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Initiation of Sporulation in *Clostridium difficile*: a Twist on the Classic Model

Adrianne N. Edwards and **Shonna M. McBride***
Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA USA

Abstract

The formation of dormant endospores is a complex morphological process that permits long-term survival in inhospitable environments for many Gram-positive bacteria. Sporulation for the anaerobic gastrointestinal pathogen *Clostridium difficile* is necessary for survival outside of the gastrointestinal tract of its host. While the developmental stages of spore formation are largely conserved amongst endospore-forming bacteria, the genus *Clostridium* appears to be missing a number of conserved regulators required for efficient sporulation in other spore forming bacteria. Several recent studies have discovered novel mechanisms and distinct regulatory pathways that control the initiation of sporulation and early sporulation-specific gene expression. These differences in regulating the decision to undergo sporulation reflects the unique ecological niche and environmental conditions that *C. difficile* inhabits and encounters within the mammalian host.

Keywords

Spores; Endospore; Anaerobe; Sporulation; Nutrition; Phosphorylation

Introduction

Clostridium difficile is a significant gastrointestinal pathogen that infects humans and other animals and is the primary cause of antibiotic associated diarrhea (AAD). *C. difficile* infection (CDI) is typically precipitated by the use of antibiotics, which disrupts the native gut microbiota, providing a niche for *C. difficile* overgrowth and toxin production. CDI and AAD have recently become an increasing health problem in hospital and nursing home settings (O'Brien *et al*., 2007, Bouza, 2012, Dubberke & Olsen, 2012, Murphy *et al*., 2012). In addition, *C. difficile* has recently been recognized as an emerging pathogen and urgent public health threat by the CDC (CDC, 2013). Although *C. difficile* is an important pathogen, its growth and lifecycle within the host remain poorly understood.

C. difficile is a strict anaerobe that forms metabolically inactive spores within the gastrointestinal tract of mammals. These dormant spores are naturally resistant to a variety of environmental and chemical insults, including exposure to oxygen, disinfectants,

*Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322. Phone: (404) 727-6192. Fax: (404) 727-8250. shonna.mcbride@emory.edu.

desiccation and extreme temperatures (Lawley *et al*., 2009). The ability for *C. difficile* to form spores is critical for survival outside of the host and for transmission from host to host (Deakin *et al*., 2012). Spore formation is a factor in *C. difficile* resistance to traditional antibiotic therapies and also contributes to recurrent infection (Deakin *et al*., 2012). Further, sporulation-specific gene expression is upregulated early in the mouse model of CDI (Janoir *et al*., 2013). Despite the importance of sporulation in the pathogenesis of *C. difficile*, the triggers and molecular mechanisms that govern the initiation of spore formation are not well understood.

Our limited understanding of *C. difficile* sporulation is primarily based on comparisons with other spore-forming bacteria. In the model organism *Bacillus subtilis*, sporulation is a complex morphological event that is tightly controlled by multiple regulators, checkpoints and feedback loops. The sporulation process is demarcated into several stages based on physiological landmarks. The physiological changes within the cell are accomplished through compartmentalized transcription programs within the mother cell and the developing endospore compartments. These transcription programs are orchestrated via the four sporulation-specific sigma factors: σ^F, σ^E, σ^G and σ^K. The *C. difficile* genome encodes the four conserved sporulation-specific sigma factors and readily forms heat-resistant spores within mammalian intestinal tracts (Fimlaid *et al*., 2013, Pereira *et al*., 2013, Saujet *et al*., 2013). Although the sporulation specific sigma factors are highly conserved in *C. difficile*, recent global studies revealed that divergent regulatory mechanisms mediate the timing of expression and activation of these sigma factors compared to other *Clostridium* and *Bacillus* species (Fimlaid *et al*., 2013, Pereira *et al*., 2013, Saujet *et al*., 2013).

In *B. subtilis*, spore formation begins as the cells transition from exponential to stationary growth phase (Stage 0). At Stage 0, gene expression shifts to support spore formation, rather than vegetative growth. Stage 0 is defined by the post-translational activation of the transcription factor, Spo0A, which upregulates sporulation-specific gene expression and serves as the master regulator of sporulation. Because spore formation is an energy-intensive process and is irreversible at specific points in spore development, the decision to initiate sporulation involves the integration of multiple environmental signals to determine whether conditions are unfavorable to support further vegetative cell growth. In *B. subtilis* and other *Bacillus* species, Spo0A activity is tightly regulated through a phosphorylation-mediated signal transduction pathway in response to nutrient availability, cell density and other signals (Sonenshein, 2000). Importantly, the *C. difficile* genome lacks the *B. subtilis* phosphorelay orthologs, and instead, Spo0A activity appears to be controlled through at least three orphan histidine kinases and potentially other unidentified factors (Paredes *et al*., 2005, Underwood *et al*., 2009).

Genomic analyses have revealed that *C. difficile* does not possess many of the conserved early stage regulatory components required for Spo0A activation and efficient sporulation in other spore forming bacteria (Paredes *et al*., 2005, Galperin *et al*., 2012). In the last few years, researchers have begun to elucidate the functions of *C. difficile* early sporulation orthologs in controlling initiation through the regulation of *spo0A* transcription and Spo0A phosphorylation. Understanding the genetic pathways and environmental conditions that

lead to Spo0A activation in *C. difficile* is key for designing targeted therapeutics to inhibit spore formation and thus, limit the spread of the disease.

Spo0A: the master regulator of sporulation

Several studies characterizing *C. difficile* sporulation have determined that some of the most conserved global regulators of sporulation play similar roles as found in other spore formers (Saujet *et al*., 2011, Antunes *et al*., 2012, Deakin *et al*., 2012). In particular, studies of Spo0A-dependent regulation have revealed that while the *C. difficile* Spo0A regulon has significant overlap with *B. subtilis*, there are differences in early sporulation gene regulation and in the role Spo0A may play in sigma factor activation and late stage gene expression (Fimlaid *et al*., 2013, Pereira *et al*., 2013, Saujet *et al*., 2013, Pettit *et al*., 2014). All sequenced *C. difficile* genomes contain the highly conserved Spo0A transcription factor and possess both the N-terminal phosphorylation and dimerization domain and the C-terminal DNA-binding domain. As in *Bacillus* species, *C. difficile spo0A* mutants are asporogenous and fail to activate stationary phase and early sporulation gene transcription (Heap *et al*., 2007, Deakin *et al*., 2012, Rosenbusch *et al*., 2012, Fimlaid *et al*., 2013, Pettit *et al*., 2014). The *C. difficile* Spo0A protein recognizes a consensus sequence similar to the 0A box defined in *B. subtilis* and directly binds with high affinity and specificity to the promoter regions of itself (*spo0A*), *sigH* (σ^H) and the early sporulation genes that control σ^F and σ^E activation (*spoIIAA*, *spoIIE* and *spoIIGA*; (Rosenbusch *et al*., 2012). Global transcriptional analyses have further defined the *C. difficile* Spo0A regulon to include additional conserved early sporulation genes, such as those encoding σ^F and σ^E, and a negative regulator of Spo0A activity, *sinR* (Pettit *et al*., 2014). The Spo0A regulon also contains genes that are unique to *C. difficile*, such as CD1492 and CD1579, which encode the orphan histidine kinases that directly or indirectly influence Spo0A phosphorylation (see below; (Underwood *et al*., 2009, Pettit *et al*., 2014). Unlike *B. subtilis*, the conditions that facilitate synchronous *in vitro* sporulation of *C. difficile* have not been identified, making it difficult to detect small changes in gene transcription during spore development (Fimlaid *et al*., 2013, Putnam *et al*., 2013, Saujet *et al*., 2013). As a result, there are likely many more transcripts in the Spo0A regulon that remain to be identified.

Spo0A also regulates physiological processes other than sporulation, including biofilm formation (Dawson *et al*., 2012, Dapa & Unnikrishnan, 2013), motility (Pettit *et al*., 2014), carbon metabolism (e.g. butyrate biosynthesis; (Pettit *et al*., 2014) and, in some cases, toxin A (TcdA) and toxin B (TcdB) production (Deakin *et al*., 2012, Mackin *et al*., 2013, Pettit *et al*., 2014). Interestingly, Spo0A can repress toxin production in *C. difficile*, but this regulation appears to occur primarily in the ribotype 027 epidemic strains and not in other evolutionarily divergent strains, such as ribotype 012 (630Δ*erm*) or 078 (JGS6133; (Deakin *et al*., 2012, Rosenbusch *et al*., 2012, Mackin *et al*., 2013). Conversely, two studies report Spo0A-dependent regulation of *tcdA* expression in 630Δ*erm* (Underwood *et al*., 2009, Pettit *et al*., 2014), but this effect is likely indirect as no Spo0A consensus sequences are identified upstream of *tcdA* (Rosenbusch *et al*., 2012). The inconsistencies observed in Spo0A-mediated regulation of toxin production can be partly attributed to differences in the growth medium used and the experimental conditions tested. These differences underscore the need

for standard assays and growth conditions for the study of *C. difficile* sporulation, and highlight the need for complementation studies in this research.

Global regulators of stationary phase control expression of early sporulation-specific genes

Along with Spo0A, the transition phase sigma factor, σ^H, shares responsibility for upregulating expression of sporulation-related genes as well as mediating the transcriptional changes that occur during the switch from exponential to stationary phase growth. *C. difficile* SigH recognizes a similar consensus sequence to that in *B. subtilis* (Saujet *et al*., 2011). SigH positively regulates transcription of some early sporulation genes in *C. difficile*, including *spo0A*, a putative Spo0A histidine kinase, the putative *sinRI* operon as well as *spo0J* and *soj*, whose gene products are likely involved in chromosomal segregation during asymmetric division (Saujet *et al*., 2011). These data suggest that SigH positively contributes to sporulation initiation through multiple pathways by directly inducing *spo0A* gene expression and positively influencing Spo0A activity through increased transcription of the Spo0A-associated kinase, CD2492. In addition, SigH has a negative effect on expression of the *app* operon, which encodes a predicted oligopeptide permease (see below; (Pereira *et al*., 2013). Finally, the reciprocal control between SigH and Spo0A creates a positive feed-forward loop, with SigH activating *spo0A* transcription and Spo0A upregulating *sigH* gene expression. This regulatory circuitry reinforces the global transcriptional changes necessary to initiate sporulation.

There are at least two additional conserved global regulators that influence sporulation in *Bacillus* and *Clostridium* species: CcpA and CodY. Both of these transcriptional regulators share a number of regulatory targets in *C. difficile*, including the regulation of toxin synthesis (Dineen *et al*., 2007, Dineen *et al*., 2010, Antunes *et al*., 2012). Catabolite control protein A (CcpA) is a DNA-binding transcriptional regulator that governs the global response to carbon availability in low G+C Gram-positive organisms. CcpA controls expression of genes involved in sugar uptake, fermentation and amino acid metabolism in the presence of preferred carbon sources such as glucose and other phosphotransferase system (PTS) sugars (Antunes *et al*., 2011, Antunes *et al*., 2012). In *B. subtilis*, CcpA activity is linked to carbon availability through the direct interaction with a corepressor, HPr-Ser-P, the phosphorylated form of HPr (Fujita *et al*., 1995). HPr phosphorylation occurs in the presence of fructose-1,6-biphosphate (FBP) which triggers HPr serine kinase/ phosphatase activity of the HprK/P protein (Deutscher & Saier, 1983). In contrast to *B. subtilis*, the DNA binding affinity of the *C. difficile* CcpA protein is enhanced by FBP *in vitro*, but not by the presence of HPr-Ser-P (Antunes *et al*., 2011). Although the molecular mechanism of this interaction is not yet understood, CcpA function in *C. difficile* appears to be regulated by the same carbon metabolite as in *B. subtilis*.

In the absence of glucose, CcpA is required for efficient sporulation in *C. perfringens* (Varga et al., 2004), but CcpA downregulates sporulation in *C. difficile* (Antunes *et al*., 2012), demonstrating that unique regulatory processes can control sporulation initiation in different *Clostridium* species. In *C. difficile*, CcpA binds to conserved recognition sequences known as catabolite responsive elements (cre_{CD}) and directly represses transcription of

many stationary phase transcripts, including *spo0A* and the *opp* operon, which encodes a predicted oligopeptide transporter system (Antunes *et al*., 2012). CcpA also represses expression of the putative Spo0A histidine kinase CD1579 and the *sinR* transcriptional regulator, but these interactions are likely indirect as no cre_{CD} motifs are apparent in the respective promoter regions (Antunes *et al*. 2012). Transcription of genes involved in later stages of sporulation are also repressed by CcpA (Antunes *et al*., 2012). It is important to note that while CcpA-mediated catabolite repression of sporulation has been observed in Bacilli and Clostridia, glucose-mediated repression of sporulation genes occurs independently of CcpA in *B. subtilis*, *C. difficile* and *C. perfringens* (Moreno *et al*., 2001, Varga *et al*., 2004, Antunes *et al*., 2012). In *B. subtilis*, the primary mediator of this physiological response appears to be the transcriptional regulator ScoC, which represses sporulation gene expression in a glucose-dependent manner (see below; (Shafikhani *et al*., 2003).

CodY is another conserved global regulator that responds to nutrient availability in *C. difficile* and other Gram-positive bacteria (Sonenshein, 2005, Dineen *et al*., 2007, Dineen *et al*., 2010). CodY, along with the cofactors GTP and branched-chain amino acids (BCAAs), represses gene expression in high nutrient conditions (Dineen *et al*., 2007, Dineen *et al*., 2010). When nutrients become limiting, such as during the entry into stationary phase, the intracellular concentrations of GTP and BCAAs decreases, thereby relieving CodY-mediated repression (Sonenshein, 2007). A global analysis that combined both *in vivo* and *in vitro* methods to define the CodY regulon revealed that CodY directly binds to and regulates expression of the CD2492 histidine kinase, a Rap phosphatase ortholog (CD2123) and the *opp* operon (Dineen *et al*., 2010). While it is clear that both CcpA and CodY affect sporulation of *C. difficile* by regulating expression of sporulation-related genes, the intricate regulatory networks that connect CcpA and CodY with the initiation of sporulation includes hundreds of directly regulated transcripts and many more indirect targets.

Post-translational activation of Spo0A

In the genus *Bacillus*, the regulatory pathway controlling Spo0A phosphorylation is known as the sporulation phosphorelay. The phosphorelay is a variant of a two-component signal transduction system (TCS) comprised of five sensor kinases (KinA-E), which respond to specific ligands, allowing multiple signals to converge and influence Spo0A activity (Perego *et al*., 1989, Burbulys *et al*., 1991, Kobayashi *et al*., 1995, LeDeaux & Grossman, 1995, LeDeaux *et al*., 1995, Jiang *et al*., 2000). KinA-E directly phosphorylate Spo0F which subsequently transfers the phosphate to Spo0A through an additional phosphotransferase, Spo0B (Burbulys *et al*., 1991). Despite the conservation of Spo0A and the similarities between Spo0A targets in *Bacillus* and *Clostridium* species, the components of the *Bacillus* phosphorelay do not appear to be encoded within *C. difficile* or the other Clostridial genomes. Based on the lack of an apparent phosphorelay, the favored hypothesis is that the sporulation initiation pathway in *Clostridium* species functions more similarly to a traditional TCS in which sporulation-associated sensor kinases recognize specific internal or environmental signals and directly phosphorylate Spo0A (Worner *et al*., 2006, Steiner *et al*., 2011). Supporting this hypothesis, three of the five orphan histidine kinases (CD1492, CD1579 and CD2492) present in the *C. difficile* genome share some sequence identity with

KinA-E (Underwood *et al*., 2009). Based on *in vitro* biochemical assays and *in vivo* sporulation studies, the CD1579 and CD2492 kinases are anticipated to directly phosphorylate Spo0A (Underwood *et al*., 2009). CD1579 is predicted to be a cytosolic protein and contains a degenerate heme-oxygen-sensing PAS domain ((Underwood *et al*., 2009, Edwards *et al*., 2012). The CD1579 histidine kinase was shown to directly phosphorylate Spo0A *in vitro*, while a CD2492 mutant exhibits a 3-fold decrease in sporulation *in vivo* (Underwood et al., 2009). These results strongly suggest that both of these histidine kinases influence *C. difficile* sporulation. CD1492 and CD2492 encode conserved autophosphorylation domains and are integral membrane proteins, suggesting that these histidine kinases may autophosphorylate upon contact with specific extracellular signals. However, no characterization of CD1492 has been published. Of the remaining two orphan histidine kinases, CD1352 has been shown to regulate a lantibiotic resistance mechanism (McBride & Sonenshein, 2011, Suarez *et al*., 2013), and CD1949 has no known function.

Control of sporulation initiation by limiting accumulation of Spo0A~P

In *B. subtilis*, Spo0A phosphorylation is further controlled through multiple phosphatases and their respective regulators. One mechanism employed by *B. subtilis* to restrict sporulation initiation is the dephosphorylation of the components within the phosphorelay, which in turn limits the accumulation of phosphorylated Spo0A. The Spo0E, YnzD and YisI aspartyl-phosphate phosphatases act directly on Spo0A (Perego & Hoch, 1991, Ohlsen *et al*., 1994, Perego, 2001), while the RapA, RapB and RapE phosphatases bind to and dephosphorylate Spo0F (Perego *et al*., 1994, Jiang *et al*., 2000). Additionally, a histidine kinase inhibitor, KipI, directly blocks the catalytic domain of KinA, preventing ATPase activity and subsequent autophosphorylation of KinA (Wang *et al*., 1997). Adding further complexity to these regulatory pathways, Rap activity is inhibited by specific small quorum sensing peptides, known as Phr peptides (Magnuson *et al*., 1994, Lazazzera *et al*., 1997, Perego, 1997), while KipI antikinase activity is inhibited by KipA (Wang *et al*., 1997). *C. difficile* encodes two orthologs to the Rap phosphatases and orthologs of KipI and its antagonist KipA, but no apparent Phr peptide or Spo0E, YnZD or YisI phosphatase orthologs are present (Paredes *et al*., 2005, Galperin *et al*., 2012). The Rap orthologs in *C. difficile* have low homology to the *B. subtilis* Rap proteins. The N-terminal protein binding and phosphatase domains are not conserved in *C. difficile*. Rather, the conserved domains present in the *C. difficile* orthologs are the tetratricopeptide (TPR) repeats which are responsible for direct interaction with the inhibitory Phr peptides (Diaz *et al*., 2012). *C. difficile* is also missing an apparent ortholog of *sda*, which encodes another histidine kinase inhibitor that prevents KinA autophosphorylation in response to defects in DNA replication initiation (Burkholder *et al*., 2001, Rowland *et al*., 2004, Paredes *et al*., 2005). It is not known if the Rap or Kip orthologs influence spore formation in *C. difficile*. Since their conserved targets within the phosphorelay are absent in *C. difficile*, the Rap and Kip orthologs may act on the putative histidine kinases or directly on Spo0A.

Bacillus species initiate sporulation in a cell density-dependent manner through the synthesis, export and uptake of small quorum sensing peptides known as Phr peptides. These five to six amino acid peptides are recognized and imported by two oligopeptide permeases,

Spo0K (Opp) and App (Perego, 1997). Once imported, Phr peptides directly bind to and inhibit the phosphatase activity of the Rap proteins previously mentioned, initiating sporulation (Magnuson *et al*., 1994, Lazazzera *et al*., 1997, Perego, 1997). *C. difficile* encodes orthologs of the Opp and App transporters but is missing orthologs to the Phr peptides (Edwards *et al*., 2014). In contrast to *B. subtilis*, an *opp app* mutant in *C. difficile* increases sporulation-specific gene expression and exhibits a hypersporulation phenotype (Edwards *et al*., 2014). Increased sporulation appears to occur in response to a decrease in general peptide uptake mediated by these oligopeptide transporters, which corresponds with an overall decrease in CcpA and CodY activity in transporter mutants. Hence, Opp and App likely indirectly influence sporulation initiation by signaling nutrient availability through CodY- and CcpA-mediated gene regulation (Edwards *et al*., 2014). Because the standard methods of inducing sporulation in other spore formers, such as *B. subtilis*, do not increase the sporulation rate of *C. difficile*, it has been unclear whether *C. difficile* sporulates in response to nutrient starvation. The observation that Opp and App function inhibits sporulation in *C. difficile* is evidence that this organism initiates sporulation in nutrient limiting conditions.

Auxiliary regulators of sporulation initiation

There are several additional regulatory factors involved in sporulation initiation in *B. subtilis* that may also play a role in controlling sporulation in *C. difficile*. ScoC, a negative transcriptional regulator of sporulation in *B. subtilis*, downregulates both *opp* and *app* transcription (Koide *et al*., 1999). Overexpression of a putative *scoC* ortholog (CD0852), divergently transcribed from the *opp* operon, decreases sporulation efficiency but does not influence gene expression of the *opp* or *app* operons in *C. difficile*, suggesting that CD0852 may negatively influence sporulation through a unique regulatory pathway (Edwards *et al*., 2014). Another gene, CD3409, was suggested to function as the ScoC ortholog in *C. difficile* (Pettit *et al*., 2014); however, this gene encodes for the HPr kinase/phosphorylase mentioned previously. These genes are often confused because in *B. subtilis* the *hpr* gene encodes ScoC, while *ptsH* and *hprK* encode HPr and HPrK/P, respectively.

In *B. subtilis*, the AbrB protein functions as a transition state regulator and represses stationary phase and sporulation genes during exponential growth. AbrB can repress its own transcription as well as expression of both *sigH* (σ^H) and the Spo0A-phosphatase, *spo0E*, and acts as an activator of *hpr* expression (encoding ScoC; (Zuber & Losick, 1987, Strauch *et al*., 1989, Perego & Hoch, 1991). Expression of *abrB* is controlled by negative autoregulation and is repressed by activated Spo0A upon entry into transition phase (Perego *et al*., 1988). There are two AbrB paralogs present in *B. subtilis*, Abh and SpoVT, which function similarly to AbrB as DNA-binding regulators although their regulons do not fully overlap (Bagyan *et al*., 1996, Dong *et al*., 2004, Yao & Strauch, 2005). The *C. difficile* genome encodes two putative *abrB* orthologs (CD1859A and CD3120) as well as a SpoVT ortholog (CD3499). Unfortunately, no experimental evidence for any of the *C. difficile* AbrB orthologs is available, so the functions of these factors remain a mystery. However, recent work demonstrated that SpoVT functions slightly differently in *C. difficile* as compared to *B. subtilis* and is necessary for the formation of mature, heat-resistant spores in *C. difficile*

(Saujet *et al*., 2013). Similar to *B. subtilis*, SpoVT does not appear to influence sporulation initiation as a *C. difficile spoVT* mutant forms phase dark spores (Saujet *et al*., 2013).

The tetrameric DNA-binding regulator SinR provides another layer of regulatory control in sporulation initiation by directly repressing *spo0A* gene expression (Mandic-Mulec *et al*., 1995). SinR repression is disrupted by the production of the antagonist, SinI, which is encoded in the same operon as *sinR* and forms an inactive heterodimeric complex with SinR (Bai *et al*., 1993). Regulation of *sinIR* gene expression in *B. subtilis* is complex as AbrB, ScoC and Spo0A all play roles in up- or downregulating transcription (Shafikhani *et al*., 2002). The *sinRI* genes are encoded in the *C. difficile* genome (CD2214 and CD2215), and although no characterization of these has been reported, there may be some regulatory circuitry conserved in *C. difficile* as Spo0A represses *sinR* gene expression (Pettit *et al*., 2014).

Finally, fidelity of DNA replication and segregation is a regulatory checkpoint for initiating sporulation in other spore formers. Together, the Soj and Spo0J proteins repress sporulation until chromosomal segregation has occurred. Soj and Spo0J are similar to the ParA/B family of proteins involved in plasmid partitioning and are required for proper chromosomal replication and segregation during vegetative growth, as well as before asymmetric division occurs during sporulation (Ireton *et al*., 1994). Soj prevents Spo0A-dependent transcription while Spo0J inhibits Soj activity (Ireton *et al*., 1994, Cervin *et al*., 1998, Quisel & Grossman, 2000). *C. difficile* contains both *soj* (CD3672) and *spo0J* (CD3671) orthologs along with an additional Spo0J-like ortholog (CD3673) encoded immediately downstream. The roles that these proteins play in DNA replication and sporulation in *C. difficile* have not been confirmed.

Conclusion/Outlook

The altered roles of conserved regulatory components and the apparent absence of several key factors (e.g. the phosphorelay transferases, Sda, Spo0E, YnzD, YisI and the Phr peptides) involved in early sporulation in the *C. difficile* genome indicate that this organism has evolved to induce sporulation specifically to survive harsh conditions within its gastrointestinal niche and outside of its host. Because of the specialized ecological niche of *C. difficile*, there are likely unknown unique regulators and molecular mechanisms controlling Spo0A activation. Compared to other characterized spore formers, *C. difficile* incorporates different environmental and intracellular signals and utilizes a novel regulatory pathway to mediate Spo0A phosphorylation and subsequent sporulation-specific gene expression. This apparently simplified phosphorylation cascade suggests that *C. difficile* may possess fewer checkpoints before initiating sporulation compared to *Bacillus* species, but it is equally plausible that there are alternative pathways and checkpoints that have yet to be discovered. With the recent advances in *C. difficile* genetics and whole transcriptomic and proteomic sequencing, genetic screens and global studies in this notoriously challenging organism have become feasible and are expected reveal a wealth of information about *C. difficile* in the coming years.

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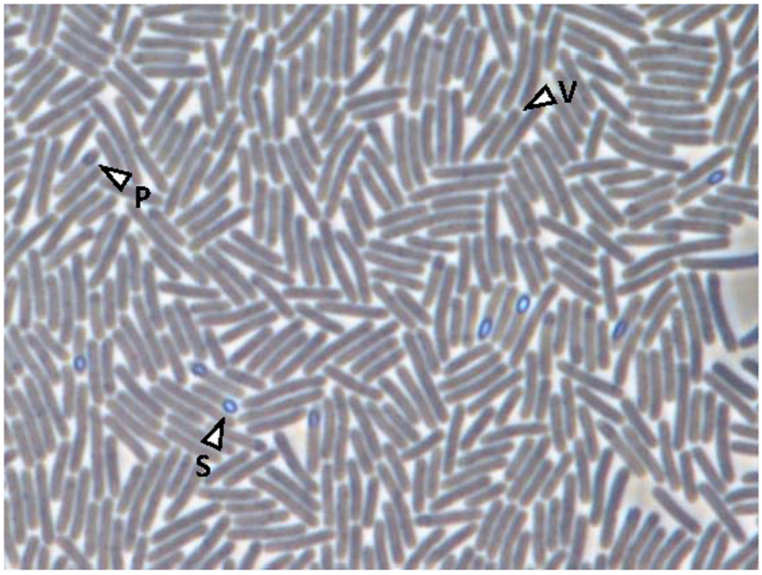

Figure 1.

Phase contrast micrograph of sporulating *C. difficile* R20291, an epidemic 027 ribotype strain (Stabler *et al*., 2009). Depicted are vegetative cells (v), phase dark prespores (p) and phase bright spores (s).

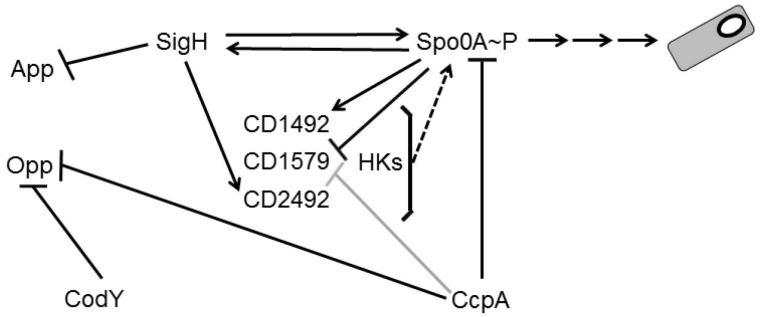

Figure 2.

Representative schematic of the putative regulatory pathway that controls sporulation initiation in *C. difficile*. Solid lines represent direct transcriptional control while dashed lines indicate post-translational modifications (phosphorylation). Black lines indicate direct regulatory interactions, and gray arrows denote indirect regulation. Regulatory interactions have been demonstrated experimentally in (Underwood *et al*., 2009, Dineen *et al*., 2010, Saujet *et al*., 2011, Antunes *et al*., 2012, Pettit *et al*., 2014).

Table 1

Known and predicted genes involved in early sporulation in *C. difficile*.

Gene name[a]	Accession number[b]	Known or predicted function	References
spo0A	CD1214	master transcriptional regulator of sporulation; active when phosphorylated	(Deakin *et al*., 2012)
sigH	CD0057	transition phase sigma factor	(Saujet *et al*., 2011)
ccpA	CD1064	carbon catabolite control protein; transcriptional regulator that responds to fructose-1,6-biphosphate	(Antunes *et al*., 2011, Antunes *et al*., 2012)
codY	CD1275	transcription regulator; requires cofactors GTP or branched chain amino acids (BCAAs)	(Dineen *et al*., 2007. Dineen *et al*., 2010)
	CD1492	putative Spo0A-specific histidine kinase; integral membrane protein	(Underwood *et al*., 2009)
	CD1579	putative Spo0A-specific histidine kinase; cytosolic protein	(Underwood *et al*., 2009)
	CD2492	putative Spo0A-specific histidine kinase; integral membrane protein	(Underwood *et al*., 2009)
oppA-F	CD0853-57	nonspecific oligopeptide permease; negatively influences sporulation indirectly	(Edwards *et al*., 2014)
appA-F	CD2670-74	nonspecific oligopeptide permease; negatively influences sporulation indirectly	(Edwards *et al*., 2014)
rapA	CD2123	putative phosphatase	
rapB	CD3668	putative phosphatase	
sinR	CD2214	putative transcriptional regulator	
sinI	CD2215	putative inhibitor of SinR activity	
kipI	CD1386	putative inhibitor of histidine kinase activity	
kipA	CD1387	putative antagonist of Kipl activity	
hpr	CD0852	putative ScoC transcriptional regulator	
spo0J	CD3671	putative inhibitor of Soj activity	
soj	CD3672	putative negative regulator of Spo0A activity	
spo0J2	CD3673	putative inhibitor of Soj activity	

[a] If uncharacterized in *C. difficile*, the gene name reflects the closest ortholog present in the *B. subtilis* 168 genome (GenBank accession no. AL009126).

[b] Accession number is based on aenomic annotation of *C. difficile* 630 (AM180355; (Monot *et al*. 2011).