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Genetic Mechanisms Governing Sporulation Initiation in *Clostridioides difficile*

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Abstract

As an anaerobe, *Clostridioides difficile* relies on the formation of a dormant spore for survival outside of the mammalian host's gastrointestinal tract. The spore is recalcitrant to desiccation, numerous disinfectants, UV light, and antibiotics, permitting long-term survival against environmental insults and efficient transmission from host to host. Although the morphological stages of spore formation are similar between *C. difficile* and other well-studied endospore-forming bacteria, the *C. difficile* genome does not appear to encode many of the known, conserved regulatory factors that are necessary to initiate sporulation in other spore-forming bacteria. The absence of early sporulation-specific orthologs suggests that *C. difficile* has evolved to control sporulation initiation in response to its unique and specific ecological niche and environmental cues within the host. Here, we review our current understanding and highlight the recent discoveries that have begun to unravel the regulatory pathways and molecular mechanisms by which *C. difficile* induces spore formation.

Graphical Abstract

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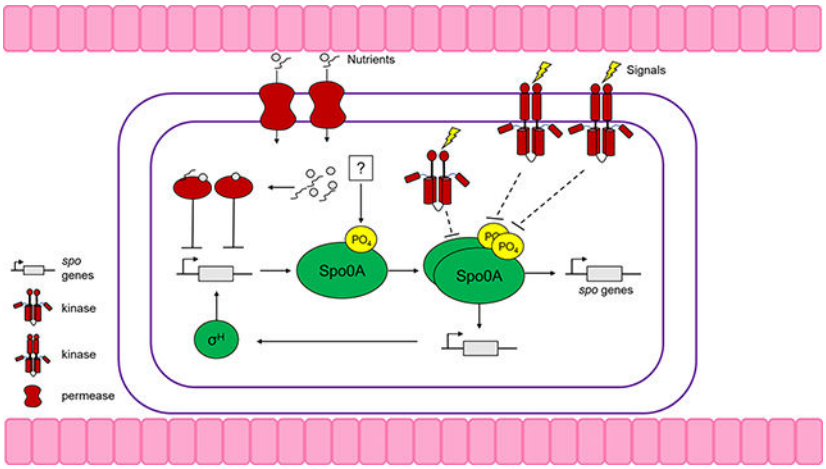

Keywords

Clostridium difficile ; *Clostridioides difficile* ; sporulation; spore; sigma factor; transcriptional regulator; regulation; anaerobe

INTRODUCTION

Clostridioides difficile is an anaerobic, Gram-positive, spore-forming bacterium that is a foremost cause of antibiotic-associated diarrhea. *C. difficile* infection (CDI) symptoms range from mild diarrhea and abdominal distress to life-threatening pseudomembranous colitis. Antibiotic usage causes gut dysbiosis by altering the gut microbiota and sensitizes individuals to *C. difficile* colonization. A key aspect of *C. difficile* pathogenesis is its ability to form spores, as they are critical for the infection cycle and also resistant to antibiotics, environmental insults, and disinfectants. While much of the general sporulation pathway in *C. difficile* is conserved with other Firmicutes, the molecular mechanisms controlling the initiation of sporulation are not conserved and remain poorly understood. This review summarizes our current understanding of regulation of *C. difficile* sporulation initiation mechanisms and outlines the identified factors that contribute to initiation through the activation of the conserved master regulator of sporulation, Spo0A. Further, we provide a summary of the nutritional and environmental regulations that promote or repress spore formation, as well as an overview of recently characterized factors that contribute to sporulation regulation through undiscovered mechanisms.

Positive Regulators of Sporulation Initiation

Spo0A—Spo0A is the master transcriptional regulator of sporulation in all endospore-forming bacteria [1]. As observed in other spore formers, Spo0A is essential for sporulation in *C. difficile*. A *spo0A* null mutant cannot initiate sporulation and was unable to transmit infection in a mouse model due to the absence of spores [2]. In addition to regulating sporulation in *C. difficile*, Spo0A impacts a myriad of cellular processes, including motility, protein transport, metabolism, cell envelope, and global gene regulation [3].

Once activated by phosphorylation, Spo0A binds to DNA at conserved binding sites known as Spo0A-boxes and induce the expression of genes required for initiating sporulation, including *sigH* and the sigma factors for early spore development, *sigF* and *sigE* [4]. In *B. subtilis*, Spo0A activity is modulated through a phosphorelay composed of a series of phosphotransfer proteins and accessory phosphatases [5]. Spo0A activation is notably different amongst the Clostridia, with most clostridial species lacking apparent orthologs to the phosphorelay kinases [1]. While *C. difficile* lacks orthologs to many of the activating proteins of Spo0A in *B. subtilis*, many of the functional and protein-interaction residues of *B.s*. Spo0A are conserved in *C.d*. Spo0A (DiCandia *et al.*, unpublished). This suggests that while the phosphorelay that controls Spo0A is not conserved in *C. difficile*, there may be factors that perform similar functions to control Spo0A activity. The absence of an obvious phosphorelay in *C. difficile* led to the hypothesis that Spo0A could be directly activated by orphan sensor histidine kinases in the Clostridia [6]. Further contributing to this view, multiple clostridial species directly phosphorylate and dephosphorylate Spo0A via orphan histidine kinases (*i.e.*, kinases that are not encoded with cognate response regulators) [7–9]. Three predicted orphan histidine kinases, PtpA (CD1492), PtpB (CD2492), and PtpC (CD1579), have been implicated in regulating early sporulation events in *C. difficile* [10, 11*, 12]. PtpA, PtpB, and PtpC repress spore production, as evidenced by the hypersporuiation pnenotype of their respective null mutants [11*–13]. The sporulation-repressing activities of PtpA, PtpB, and PtpC strongly suggest they do not function as Spo0A kinases, thus, the activating kinase for Spo0A in *C. difficile* remains unidentified.

SigH—SigH is an alternative sigma factor and a key regulator of transition phase and sporulation initiation in *C. difficile* and other firmicutes. SigH regulates over 700 genes involved in sporulation, cellular division, motility, virulence, and metabolism [14]. As the primary transition phase sigma factor, SigH allows *C. difficile* to rewire metabolism to adapt to limited availability of nutrients. SigH regulates hundreds of genes involved in energy production, metabolite transport, amino acid synthesis, and carbon fixation, therein linking metabolism with the initiation of sporulation. SigH-RNA polymerase holoenzyme transcribes several genes known to impact sporulation initiation, including *spo0A*, the phosphotransfer protein PtpB, and the *spo0J*, *soj*, and *spoIIP* genes involved in chromosomal segregation and prespore engulfment. SigH is required for both the initiation and progression of spore formation, and consequently, a *sigH* mutant is asporogenic. The transcription of *sigH* and *spo0A* are intertwined, with both factors promoting positive feedback expression of each other [4,14]. In addition, SigH positively regulates genes involved in the later stages of sporulation, including the *spoIIIA* operon, *spolllD*, *spolVA*, *spoVD*, *spoVE*, *spoVG*, and *spoVS* [14].

RstA—Another regulator that modulates Spo0A activity in *C. difficile* is RstA, a highly conserved and multifunctional protein [15]. RstA shares some sequence similarity to the *Bacillus* genus' Rap phosphatases, which directly dephosphorylate Spo0F to inhibit the transfer of phosphate to Spo0A and impede sporulation. RstA belongs to the RRNPP protein family. RRNPP proteins possess an N-terminal DNA-binding domain and/or protein-binding domain, followed by multiple C-terminal tetratricopeptide repeat (TPR) domains that encompass a small quorum-sensing-binding domain. RstA positively influences sporulation

and directly represses toxin production and motility through two distinct domains, indicating that RstA employs different molecular mechanisms to regulate these processes [16**]. A null mutation in *rstA* reduces sporulation frequency by ~20-fold and decreases Spo0A phosphorylation through an unknown mechanism [15,17]. RstA does not appear to directly bind Spo0A, but is hypothesized to interact with PtpA and PtpB to influence Spo0A phosphorylation [13]. Interestingly, there are some strain-dependent effects on RstA regulation between the historical 630Δ*erm* and the epidemic R20291 strains. RstA exhibited stronger regulation of sporulation and toxin production in R02921 compared to 630Δ*erm*, and surprisingly, the loss of *rstA* did not affect R20291 motility [17].

The DNA-binding and/or protein-binding activities of RRNPP proteins are controlled by the interaction of quorum-sensing peptides with the C-terminus of the protein [18]. It is unclear whether RstA is regulated by quorum-sensing peptides. But, evidence suggests that RstA activity is controlled by a cofactor, as purified RstA is unable to bind DNA and substitution of the C-terminal quorum-sensing-binding domain with other species' RstA orthologs abolishes RstA DNA-binding activity [17]. Determining how RstA functions to promote *C. difficile* sporulation may uncover additional sporulation factors and environmental cues that regulate Spo0A phosphorylation and activation.

Negative Regulators of Sporulation Initiation

Spo0E—The Spo0E class of proteins are a family of small aspartyl-phosphate phosphatases that dephosphorylate and inactivate Spo0A in *B. subtilis*, but their functions are poorly understood in *C. difficile* or other anaerobes [19,20]. Comparative genomics identified putative Spo0E orthologs in several Clostridia species by probing candidate genomes for the "SQELD" phosphatase motif identified in *B. subtilis* [21, 22**, 23]. The clostridial Spo0E-like proteins cluster separately from aerobic Spo0E orthologs with no conserved synteny between anaerobic and aerobic Spo0E clusters [22], suggesting that clostridial Spo0E proteins have evolved independently of the *Bacillus* Spo0E orthologs.

C. difficile encodes at least one putative Spo0E ortholog (CD3271) that contains a loosely conserved functional motif (SKKID). Recent work reveals that a *C. difficile spo0E* mutant exhibits increased sporulation, similar to observations in *B. subtilis* (DiCandia *et al.*, unpublished). In addition, co-immunoprecipitation of recombinant Spo0A revealed that Spo0E co-purifies with Spo0A, suggesting that Spo0E in *C. difficile* directly dephosphorylates Spo0A to negatively regulate sporulation initiation (DiCandia *et al.*, unpublished). However, the *C. difficile spo0E* mutant has additional phenotypes that were not observed in *B. subtilis*, including hypermotility, increased toxin expression, and mucoid colony morphology, which are not associated with Spo0A regulation. These results suggest that the Spo0E of clostridial species have evolved additional regulatory functions outside of the sporulation pathway, though the additional mechanisms remain to be determined.

PtpA and PtpB—In other sporulating species, phosphorylation of Spo0A occurs through a phosphorelay initiated by the activation of orphan histidine kinases [1,8,24,25]. *C. difficile* encodes five predicted orphan histidine kinases; PtpA (CD1492), PtpB (CD2492), PtpC (CD1579), CprK (CD1352), and CD1949. CprK and CD1949 have no involvement in

sporulation initiation [13,26,27]. PtpA and PtpB repress sporulation initiation, though it is not known whether PtpA or PtpB control Spo0A phosphorylation directly, as observed in other Clostridia [7,8]. Deletion of *ptpA* increases sporulation frequency ~2.4-fold (strain 630Δ*erm*), suggesting that PtpA represses sporulation initiation. The hypersporulation phenotype of the *ptpA* mutant suggests that PtpA does not phosphorylate Spo0A, but instead acts as a phosphatase or an indirect regulator of Spo0A function. The *ptpA* mutant also produces less toxin and is less virulent than wild-type, likely due to reduced expression of *sigD*, which induces toxin production, or through reciprocal regulation with RstA [11**].

Results from an early examination of PtpB suggested that this predicted kinase promotes sporulation, since disruption of *ptpB* appeared to reduce sporulation. However, that study did not complement the mutation, and the assay used to examine sporulation was unconventional and did not include important controls [10]. Further examination of a *ptpB* mutant revealed a hypersporulation phenotype, similar to that of a *ptpA* mutant, suggesting that PtpB is also a negative regulator of sporulation initiation [12]. Additionally, a *ptpA ptpB* double mutant results in the same sporulation phenotype as the single mutants, suggesting both proteins function together in the same regulatory pathway [12]. Surprisingly, the conserved histidine residue necessary for phosphate transfer is required for PtpA function, but not for PtpB function, suggesting that although these proteins function in the same pathway, they may have divergent roles [13]. Finally, the inverse pattern of gene expression and phenotypes for the *ptpA*, *ptpB*, and *rstA* mutants suggests that PtpA, PtpB, and RstA function within the same regulatory pathway to influence sporulation in *C. difficile* [11**]. Although PtpB and PtpA clearly play a role in sporulation regulation, their direct targets and phosphotransfer functions remain to be determined.

PtpC—PtpC (CD1579) is one of the predicted orphan histidine kinases originally hypothesized to promote sporulation by phosphorylating Spo0A [1,10]. PtpC was shown to phosphorylate Spo0A *in vitro*; however, it is unclear whether this is the preferential direction of phosphate transfer between these proteins *in vivo* [10]. Although it was presumed that PtpC would activate sporulation, deletion of *ptpC* results in increased, but variable, sporulation, indicating that PtpC represses sporulation in *C. difficile* [13]. The mechanism of PtpC activation is not known, but *ptpC* transcription is influenced by other sporulation factors, including RstA, PtpA, and PtpB [11**,15]. Further characterization of PtpC is needed to define the role of PtpC in Spo0A phosphoregulation.

SigB—Similar to other Gram-positive species, SigB transcribes factors in *C. difficile* to aid in survival during stress-inducing conditions, including nitrosative and oxidative conditions, acidic environments, and thiol homeostasis [28–31]. In *C. difficile*, a *sigB* mutant displays a 10-fold increase in spore formation relative to wild-type, indicating that SigB has a negative effect on sporulation [29]. However, as a sigma factor, SigB is a direct positive regulator of transcription, thus the negative effects of SigB on sporulation are expected to be indirectly mediated through transcription of genes whose product(s) impedes the initiation process [32]. Candidate SigB-dependent factors that may impede sporulation initiation were identified by transcriptional analysis of a *sigB* mutant [29]. These factors include the aforementioned phosphotransfer protein PtpA and the Spo0J-soj system, which is involved

in chromosome partitioning. Whether through the expression of these or other sporulation suppressing factors, SigB allows *C. difficile* to repress sporulation under conditions of cellular stress that do not support spore formation.

CcpA—The global regulator catabolite control protein, CcpA, is an acI/GalR transcriptional repressor that is responsible for transcriptional carbon catabolite repression (CCR) in Gram-positive bacteria [33]. Antunes *et al.* investigated the role of CcpA in the CCR of *C. difficile* and found that CcpA directly represses expression of genes encoding the key early sporulation regulators *spo0A* and *sigF* [34*]. Relief of CcpA repression in a *ccpA* null mutant resulted in a higher sporulation frequency compared to the parental strain in the absence of glucose, but at later time points, the sporulation frequencies were similar [34*]. The addition of glucose substantially reduced sporulation to a similar level in both the wild-type and *ccpA* mutant, indicating that glucose-mediated CCR of sporulation is independent of CcpA, as observed in *C. perfringens* [35]. However, the mechanism for CcpA-independent glucose-mediated repression of sporulation has not been determined.

CodY—CodY is a nutrient-sensing global regulator that was discovered in *B. subtilis* [36]. CodY acts as a sensor of branched-chain amino acids (BCAAs) and GTP concentrations within the cell and modulates the expression of CodY-dependent genes [37,38**]. CodY acts primarily as a repressor of transcription during exponential phase, when BCAAs and GTP are abundant [37,39]. When GTP and BCAA levels are low in later growth phases, CodY repression is alleviated and the transcription of genes involved in amino acid biosynthesis, virulence, and sporulation increase [39]. CodY represses transcription of the toxin genes, which results in increased toxin production in low nutrient conditions [37]. Deletion of *codY* in the epidemic strain UK1 (RT027) resulted in more than a 1000-fold increase in spore formation [40*]. The mechanism by which CodY represses sporulation is not clear [38**,40*]. Since CodY represses the expression of several regulatory factors, many genes that are derepressed in a *codY* mutant are not directly regulated by CodY. However, several genes that impact sporulation initiation have predicted CodY binding sites or are enriched in CodY-DNA binding experiments, including the phosphotransfer protein PtpC (CDR20291_1476), *spo0E*, and *sinR/sinR'* [38**]. Although evidence indicates that CodY represses sporulation initiation in *C. difficile*, the specific mechanism(s) through which this is accomplished is not clear.

Other factors that influence sporulation frequency

Agr—The accessory gene regulator (Agr) system is a quorum-sensing system found in most Gram-positive bacteria [41–43]. The Agr system controls many cellular processes, including the expression of genes involved in colonization and virulence. The typical Agr system includes an autoinducer peptide (AIP), AgrD, which is processed and exported by AgrB. The AgrD peptide is sensed exogenously by AgrC, a histidine kinase that activates a response regulator, AgrA, to directly regulate gene expression. There are three identified Agr systems in *C. difficile*: Agr1, Agr2 and Agr3. All sequenced *C. difficile* strains contain a partial Agr1 system, encoding only *agrB1* and *agrD1* in a single operon [44,45]. A complete Agr2 system is found in the R20291 (RT027) [46], while the Agr3 system, lacking the response regulator, is encoded in RT078 strains [47]. Deletion of *agrB1D1* in *C. difficile*

strain 630 results in significantly reduced sporulation [48**]. Sporulation of the *agrB1D1* mutant was recovered by providing the supernatant of stationary phase cultures from the parent strain. These data are the first evidence that quorum sensing regulates *C. difficile* sporulation; however, because a cognate histidine kinase (AgrC) and response regulator (AgrA) are missing from strain 630, the signaling pathway that responds to the AgrB1D1 AIP remains unknown.

Opp/App Permeases—Opp and App are oligopeptide permease systems that are hypothesized to import small peptides into *C. difficile*. Loss of Opp and App results in earlier and increased spore formation, increased expression of the SinR orthologs, *CD2214* and *CD2215*, as well as expression of other CodY and CcpA-dependent genes [49]. Loss of Opp and App also results in increased virulence in a hamster model of infection [49]. The data suggest that Opp and App indirectly repress sporulation through importation of small peptides, but the specific cargo that they transport has not been verified. OppA resembles a nickel-uptake receptor, while AppA is structurally similar to the oligopeptide-binding protein CtaP (cysteine transport-associated protein) from *L. monocytogenes*, which binds a restricted set of peptides, suggesting that *C. difficile* AppA may also bind a restricted set of peptides [50–52*].

SinR orthologs—The SinR orthologs (CD2214 and CD2215) impact sporulation, motility, and toxin production in *C. difficile* strain R20291 [53*]. A mutant that does not transcribe either *CD2214* or *CD2215* is asporogenic [53*]. Specifically, CD2214 promotes sporulation, motility, and toxin production, while CD2215 decreases sporulation, motility, and toxin production indirectly by binding and preventing CD2214 from influencing target genes [53*]. CD2214-CD2215 binding is mediated through the multimerization domain of CD2215, which was shown to complement the sporulation, motility, and toxin production phenotypes seen in the R20291 *CD2215 (CDR20291_2122)* mutant [54]. However, the CD2215 helix-turn-helix domain was dispensable for complementation, and its function remains unknown [54]. CD2214 promotes sporulation in *C. difficile* through a currently unknown mechanism, but likely through DNA-binding and regulation of target genes [55].

CsiA (CD2589)—*CD2589(0)* was identified as a genomic signature for *C. difficile* sporulation in the gastrointestinal tract [56]. Deletion of *CD25890* resulted in increased sporulation in SM broth, but not 70:30 medium, suggesting that CD25890 has a nutritional function or impact [57]. Notably, expression of the *sin* genes was also increased ~five-fold in the *CD25890* mutant (strain 630Δ*erm*). Though the data suggest that CD25890 decreases sporulation in response to nutritional cues, the mechanism is currently unknown.

c-di-GMP—The nucleotide-based second messenger signaling molecule, c-di-GMP, modulates several physiological processes in *C. difficile* important for pathogenesis and colonization. Two recent studies revealed that overexpression of a diguanylate synthase reduces sporulation and the regulated production of a phosphodiesterase increases sporulation in *C. difficile* [12,58], Altogether, these studies support that c-di-GMP inhibits *C. difficile* sporulation, although the molecular mechanisms and regulatory pathways that mediate this response are unknown.

Orthologs of unknown influence—Below is a summary of *C. difficile* orthologs to sporulation initiation proteins from *B. subtilis* that have not been investigated: The KipI-KipA system in *B. subtilis* regulates sporulation initiation by inhibiting phosphorylation of KinA. Orthologs of KipIA may play a similar role in *C. difficile*, though in the absence of a KinA ortholog, their target kinase is not apparent [59–61].

Soj (ParA) inhibits *B. subtilis* sporulation by preventing early sporulation gene transcription [62]. Spo0J (ParB) is responsible for chromosome segregation in *B. subtilis* and allows Soj to dissociate from DNA, to enable transcription of early sporulation genes [63]. *C. difficile* encodes similar proteins, but they have not been characterized.

Summary

Successful sporulation initiation is critical to *C. difficile* transmission and survival within an aerobic environment. This review highlights our current understanding of sporulation initiation in *C. difficile*; however, sporulation initiation in this species is complex, and many details of the process remain to be determined. It is clear that the sporulation initiation mechanisms of Clostridia, including *C. difficile*, vary considerably from the *Bacillus* paradigm and from each other [1,7–9,64]. Other Clostridia encode predicted kinases that directly phosphorylate or dephosphorylate Spo0A, rather than indirectly via a phosphorelay. But, each of the clostridial initiation pathways that have been characterized appear to have evolved distinct Spo0A regulatory mechanisms with regulators that bear limited similarity to each other [1]. Thus far, the majority of factors that are characterized in the initiation pathway of *C. difficile* repress Spo0A activity and spore formation. Although direct activators of Spo0A have not been identified, the presence of multiple Spo0A inactivating factors in *C. difficile* alludes to the existence of a mechanism for deliberate Spo0A phosphorylation. Most likely, there are unidentified factors involved in Spo0A activation. Identification and characterization of these additional initiation factors will be critical for piecing together the puzzle of the sporulation initiation program.

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HIGHLIGHTS

- The master transcriptional regulator of sporulation in *C. difficile* is Spo0A.
- Spo0A activity depends on phosphorylation.
- Diverse regulators translate environmental signals to impact Spo0A phosphorylation.
- This complex regulatory network ensures sporulation initiates only when required.

Table 1

Sporulation genes of interest within *C. difficile.*

Gene name	Locus tag[a]	Known or predicted function	References
spo0A	CD1214	Master transcriptional regulator of sporulation	[2,3]
sigH	CD0057	Transition/stationary-phase sigma factor	[14]
rstA	CD3668	Multifunctional regulator; promotes sporulation	[14, 15, 16**]
spo0E	CD3271	Putative Spo0A phosphatase; sporulation inhibition	
PtpA	CD1492	Sporulation inhibition	[11*]
PtpB	CD2492	Sporulation inhibition	
PtpC	CD1579	Sporulation inhibition	
sigB	CD0011	Alternative sigma factor; stress responses; sporulation inhibition	[29–31]
ccpA	CD1064	Carbon catabolite control, transcriptional regulator; cofactor: fructose-1,6-bisphosphate; sporulation inhibition	[34*]
codY	CD1275	Transcriptional regulator; cofactors: GTP and BCAA; sporulation inhibition	[38**–40*]
agrB1D1	CD27491–50	quorum-sensing related sporulation inhibition	[48**]
oppA-F	CD0853–57	Putative peptide permease; sporulation inhibition	[49]
appA-F	CD2670–74	Putative peptide permease; sporulation inhibition	[49]
sin	CD2214–15	sporulation	[53*,54]
csiA	CD2589	Conditional repression of sporulation	[57]
kipI-kipA	CD1386–87	Putative inhibitor of histidine kinase	
spo0J-soj-spo0J2	CD3671–73	Putative inhibitor of sporulation gene transcription	

[a]Locus tag number based on *C. difficile* 630 reference genome (GenBank: AM180355)