Investigation of the Factors that Regulate Spo0A Activity in *Clostridioides difficile*

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Abstract

Clostridioides difficile is an anaerobic, Gram-positive pathogen that is responsible for *C. difficile* infection (CDI). To survive in the environment and spread to new hosts, *C. difficile* must form metabolically dormant spores. The highly conserved transcription factor, Spo0A, is the master transcriptional regulator of sporulation initiation in all spore-forming bacteria and is activated through phosphorylation at a highly conserved aspartate residue in the Spo0A receiver domain. Though the sporulation initiation pathway has been well characterized in the model spore-former *Bacillus subtilis*, the positive and negative regulators of Spo0A in *C. difficile* remain unidentified. To gain insight into the molecular mechanisms that govern sporulation initiation in *C. difficile*, we performed site-directed mutagenesis of conserved Spo0A receiver domain residues and examined the effects on sporulation frequency. As Spo0A shares high sequence similarity in the receiver domain between *B. subtilis* and *C. difficile*, we chose to mutate conserved Spo0A residues that are functionally important for interaction with sporulation regulatory proteins in *B. subtilis*. Our data demonstrate that mutation of conserved Spo0A residues significantly impacts sporulation frequency, suggesting these sites are likewise important for sporulation in *C. difficile*. Additionally, we have isolated suppressor mutants from low-sporulating Spo0A site-directed mutants that display an increase in sporulation frequency. Characterization of the suppressor mutations responsible for the increase in sporulation frequency in these compensatory mutants is expected to reveal key regulators of Spo0A. Lastly, we have identified putative binding partners of purified Spo0A using LC-MS/MS. Characterization of these potential interactions will further illuminate the regulation of sporulation initiation in *C. difficile*.

Background

- *C. difficile* is a Gram-positive, spore-forming obligate anaerobe that causes severe diarrheal disease (CDI), often after the administration of antibiotics.
- Complications from CDI results in about 29,000 deaths and $4.8 billion in increased healthcare costs annually in the United States.[1]
- *C. difficile* is able to survive outside of the intestine and transmit infection from host to host through formation of oxygen-resistant endospores that are shed in host feces.
- Spo0A is a highly conserved response regulator and the master regulator of sporulation initiation in *C. difficile* and other spore-forming bacteria.
- *C. difficile* Spo0A shares sequence homology with Spo0A of the model spore-forming bacterium, *Bacillus subtilis*; but, unlike *B. subtilis*, the mechanisms of *C. difficile* Spo0A regulation are unknown.
- In *C. difficile*, it remains unknown which factors interact with Spo0A to regulate its function and impact sporulation initiation.

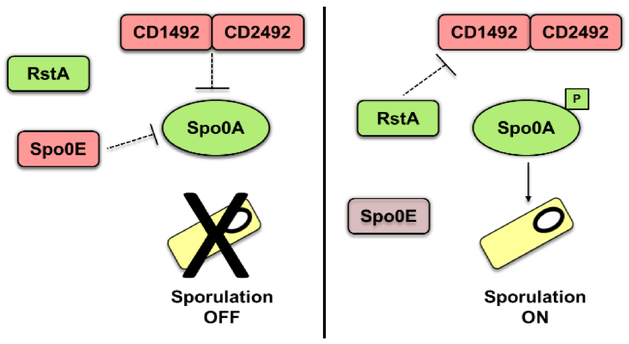

Figure 1. Predicted Partial Model of Sporulation Initiation in *C. difficile*. In the sporulation OFF state (left), the histidine kinases CD1492 and CD2492 prevent Spo0A activation, while the phosphatase Spo0E directly dephosphorylates Spo0A. In the sporulation ON state (right), RstA precludes CD1492 and CD2492 from inhibiting Spo0A, allowing for activation of Spo0A via phosphorylation. The activating phosphodonors(s), and the conditions that mediate Spo0A activation remain to be identified.

Results

Figure 2. Alignment of Spo0A Receiver Domain. Alignment of Spo0A N-terminal receiver domain from *C. difficile* 630Δerm (top) and *B. subtilis* (bottom). The highly conserved aspartate residue (D61) hypothesized to be the site of phosphorylation in *C. difficile* is marked by a blue star. Predicted secondary structure was generated using the Phyre2 program.[2] Pointed arrows represent β-strands, and solid ovals represent α-helices. Sites selected for targeted mutagenesis are outlined in **Figure 3.**

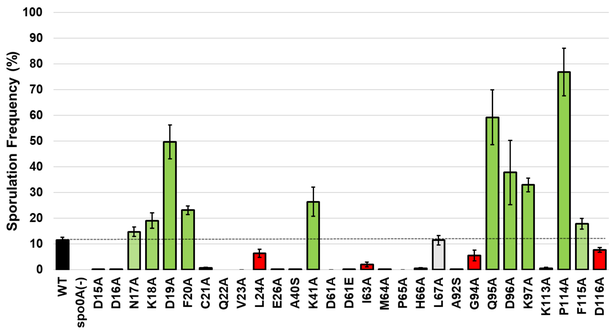

Figure 3. Spo0A Site-directed Mutants and their Associated Sporulation Frequencies. Sporulation efficiency was determined as previously described.[3] Active *C. difficile* cultures were grown to an OD_{600} = 0.5, plated onto 70:30 agar with 2 mg/mL thiamphenicol and incubated anaerobically for 24 h. Ethanol-resistant spores were enumerated on BHIS plates supplemented with 0.1% taurocholate. Sporulation assays were performed at least four times for each strain. Hypersporulating (green columns), hyposporulating (red columns), asporogenous (red columns), and no change (gray columns) phenotypes were observed.

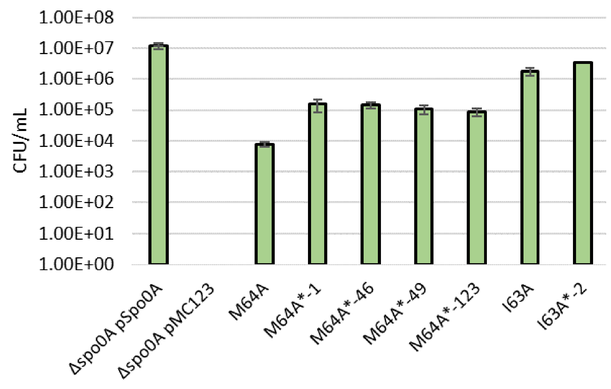

Figure 5. Suppressors of Spo0A Site-directed Mutants Increase Sporulation. Suppressor mutants were generated then screened for an increase in sporulation frequency either following ethyl methanesulfonate (EMS) treatment or isolated spontaneously, as previously described.[4,5] Suppressor mutants were generated from the progenitor Spo0A site-directed mutant strains I63A and M64A. Sporulation assays were performed at least twice, with the exception of I63A*-2, which was done once.

Strain	Mutation Location	Mutation Annotation	Gene Function
M64A*-1	CD3673	Coding region (L166F)	Putative Spo0J-like protein *noc*
M64A*-1	CD1579-CD1580	225bp deletion, intergenic	Two-component sensor histidine kinase/transcription regulator
M64A*-1	*nagA*	Coding region (C3Y)	N-acetylglucosamine-6-phosphate deacetylase
M64A*-1	*clpP1*	Coding region (D57N)	ATP-dependent Clp protease proteolytic subunit 1
M64A*-46	CD3267-CD3268	Intergenic, new junction	Two-component sensor histidine kinase/ABC transporter
M64A*-49	CD3267-CD3268	Intergenic, new junction	Two-component sensor histidine kinase/ABC transporter
M64A*-123	CD0567-CD0577	225bp deletion, intergenic	Two-component sensor histidine kinase/hypothetical protein
M64A*-123	CD2047-CD2048	Intergenic, new junction	Two-component response regulator/transcription regulator
I63A*-2	*rumA*	36bp deletion coding (1329-1365)	23S rRNA mthyltransferase
I63A*-2	*flgL*	Coding region (D252N)	Flagellar hook-associated protein (HAP3)

Table 1. Mutations Identified through Whole Genome Sequencing in Spo0A Site-directed Mutant Suppressor Mutants. Genomic DNA was extracted and prepared for whole genome sequencing. Library preparation of DNA was performed by the Microbial Genomic Sequencing Center (MiGS). The Breseq pipeline was used for bioinformatic analysis and mutation prediction. Suppressor mutants were compared to the reference strain (*C. difficile* 630Δerm) and the Spo0A site-directed mutant complement strain (Δ*spo0A* p*spo0A*).

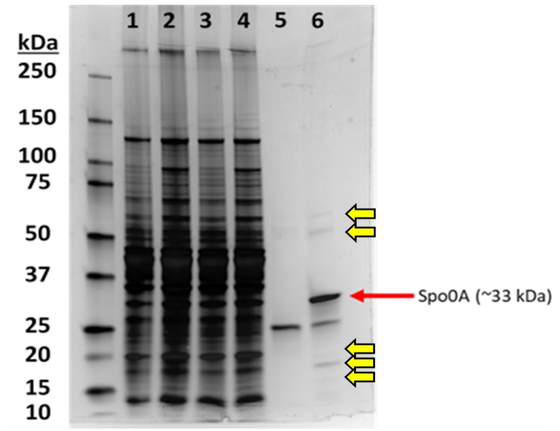

Figure 4. Co-IP of Spo0A-FLAG. Sporulating cultures of *C. difficile* 630Δerm expressing Spo0A-FLAG or Spo0A-empty vector control grown on 70:30 agar were harvested at H_{12} and processed for silver staining using M2 α-FLAG magnetic beads. **Lane 1** = empty vector total cell lysate; **Lane 2** = Spo0A-FLAG total cell lysate; **Lane 3** = empty vector lysate post-incubation with α-FLAG beads; **Lane 4** = Spo0A-FLAG lysate post-incubation with α-FLAG beads; **Lane 5** = empty vector elution heated to 65°C; **Lane 6** = Spo0A-FLAG elution heated to 65°C. Red arrow highlights purified Spo0A-FLAG. Yellow arrows represent potential binding partners that co-immunoprecipitate with Spo0A-FLAG.

Locus Tag	Function
CD1579	Orphan histidine kinase
CD0553	RNA methyltransferase
CD3271	Putative Spo0E protein
CD1038	DNA helicase
CDp100	Transcriptional regulator
CD1492	Orphan histidine kinase
CD2216	Hypothetical protein
CD1164	GTPase Obg
CD3088	Cellobiose degrading protein
CD1247	Hypothetical protein
CD2492	Orphan histidine kinase

Figure 5. Potential Spo0A Binding Partners Identified Using LC-MS/MS. Spo0A-FLAG and negative control eluates were analyzed for putative Spo0A-FLAG binding partners using a Q-Exactive Orbitrap mass spectrometer at the Emory Proteomics Core facility. The proteins shown represent those with the greatest signal intensity in the Spo0A-FLAG eluate compared to the negative control. Red locus tags represent regulatory proteins that are hypothesized to interact with Spo0A to regulate Spo0A activity.

Summary

- Alignment of *C. difficile* Spo0A with *B. subtilis* Spo0A allowed for prediction of conserved Spo0A residues that are important for sporulation initiation.
- Through targeted mutagenesis of conserved *C. difficile* Spo0A residues, important sites have been identified that impact sporulation frequency and are likely to have a function in sporulation initiation.
- Generation and isolation of suppressor mutants that exhibit increased sporulation frequency from low-sporulating Spo0A site-directed mutants are expected to uncover novel regulators of sporulation.
- Co-immunoprecipitation of Spo0A-FLAG and subsequent LC-MS/MS analysis has identified potential Spo0A regulatory proteins.

Future Directions

- Elucidate compensatory mechanisms of identified Spo0A suppressor mutations that result in increased sporulation frequency.
- Western blotting to assess stability of Spo0A site-directed mutations.
- Characterize proteins that directly interact with Spo0A using genetic and biochemical approaches to understand Spo0A regulatory network in *C. difficile*.
- Confirm direct protein-protein interactions with Spo0A

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