent transcriptome models to obtain p-values (see Text S1).

Table S1 Quantitation of sporulating cell phenotypes. C. difficile strains JIR8094 (WT), sig$F^-$, sig$E^-$, sig$G^-$, and sig$K^-$ exhibit asynchronous sporulation when grown on sporulation induction media for 18 hours. Phase-contrast microscopy and fluorescence light microscopy using the membrane stain FM4-64 and the nucleic acid dye Hoechst was used to analyze sporulation in the indicated strains. A cell was deemed positive for sporulation if it fell into one of five criteria: (1) a polar septum was detected by FM4-64, but the forespore did not stain with Hoechst; (2) Polar septum was detected by FM4-64, and the forespore stained with Hoechst; (3) a phase-dark forespore stained with both FM4-64 and Hoechst; (4) A phase-dark forespore stained with FM4-64 but not Hoechst, or (5) a phase-bright forespore was visible, but it failed to stain with either FM4-64 or Hoechst. The percent of total sporulating cells reflects the number of events that fall within the stated criteria relative to the total number of cells. A total of 200 cells were counted for each strain. spo0A$^-$ cells were not evaluated for sporulation staining.

Table S2 Summary of RNA-Seq data analysis. Strain.Rep refers to the strain name followed by the replicate number. Three biological replicates were processed for RNA-Seq analyses for each strain, WT refers to the parental JIR8094 strain. The total number of reads obtained and mapped to the genome is indicated. % mapped refers to the percentage of reads that mapped to the C. difficile genome. $>$90% of the unmapped reads did not map to sequences in the NCBI database and appear to derive from spurious amplification products during library construction.

Table S3 Spo0A-dependent (activated) genes. † Two factors are listed in the table for genes whose expression was dependent on both $\sigma^E$ and $\sigma^G$ (adjusted p-value=0.05, log$_2$FC$\leq-2$). Dep. indicates the most downstream sigma factor on which gene expression depends upon. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log$_2$FC denotes log$_2$-fold-change. A negative value indicates that the gene was downregulated relative to wild type. * Indicates that gene product was detected in Lawley et al. proteomic analysis of purified spores [70]. –Inf indicates that no transcript was detected in the mutant relative to wild type. See Text S2 for the references.

Table S5 $\sigma^F$-dependent genes. † Two factors are listed in the table for genes whose expression was dependent on both $\sigma^E$ and $\sigma^G$ (adjusted p-value=0.05, log$_2$FC$\leq-2$). Dep. indicates the most downstream sigma factor on which gene expression depends upon. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log$_2$FC denotes log$_2$-fold-change. A negative value indicates that the gene was downregulated relative to wild type. * Indicates that gene product was detected in Lawley et al. proteomic analysis of purified spores [70]. –Inf indicates that no transcript was detected in the mutant relative to wild type. See Text S2 for the references.

Table S6 $\sigma^G$-dependent genes. † Two factors are listed in the table for genes whose expression was dependent on both $\sigma^E$ and $\sigma^G$ (adjusted p-value=0.05, log$_2$FC$\leq-2$). Dep. indicates the most downstream sigma factor on which gene expression depends upon. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log$_2$FC denotes log$_2$-fold-change. A negative value indicates that the gene was downregulated relative to wild type. * Indicates that gene product was detected in Lawley et al. proteomic analysis of purified spores [70]. –Inf indicates that no transcript was detected in the mutant relative to wild type. See Text S2 for the references.

Table S7 $\sigma^K$-dependent genes. † Two factors are listed in the table for genes whose expression was dependent on both $\sigma^E$ and $\sigma^G$ (adjusted p-value=0.05, log$_2$FC$\leq-2$). Dep. indicates the most downstream sigma factor on which gene expression depends upon. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log$_2$FC denotes log$_2$-fold-change. A negative value indicates that the gene was downregulated relative to wild type. * Indicates that gene product was detected in Lawley et al. proteomic analysis of purified spores [70]. –Inf indicates that no transcript was detected in the mutant relative to wild type. See Text S2 for the references.

Table S8 $\sigma^L$-dependent genes. † Two factors are listed in the table for genes whose expression was dependent on both $\sigma^E$ and $\sigma^G$ (adjusted p-value=0.05, log$_2$FC$\leq-2$). Dep. indicates the most downstream sigma factor on which gene expression depends upon. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log$_2$FC denotes log$_2$-fold-change. A negative value indicates that the gene was downregulated relative to wild type. * Indicates that gene product was detected in Lawley et al. proteomic analysis of purified spores [70]. –Inf indicates that no transcript was detected in the mutant relative to wild type. See Text S2 for the references.
Table S10 Genes encoding hypothetical proteins induced during sporulation. Two factors are listed in the table for genes whose expression was dependent on both σ^52 and σ^54 (adjusted p-value ≤ 0.05, log2FC ≤ −2). Dep. indicates the most downstream sigma factor on which gene expression depends upon. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log2FC denotes log2-fold-change. A negative value indicates that the gene was downregulated relative to wild type. ∆ indicates that gene product was detected in Lawley et al. proteomic analysis of purified spores [70]. —inf indicates that no transcript was detected in the mutant relative to wild type. See Text S2 for the references.

Table S11 E. coli strains used in this study.

Table S12 Plasmids used in this study.

Table S13 Primers used in this study.

Table S14 Genes whose expression is increased in the absence of spo0A. BM refers to base mean, the mean of the counts after they were divided by the size factors to adjust for different sequencing depths. This value is the mean for the sample relative to wild type. log2FC denotes log2-fold-change. A positive value indicates that the gene was upregulated in the spo0A^− mutant relative to wild type. +inf indicates that no transcript was detected in wild type relative to the spo0A^− mutant.

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