

## RESEARCH ARTICLE

# The small acid-soluble proteins of *Clostridioides difficile* are important for UV resistance and serve as a check point for sporulation

Hailee N. Nerber , Joseph A. Sorg \*

Department of Biology, Texas A&amp;M University, College Station, Texas, United States of America

\* [jsorg@bio.tamu.edu](mailto:jsorg@bio.tamu.edu) OPEN ACCESS

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## Abstract

*Clostridioides difficile* is a nosocomial pathogen which causes severe diarrhea and colonic inflammation. *C. difficile* causes disease in susceptible patients when endospores germinate into the toxin-producing vegetative form. The action of these toxins results in diarrhea and the spread of spores into the hospital and healthcare environments. Thus, the destruction of spores is imperative to prevent disease transmission between patients. However, spores are resilient and survive extreme temperatures, chemical exposure, and UV treatment. This makes their elimination from the environment difficult and perpetuates their spread between patients. In the model spore-forming organism, *Bacillus subtilis*, the small acid-soluble proteins (SASPs) contribute to these resistances. The SASPs are a family of small proteins found in all endospore-forming organisms, *C. difficile* included. Although these proteins have high sequence similarity between organisms, the role(s) of the proteins differ. Here, we investigated the role of the main  $\alpha/\beta$  SASPs, SspA and SspB, and two annotated putative SASPs, CDR20291\_1130 and CDR20291\_3080, in protecting *C. difficile* spores from environmental insults. We found that SspA is necessary for conferring spore UV resistance, SspB minorly contributes, and the annotated putative SASPs do not contribute to UV resistance. In addition, the SASPs minorly contribute to the resistance of nitrous acid. Surprisingly, the combined deletion of *sspA* and *sspB* prevented spore formation. Overall, our data indicate that UV resistance of *C. difficile* spores is dependent on SspA and that SspA and SspB regulate/serve as a checkpoint for spore formation, a previously unreported function of SASPs.

## Author summary

*C. difficile* infections remain problematic and elimination of spores within an environment is essential to limit person-to-person spread. A deeper understanding of how spores resist cleaning efforts could lead to better strategies to eradicate the spores in a contaminated environment. The small acid-soluble proteins (SASPs), found in all endospore-

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forming organisms, are one mechanism that allows for spore resilience. In the model organism, *B. subtilis*, the SASPs are thought to non-specifically bind to DNA to force the DNA into a conformation that prevents the formation of thymidine dimers and, instead, result in the formation of the spore photoproduct. Here, we investigated the roles of the SASPs in *C. difficile* find that *C. difficile* SspA and SspB protect against UV light. Unexpectedly, we found that these SASPs also regulate spore formation, a role not described for any SASP to date. Our data suggest that *C. difficile* SASPs may bind specific DNA sequences to regulate spore formation.

## Introduction

*Clostridioides difficile* is the leading cause of antibiotic associated diarrhea with ~224,000 annual infections in the United States [1–3]. Prior antibiotic treatment is the greatest risk factor for *C. difficile* infection due to their broad-spectrum activity that can lead to a dysbiotic colonic microbial community [4,5]. Upon inoculation into a susceptible host, *C. difficile* spores germinate to the toxin-producing vegetative form [4]. These toxins lead to the disruption of the colonic epithelium and the common symptoms of disease (e.g., diarrhea and colitis) [4]. Vancomycin and fidaxomicin are the recommended treatments for *C. difficile* infection [4,6]. While these antibiotics treat the infection by targeting the actively-growing vegetative cells, the spore form is resistant to antibiotics [5,7]. In contrast to the anaerobic nature of the *C. difficile* vegetative cell, spores can survive the oxygen-rich environment outside of a host, and are considered the transmissible form [5,8,9].

Endospores are dormant forms of bacteria that can withstand extreme environmental conditions and chemical exposures [10]. In all endospore-forming bacteria, sporulation is controlled by the master transcriptional regulator, Spo0A [9]. Activation of Spo0A by phosphorylation results in the global activation of gene expression with the goal of optimizing/surviving post-exponential phase growth [11–13]. Upon the initiation of sporulation, the vegetative cell divides asymmetrically, resulting in a large mother cell compartment and a smaller forespore compartment [12]. Subsequently, a cascade of sigma factor activation occurs and leads to the development of a dormant endospore [12]. In *Bacillus subtilis*, a model organism for studying sporulation and germination, the activation of  $\sigma^F$  in the forespore leads to  $\sigma^E$  activation in the mother cell. The activation of  $\sigma^E$  in the mother cell, in turn, leads to activation of  $\sigma^G$  in the forespore and then results in the activation of  $\sigma^K$  in the mother cell. The result of this crisscross sigma factor activation cascade is the engulfment of the forespore by the mother cell, maturation of the forespore, and the eventual release of the spore by lysis of the mother cell. The same sigma factors drive *C. difficile* sporulation, but the crisscross activation across compartments does not occur [11,12,14–16]. The resulting spores are extremely resistant to environmental conditions and common cleaning methods [10,17]. Thus, with a deeper understanding of the resistance properties of spores, and the mechanisms that confer this resistance, novel interventions could be developed to clean contaminated environments.

The small acid-soluble proteins (SASPs) confer resistance to spores [10,18]. The SASPs are a family of proteins that are less than 100 amino acids in length and are conserved among all endospore-forming organisms [18]. They are produced late in sporulation under the forespore-specific sigma factor,  $\sigma^G$ , and account for approximately 20% of the total spore protein content [10,15,18]. Most spore-forming bacteria encode the two major  $\alpha/\beta$ -type SASPs (SspA and SspB), however there are other minor SASPs that vary in number depending on the organism [18–22]. *B. subtilis* also encodes a  $\gamma$ -type SASP that is hypothesized to serve as an amino

acid reservoir for use upon outgrowth of the vegetative cell from the germinated spore [23]. Clostridial species, to date, have not been found to contain  $\gamma$ -type SASPs [19,20]. In *C. difficile*, the R20291 strain encodes *sspA* and *sspB* orthologues and two genes annotated as putative SASPs, *CDR20291\_1130* and *CDR20291\_3080*. The 4 SASPs annotated in *C. difficile* R20291 are also encoded within the *C. difficile* CD630 genome (ribotype 012) and have 100% identity for SspA, CD630\_12900 (*CDR20291\_1130*), and CD630\_32201 (*CDR20291\_3080*) and 99% identity for SspB compared to the *C. difficile* R20291 strain (ribotype 027). SASPs are well-conserved across genera with 70% similarity between *C. difficile* and *B. subtilis* SspA and SspB [24]. However, the annotated SASPs have less similarity to *C. difficile* or *B. subtilis* SspA and SspB, ranging from 40–60%. Interestingly, *CDR20291\_3080* shares 80% similarity to *C. perfringens* Ssp4, a novel SASP, which protects the spores from nitrous acid and extreme heat associated with food processing [22,25].

In *B. subtilis*, the  $\alpha/\beta$ -type SASPs contribute to spore resistance against several chemicals, such as nitrous acid, formaldehyde, glutaraldehyde, iodine, or hydrogen peroxide [17,25–33]. Moreover, *B. subtilis*  $\Delta$ *sspA* mutants completely lose viability after 3 minutes of exposure to 254 nm UV light, and *B. subtilis*  $\Delta$ *sspB* mutants have 10% survival after 7 minutes of exposure [34]. Interestingly, a double mutant was even more sensitive to UV exposure than were vegetative cells, highlighting the importance of these proteins in spore survival [34]. These UV-sensitive phenotypes could be complemented by expressing, *in trans*, either *sspA* or *sspB* [35]. Moreover, a SASP from *B. megaterium* complemented the phenotype, suggesting that they could play interchangeable roles in UV resistance [30,35].

In other spore-forming bacteria, the role of the SASPs vary among organisms. In *C. botulinum*, SASPs were found to be necessary for protection against nitrous acid, similar to what is observed in *B. subtilis* [27]. However, they are not necessary for protection against hydrogen peroxide or formaldehyde, contrary to what is observed in *B. subtilis* [27]. In *C. perfringens* isolates that cause food poisoning, Ssp4 was necessary for spores surviving food processing events (e.g., high heat and use of nitrites) [22,25]. Other *C. perfringens* SASPs were found to protect spores against UV light, hydrogen peroxide, nitrous acid, formaldehyde, and hydrochloric acid [28,36,37].

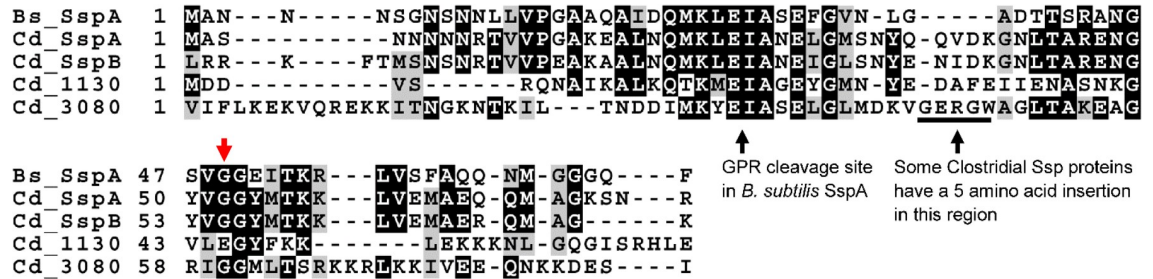
The  $\alpha/\beta$ -type SASPs of *B. subtilis*, *C. perfringens*, and *C. acetobutylicum* all bind to DNA *in vitro* [36,38–40]. In *B. subtilis*, the binding of these proteins changes the conformation of the DNA to one between an A and a B form [38,40–42]. In this unique conformation, an alternative form of UV damage is induced, a thymidyl-thymidine adduct, called the spore photoproduct. The spore photoproduct is repaired by the spore photoproduct lyase, SPL, during germination and outgrowth [24,35,43–46].

Here, we investigate the functions of *C. difficile* *sspA*, *sspB*, and the annotated SASPs, *CDR20291\_1130* and *CDR20291\_3080*. We found that *C. difficile* SspA is the major contributor to UV resistance of the spores and that SspB is minorly involved in UV resistance. *CDR20291\_1130* and *CDR20291\_3080* do not contribute to UV resistance. Additionally, some of the SASPs minorly contribute to nitrous acid spore resistance while they do not contribute to other chemicals in the concentrations and exposure times tested. Surprisingly, we found that the deletion of both *sspA* and *sspB* prevented spore formation. Our results indicate that, in addition to providing UV resistance to spores, the major *C. difficile* SASPs are involved in spore formation.

## Results

### Conservation of SASPs in *C. difficile*

The main  $\alpha/\beta$  type SASPs are conserved in spore-forming bacteria [18]. Within *C. difficile* R20291, SspA and SspB are 85% identical. The putative SASPs are approximately 40% identical



**Fig 1. SASP homology.** Alignment of *B. subtilis* SspA with *C. difficile* SspA, SspB, CDR20291\_1130 and CDR20291\_3080. The site of GPR cleavage is indicated. Also illustrated is the site of insertion in Clostridial Ssp proteins. The red arrow indicates SspA<sub>G52</sub>.

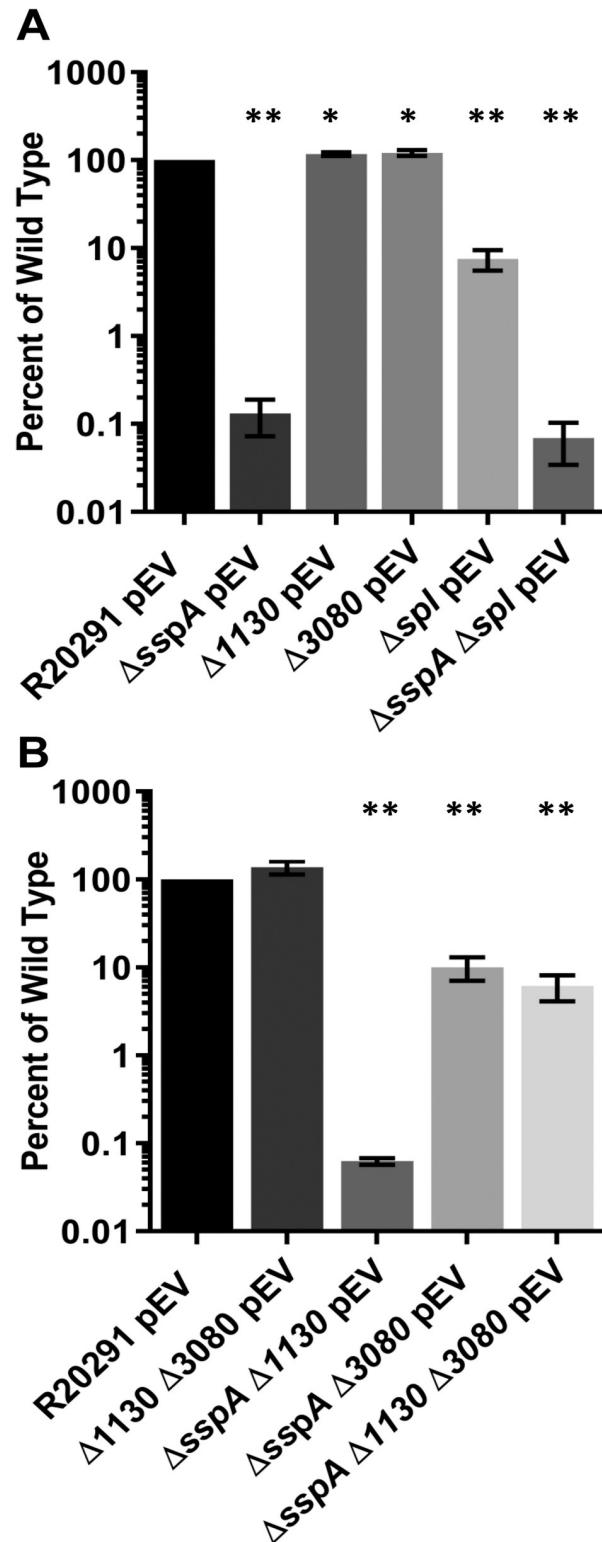
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to the main  $\alpha/\beta$  type SASPs. All 4 contain the conserved “EIA” sequence that is cleaved by the germination protease (GPR) upon germination (Fig 1) [47–49]. Even with strong sequence similarity/identity, SASPs have varying roles between spore-forming organisms. To investigate the roles of *C. difficile* SspA, SspB, CDR20291\_1130, and CDR20291\_3080 in UV resistance, outgrowth, and chemical resistance, single gene deletions and pairwise deletions were generated using CRISPR-Cas9 genome editing [50]. Total amounts of spores produced and the sporulation frequency by the single deletion strains, *C. difficile*  $\Delta$ sspA, *C. difficile*  $\Delta$ CDR20291\_1130, and *C. difficile*  $\Delta$ CDR20291\_3080 were indistinguishable from the wildtype *C. difficile* R20291 parental strain using heat or ethanol based sporulation assays (S1 and S2 Figs, respectively). Surprisingly, the *C. difficile*  $\Delta$ sspB strain did not make spores and will be discussed later. These results indicate that the single deletions of *C. difficile* sspA, *C. difficile* CDR20291\_1130 and *C. difficile* CDR20291\_3080 do not impact *C. difficile* sporulation.

### sspA is required for spore UV resistance

Because of the strong phenotype of *B. subtilis* SASPs in UV resistance, we hypothesized that *C. difficile* SspA, and/or the two annotated SASPs (CDR20291\_1130 and CDR20291\_3080) may function similarly. The viability of SASP mutants after a 10 minute exposure to 302 nm UV light was tested. Viable spores were quantified by colony formation on media supplemented with taurocholate (a *C. difficile* spore germinant) and then compared to spores derived from the wildtype parental strain [51]. Exposure of the *C. difficile*  $\Delta$ sspA strain to UV light resulted in a ~1,000x decrease in spore survival. Spores derived from the *C. difficile*  $\Delta$ CDR20291\_1130 and *C. difficile*  $\Delta$ CDR20291\_3080 mutants had a statistically significant difference in UV resistance compared to spores derived from the wildtype strain, but this difference is likely not biologically relevant (Fig 2A). These results indicate that *C. difficile* SspA is the most important SASP for UV resistance while the genes annotated as putative SASPs are not involved in UV resistance. In *B. subtilis*, SASP binding to DNA helps to protect from UV by encouraging the formation of spore photoproducts (SP) instead of cyclobutane thymine dimers [17,52]. The spore photoproduct lyase, SPL, repairs the SP [43–45,53]. To understand the impact of SPL on UV resistance in *C. difficile*, we engineered a deletion in the *C. difficile* spl gene. Spores derived from *C. difficile*  $\Delta$ spl strain have approximately 10x reduced survival compared to spores derived from the wildtype *C. difficile* R20291 strain (Fig 2A). Unsurprisingly, the *C. difficile*  $\Delta$ sspA  $\Delta$ spl double mutant remains at the level of sspA mutant alone (0.1% of wildtype), suggesting that SPL function depends on SspA.

To determine if the putative SASPs have redundant roles in UV resistance, we generated deletions of all pairwise combinations of *C. difficile* CDR20291\_1130, *C. difficile* CDR20291\_3080, and *C. difficile* sspA. After 10 minutes of UV exposure, spores derived from



**Fig 2. *C. difficile* sspA is necessary for UV protection.** Spores were exposed to 302 nm UV light for 10 minutes. After exposure, they were serially diluted and plated onto rich medium supplemented with germinants and CFUs were enumerated. All data was normalized to  $T_0$  then the ratio of the mutants was normalized to the ratio of wildtype. A.) Spores derived from wild type R20291 with the indicated deletion strains. B.) spores derived from pairwise deletions of *C. difficile*  $\Delta$ sspA,  $\Delta$ CDR20291\_1130, and  $\Delta$ CDR20291\_3080. pEV indicates an empty vector. All data points represent

the average from three independent experiments and the standard error from the mean. Statistical analysis was performed by one way ANOVA with Dunnett's multiple comparisons test in comparison to wildtype. \*  $p < 0.01$ , \*\*  $p < 0.0001$ .

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the *C. difficile*  $\Delta$ CDR20291\_1130  $\Delta$ CDR20291\_3080 were as resistant as wildtype spores, indicating that these annotated SASPs are not compensating for each other during UV exposure (Fig 2B). Spores derived from the *C. difficile*  $\Delta$ sspA  $\Delta$ CDR20291\_1130 mutant had no further reduction in survival compared to the *C. difficile*  $\Delta$ sspA mutant alone. However, spores derived from the *C. difficile*  $\Delta$ sspA  $\Delta$ CDR20291\_3080 double mutant and the  $\Delta$ sspA  $\Delta$ CDR20291\_1130  $\Delta$ CDR20291\_3080 triple mutant were not as sensitive to UV light as the *C. difficile*  $\Delta$ sspA or the *C. difficile*  $\Delta$ sspA  $\Delta$ CDR20291\_1130 strains (Fig 2B).

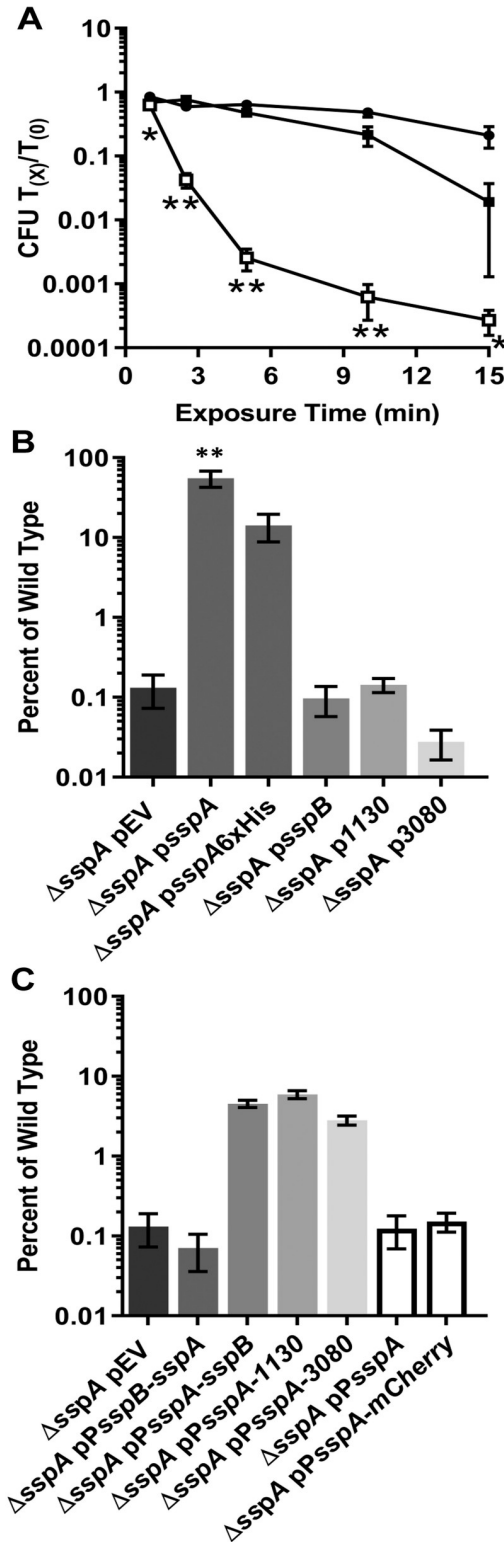
### The *sspA* promoter is necessary for complementation of *C. difficile* $\Delta$ sspA UV resistance

To understand the extent of protection that *C. difficile* SspA provides against UV damage, we quantified the viability of spores exposed to UV over time. Spores derived from the *C. difficile*  $\Delta$ sspA strain showed a 10% loss of viability after 2.5 minutes of UV exposure. This loss increased to approximately 3  $\log_{10}$  after 15 minutes of exposure. Expression of *sspA* from a plasmid under the control of its native promoter restored viability to the *C. difficile*  $\Delta$ sspA strain (Fig 3A).

To further understand the role of *C. difficile* SspA in UV resistance, we tested the impact of different *sspA* expression constructs on spore survival. The *sspA* complement consisting of the *sspA* gene under its native promoter region resulted in restoration of spore viability. A 6x-histidine tag inserted on the C-terminus of the *sspA* gene also resulted in restoration of spore viability upon UV exposure (Fig 3B). In *B. subtilis*, SASP genes can cross-complement a SASP mutant [35]. Therefore, plasmids were constructed that consisted of *C. difficile* *sspB*, *CDR20291\_1130*, or the *CDR20291\_3080* genes, driven by their native promoter regions, introduced into the *C. difficile*  $\Delta$ sspA mutant strain. After 10 minutes of exposure to UV light, spores derived with these plasmid constructs revealed that *C. difficile* *sspB*, *CDR20291\_1130*, or *CDR20291\_3080* were unable to restore the UV resistance to spores derived from the  $\Delta$ sspA mutant strain (Fig 3B). To determine if this is an issue with differences in expression, the promoter regions were changed and these genes were again tested for their ability to complement the *C. difficile*  $\Delta$ sspA strain. When the *sspA* gene was placed under control of the *sspB* promoter region, complementation no longer occurred. This further supported our hypothesis that the SASPs have different expression levels. Swapping the promoter regions of *C. difficile* *sspB*, *CDR20291\_1130*, or *CDR20291\_3080* complementation plasmids for the *C. difficile* *sspA* promoter region resulted in a restoration to approximately 5% of wildtype levels (Fig 3C). However, as negative controls, the *sspA* promoter region alone, or the *sspA* promoter driving the gene encoding mCherry, could not complement the UV phenotype. These results suggest that despite what is observed for cross-complementation in other organisms, *C. difficile* *sspB*, *CDR20291\_1130*, or *CDR20291\_3080* cannot fully complement the *C. difficile*  $\Delta$ sspA strain phenotype, and to provide any complementation, they must be expressed from the *sspA* promoter.

### *C. difficile* SASPs have redundant functions during outgrowth

*C. difficile* *CDR20291\_1130* and *CDR20291\_3080* do not contribute to spore UV resistance, but it is possible that their main role is to protect against other harsh environmental conditions



**Fig 3. Complementation of the *C. difficile*  $\Delta$ sspA strain.** A) Spores derived from wildtype *C. difficile* R20291 pEV ●, the *C. difficile*  $\Delta$ sspA pEV deletion strain □, and the *C. difficile*  $\Delta$ sspA psspA strain ■ were exposed to UV for varying times and the viability assessed by normalizing to  $T_0$ . B) Spores from the *C. difficile*  $\Delta$ sspA mutant strain containing genes under control of the *sspA* promoter were exposed to 302 nm UV light for 10 minutes. After exposure, the spores were serially diluted and plated onto rich medium supplemented with germinants. The CFUs were enumerated and

then normalized to  $T_0$  CFUs. The ratio of the complemented *C. difficile*  $\Delta$ *sspA* mutant strains were normalized to the ratio of the wildtype strain. C) Spores from the *C. difficile*  $\Delta$ *sspA* mutant strain containing genes under the control of the *sspA* or *sspB* promoter regions were exposed to UV for 10 minutes. pEV indicates an empty vector. All data represents the average of three independent experiments and the standard error from the mean. Statistical analysis was performed by ANOVA with Dunnett's multiple comparison. A) two way, in comparison to wildtype, B) and C) one way, in comparison to *C. difficile*  $\Delta$ *sspA* pEV. \*  $p < 0.05$ , \*\*  $p < 0.0001$ .

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or to serve as amino acid reservoirs (like  $\gamma$ -type SASPs of *B. subtilis*) during outgrowth of a vegetative cell from the germinated spore [23]. To determine if these annotated SASPs contribute to outgrowth, the  $OD_{600}$  of spores derived from wildtype and mutant strains were analyzed over 12 hours in complex medium supplemented with germinants [51,54]. No difference in the outgrowth of vegetative cells was observed for the mutant strains, compared to the parental strain.

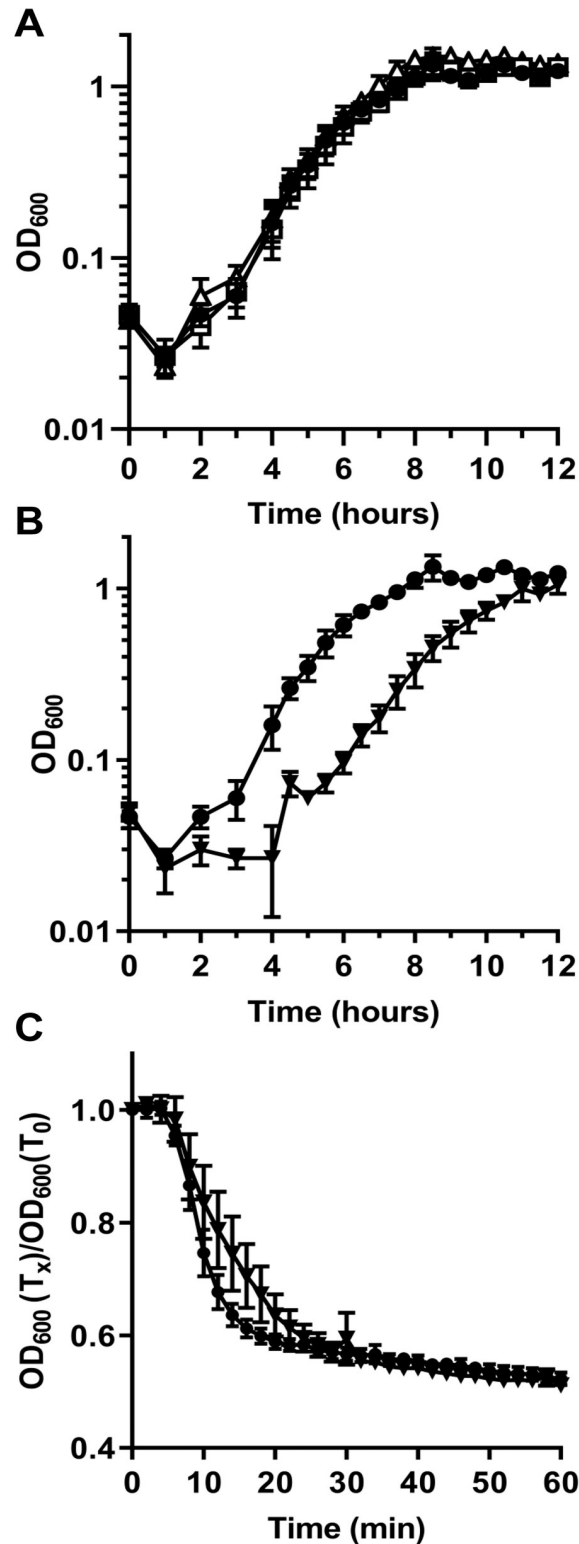
It is possible that the complex medium masked the hypothesized phenotype due to the sheer abundance of nutrients in the medium eliminating the need for the amino acids derived from the SASPs. Instead, we tested if outgrowth of a spore in minimal medium (CDMM) would be influenced in these mutant strains. Unfortunately, using a minimal medium resulted in an extreme delay in outgrowth that was not possible to practically measure. Therefore, spore outgrowth was analyzed in half-strength complex medium. In addition, to eliminate the possibility of extra resources being packaged into the spore when grown on a complex medium, and thus reducing the need for SASPs during outgrowth, spores were generated on minimal medium. Again, despite spore production on minimal medium and using half-strength complex medium during the assay, there were no differences between the outgrowth of wildtype spores or spores derived from the *C. difficile*  $\Delta$ *sspA* or the *C. difficile*  $\Delta$ *CDR20291\_1130  $\Delta$ *CDR20291\_3080* double mutant strain (Fig 4A).*

Due to the possibility that the SASPs can compensate for the deletion of one, a triple mutant was generated of *sspA*, *CDR20291\_1130*, and *CDR20291\_3080*. These spores were also generated on minimal medium and outgrowth analyzed in half-strength complex media. Spores derived from the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *CDR20291\_1130  $\Delta$ *CDR20291\_3080* triple mutant had an approximate 2-hour delay in outgrowth compared to the wildtype strain (Fig 4B). To eliminate the possibility that the difference in outgrowth is due to a germination defect in this triple mutant, we monitored germination by  $OD_{600}$  in buffer supplemented with germinants [51,54]. During the very early events of endospore germination, the dormant, phase-bright, spore transitions to a phase-dark, germinated spore. Mutant spores germinated similarly to wildtype, suggesting no defect in germination (Fig 4C). These results suggest that *C. difficile* SspA, *CDR20291\_1130*, and *CDR20291\_3080* could be used as a nutrient/amino acid source during outgrowth of a germinated spore.*

### The *C. difficile* SASPs do not contribute to chemical resistance

To further characterize the role of these proteins in the spore, spores were exposed to various chemicals. Spores derived from the single deletion of *C. difficile* *sspA*, *CDR20291\_1130*, and *CDR20291\_3080* and their complements, when necessary, were exposed to chemicals for 1 minute, 5 minutes, 10 minutes, and 30 minutes and spore viability was assessed by plating onto rich medium supplemented with germinant. Colony forming units were compared to  $T_0$  and then this ratio compared to the ratio of wildtype survival. Spores exposed to 3%  $H_2O_2$ , 75% EtOH, 0.25% glutaraldehyde, 1 M HCl, 0.05% hypochlorite, and 2.5% formaldehyde did not exhibit reduced viability in comparison to spores derived from the wildtype strain (S3A–S3F Fig). The mutant strains did have a slight reduction in viability after 5 or 10 minutes of exposure to 250 mM nitrous acid (S3G Fig).





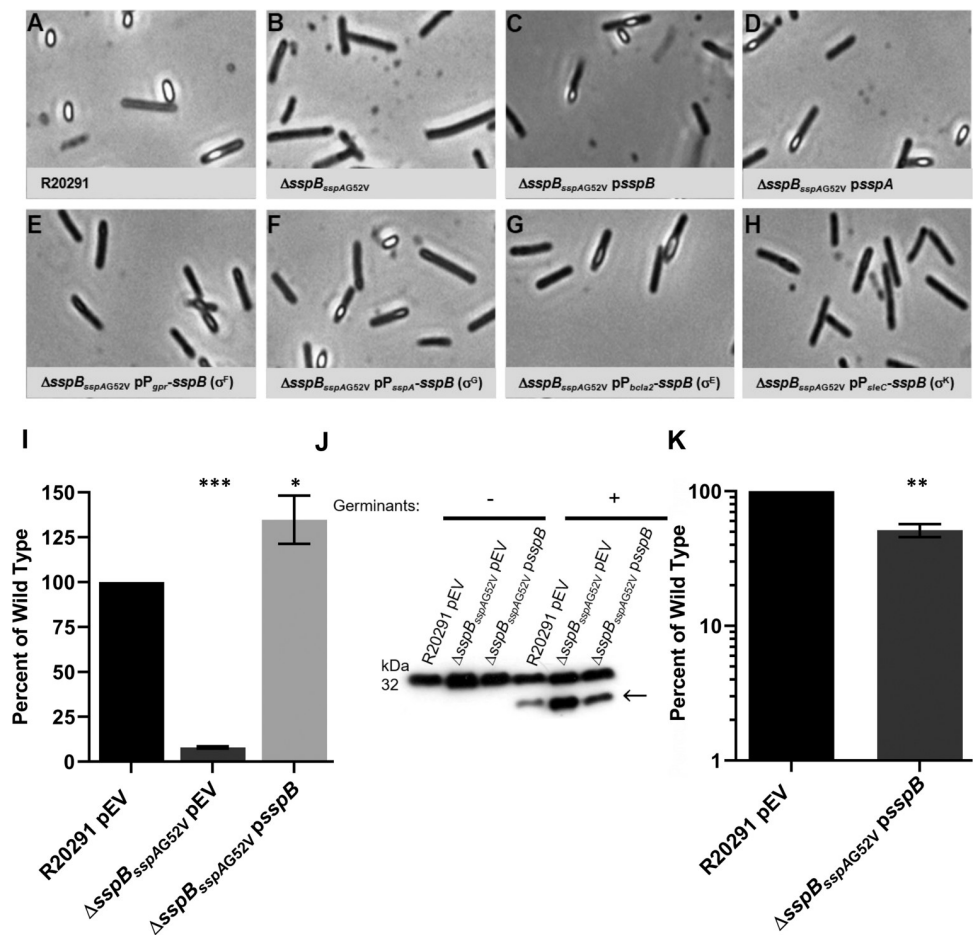
**Fig 4. *C. difficile*  $\Delta$ sspA  $\Delta$ CDR20291\_1130  $\Delta$ CDR20291\_3080 affects spore outgrowth.** Outgrowth was determined by monitoring the OD<sub>600</sub> of spores inoculated into half-strength complex medium supplemented with germinants (10 mM taurocholic acid and 30 mM glycine). All spores used for outgrowth were generated on minimal medium. A) Outgrowth of *C. difficile* R20291 pEV ●, *C. difficile*  $\Delta$ sspA pEV □ and *C. difficile*  $\Delta$ CDR20291\_1130  $\Delta$ CDR20291\_3080 pEV Δ. B) Outgrowth of wildtype R20291 pEV ● and triple mutant spores of *C. difficile*  $\Delta$ sspA  $\Delta$ CDR20291\_1130  $\Delta$ CDR20291\_3080 pEV ▼. C) Spore germination was monitored at OD<sub>600</sub> upon exposure of spores

to germinants taurocholic acid and glycine in a buffered solution. The OD<sub>600</sub> was normalized to T<sub>0</sub>. R20291 pEV ● and triple mutant spores of *C. difficile* Δ*sspA* Δ*CDR20291\_1130* Δ*CDR20291\_3080* pEV ▼. pEV indicates an empty vector. All data points represent the average from three independent experiments and the standard error from the mean. Statistical analysis by two way ANOVA with Dunnett's multiple comparisons test. A) *C. difficile* Δ*sspA*, p < 0.001 at 11 minutes. B) *C. difficile* Δ*sspA* Δ*CDR20291\_1130* Δ*CDR20291\_3080* p < 0.05 at 6.5, 6, 8, 9 and 10.5 hours. p < 0.01 at 7.5 hours.

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### *C. difficile* spore formation is influenced by SASPs

When generating the *C. difficile* Δ*sspB* mutant strain, we found that spores derived from this strain were phase-gray and were not released from the mother cell, compared to the wildtype strain (Fig 5A and 5B). Surprisingly, whole genome resequencing of the *C. difficile* Δ*sspB* strain revealed an additional, single nucleotide mutation in *sspA*. This mutation resulted in an



**Fig 5. *C. difficile* Δ*sspB*<sub>sspAG52V</sub> results in phase-gray engulfed spores.** A-H) Day 6 cultures of the indicated strains were fixed in 4% formaldehyde and 2% glutaraldehyde before imaging on a Leice DM6B microscope. I) DPA content was determined by boiling spore solutions and analyzing approximately 2.5 x 10<sup>7</sup> spores by terbium fluorescence. J) Western blot of spores exposed to 10 mM taurocholic acid in a rich medium for 1 hour and blotted against SleC. Arrow indicates processing of pro-SleC (above) to the active SleC form. K) Spores were exposed to 302 nm UV light for 10 minutes before serial dilution and plating on rich medium supplemented with germination. All values were normalized to T<sub>0</sub> and then those ratios were normalized to the ratio of wildtype survival. pEV indicates an empty vector. All data points represent the average from three independent experiments and the standard error from the mean. I) Statistical analysis by one way ANOVA with Dunnett's multiple comparisons test. K) Statistical analysis by unpaired t test. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

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*sspA*<sub>G52V</sub> allele, in addition to the *sspB* deletion (referred to as  $\Delta$ *sspB*<sub>*sspAG52V*</sub>). This phenotype could be complemented by expression of the *sspB* gene under the control of the *sspB* promoter region (Fig 5C) or the *sspA* gene under control of the *sspA* promoter region (Fig 5D). However, only a  $\sigma^G$ -controlled promoter was able to complement the phenotype, other  $\sigma$ -factor controlled promoters could not complement (Fig 5E–5H). The rate of sporulation of the *C. difficile*  $\Delta$ *sspB* mutant was also 5 log<sub>10</sub> less than the wildtype strain by heat sporulation assay and reduced by more than 4 log<sub>10</sub> with an ethanol based assay (S4 and S2 Figs). This was complemented by expression of *sspB* from a plasmid (S4 and S2 Figs).

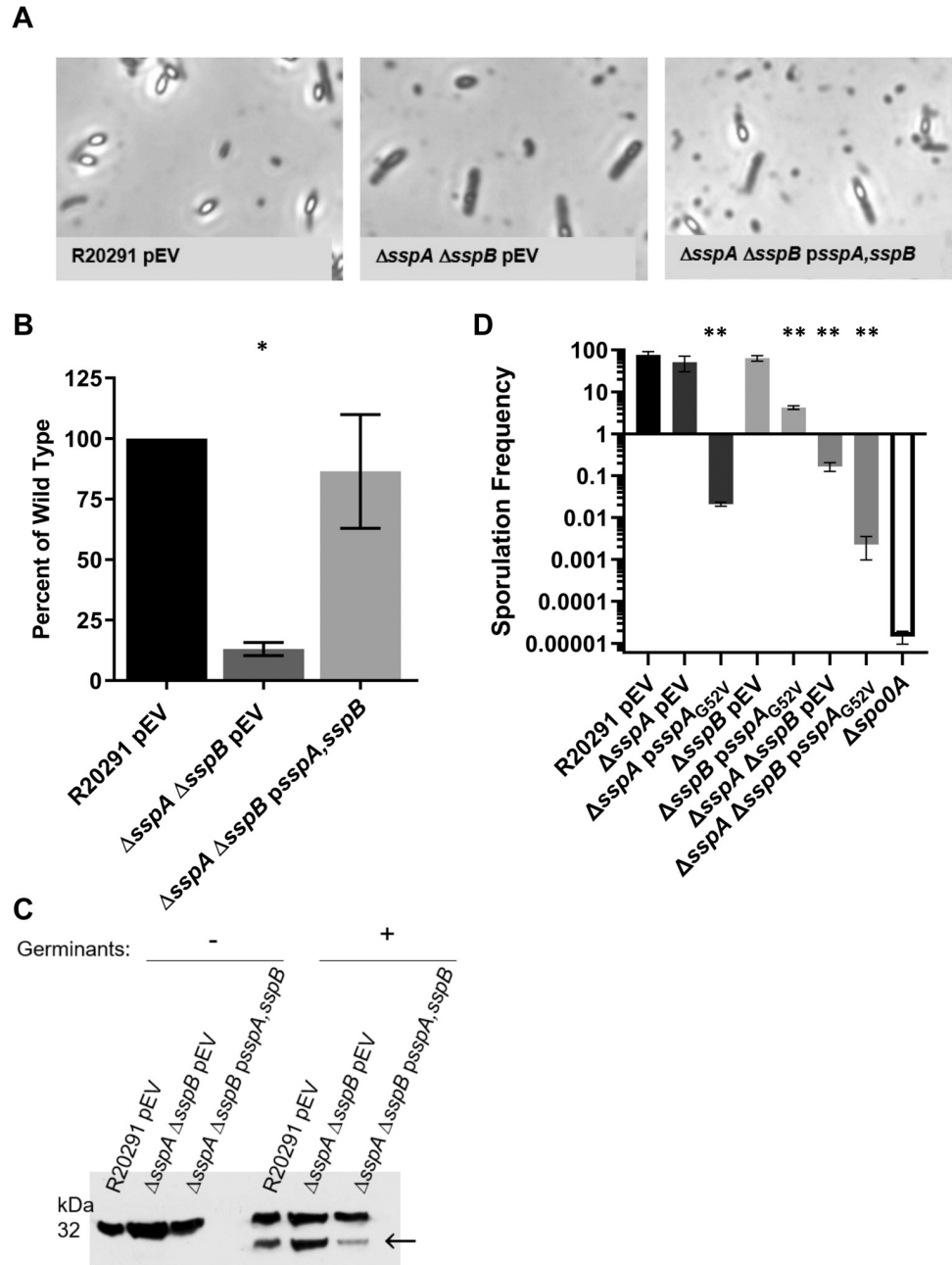
Due to the phenotype of this strain, normal spore purification processes were unsuccessful. To encourage release of the immature spores from the mother cells, cultures from sporulating cells were incubated with lysozyme before resuming normal spore purification steps. This encouraged the spores to release from the mother cells and the recovery of a limited number of phase-gray spores. The phase-bright phenotype of wildtype spores is partially attributed to the dipicolinic acid (DPA) content of the spore core. Approximately 2.5 x 10<sup>7</sup> spores of the *C. difficile* wildtype,  $\Delta$ *sspB*<sub>*sspAG52V*</sub>, and  $\Delta$ *sspB*<sub>*sspAG52V*</sub> *psspB* strains were boiled and the DPA levels determined by Tb<sup>3+</sup> fluorescence [55]. The DPA content of the *C. difficile*  $\Delta$ *sspB*<sub>*sspAG52V*</sub> strain was significantly lower than wildtype content and this was able to be complemented by expression of the *sspB* gene from its native promoter region (Fig 5I).

Because germination assays rely heavily on the release of DPA during germination (phase-bright to phase-dark transition), we were unable to use these assays to determine if this deletion and SNP combination altered germination capabilities [55,56]. Instead, the spores were exposed to germinants and the processing of spore proteins during germination were analyzed by immunoblot. In response to germinants, spores derived from the *C. difficile*  $\Delta$ *sspB*<sub>*sspAG52V*</sub> strain processed proSleC to its active form, indicating that they are capable of receiving the germinant signals (Fig 5J) [57–59].

We also tested the ability of spores derived from the *C. difficile*  $\Delta$ *sspB*<sub>*sspAG52V*</sub> *psspB* strain to resist UV damage. The *C. difficile*  $\Delta$ *sspB*<sub>*sspAG52V*</sub> alone was not assessed due to the difficulty in producing and purifying spores. In the assay, the *C. difficile*  $\Delta$ *sspB*<sub>*sspAG52V*</sub> *psspB* strain was complemented to wildtype levels, revealing that the *sspA*<sub>G52V</sub> allele is not impaired in its ability to protect against UV damage but SspA may regulate *C. difficile* sporulation with SspB (Fig 5K).

### ***C. difficile* *sspA* and *sspB* are required for spore formation**

Due to the G52V mutation in *sspA*, we created a clean *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* strain to determine if the double mutant had the same phenotype as the *C. difficile*  $\Delta$ *sspB*<sub>*sspAG52V*</sub> strain. Similar to above, the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* strain produced phase-gray, immature spores (Fig 6A). The rate of sporulation was also approximately 1,000x less than wildtype and could be complemented with expression of both *sspA* and *sspB* from a plasmid (S4 and S2 Figs). Moreover, analysis of the DPA content also showed little DPA in the double mutant strain, in comparison to wildtype (Fig 6B). Finally, to evaluate whether these spores can germinate, we monitored SleC activation. These double mutant spores also processed proSleC to the active SleC form, showing that they are still able to germinate (Fig 6C) [57–59]. Again, due to the difficulty in purifying this strain and despite density gradient purification, spore preparations still contained debris making it difficult to quantify the spores by microscopy. Therefore, we were forced to use approximate spore counts. The Coomassie-stained SDS PAGE gel shows that more protein is present in the double mutant sample than in wildtype or the complemented strains, even though the SleC bands detected by western indicate similar concentrations (S5 Fig). This is possibly due to the presence of contaminating vegetative cells in the double



**Fig 6. Phenotypic characterization of *C. difficile*  $\Delta$ sspA  $\Delta$ sspB double mutant.** A) Day 6 cultures were fixed in 4% formaldehyde and 2% glutaraldehyde before imaging on a Leica DM6B microscope. B) DPA content was determined by boiling spore solutions and analyzing approximately  $2.5 \times 10^7$  spores by terbium fluorescence. C) Western blot of spores exposed to 10 mM taurocholic acid (TA) in a rich medium for 1 hour and blotted against SleC. Arrow indicates processing of pro-SleC (above) to the active SleC form. pEV indicates an empty vector. D) Sporulation frequency was calculated by dividing the number of spores by total number of cells (spores + vegetative cells). Spore totals were determined by treating cultures with a final concentration of 28.5% ethanol and plating on BHIS supplemented with TA. Untreated cultures were plated onto BHIS without germinant to determine the number of vegetative cells. *C. difficile*  $\Delta$ spo0A was set to the limit of detection to determine sporulation frequency. Data represents the average of three independent experiments and the standard error from the mean. Statistical analysis was performed by one way ANOVA with Dunnett's multiple comparison test. \*  $p < 0.01$ , \*\*  $p < 0.001$ .

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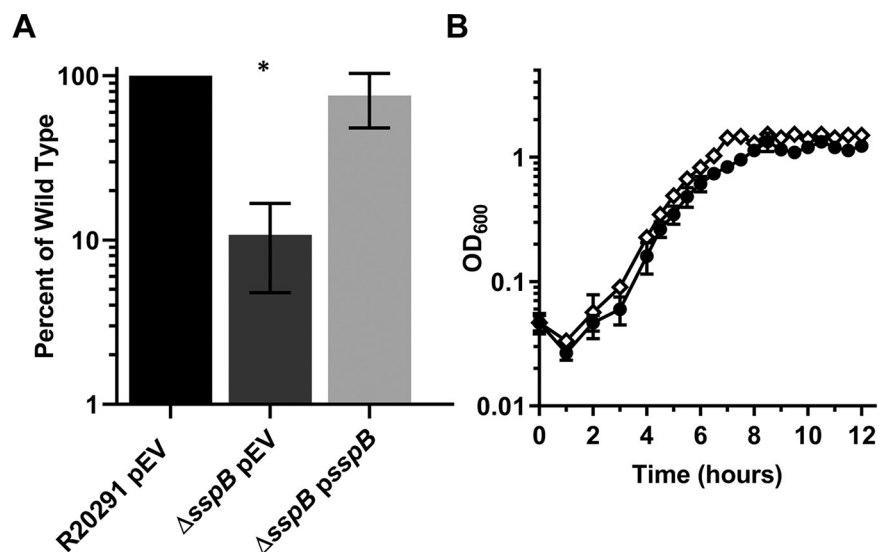
mutant preparation because of the difficulty in purifying or the spore counts could have been underestimated.

To understand how the *sspA*<sub>G52V</sub> allele affects sporulation, a plasmid containing the *sspA* promoter region and the *sspA*<sub>G52V</sub> allele was introduced into the *C. difficile*  $\Delta$ *sspA*, *C. difficile*  $\Delta$ *sspB*, or the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* double mutant strains. As expected, expression of the *sspA*<sub>G52V</sub> allele in the *C. difficile*  $\Delta$ *sspA* mutant strain led to a nearly 4 log<sub>10</sub> reduction in spore formation. Surprisingly, expression of *sspA*<sub>G52V</sub> in the *C. difficile*  $\Delta$ *sspB* strain (which encodes a wildtype chromosomal *sspA* allele) caused a >10x reduction in sporulation. Finally, sporulation frequency in the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* double deletion, which already has a sporulation defect, was further decreased upon expression of the *sspA*<sub>G52V</sub> allele (Fig 6D). These data further confirm that the *sspA* significantly contributes to the sporulation phenotype.

### *C. difficile* *sspB* plays a minor role in UV resistance and does not play a role in outgrowth or chemical resistance

After discovering the SNP in the *C. difficile*  $\Delta$ *sspB* strain, we generated a clean *sspB* deletion that produces phase-bright, released spores. The *C. difficile*  $\Delta$ *sspB* strain had a sporulation frequency identical to wildtype (S4 and S2 Figs).

Since a clean *sspB* deletion produces spores, the UV resistance could be determined. Spores derived from the wildtype *C. difficile* R20291, *C. difficile*  $\Delta$ *sspB* mutant, and *C. difficile*  $\Delta$ *sspB* *psspB* strains were exposed to UV light for 10 minutes before plating on media with germinants to determine CFUs. Spores derived from the *C. difficile*  $\Delta$ *sspB* strain had approximately 10% of the resistance observed for the wildtype strain. This phenotype was complemented by expression of *sspB* from its native promoter *in trans* (Fig 7A). Therefore, *C. difficile* SspB plays a minor role in UV resistance.



**Fig 7. *C. difficile*  $\Delta$ *sspB* has a minor UV defect and no role in outgrowth.** A) Spores derived from *C. difficile* R20291 pEV, *C. difficile*  $\Delta$ *sspB* with pEV, and *C. difficile*  $\Delta$ *sspB* *psspB* were exposed to UV light for 10 minutes. The spores were serially diluted and plated onto medium containing germinants. Strains were normalized to CFUs at  $T_0$ . The ratios were then normalized to the ratio of wildtype survival. B) Outgrowth of spores from *C. difficile* R20291 pEV ● and *C. difficile*  $\Delta$ *sspB* pEV ◇ was monitored at OD<sub>600</sub> over 12 hours. Starting OD was 0.05 into half-strength complex medium supplemented with germinants. pEV indicates an empty vector. All data represents the average of three independent experiments and the standard error from the mean. A) Statistical analysis by one way ANOVA with Dunnett's multiple comparison test. \*  $p < 0.001$ . B) Statistical analysis by two way ANOVA with Dunnett's multiple comparison test,  $p < 0.0001$  at 10 hours.

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Next, we tested the outgrowth of spores derived from the *C. difficile*  $\Delta$ sspB strain. Again, the spores were generated on a minimal medium and the outgrowth was tested in half-strength complex media supplemented with germinants. Similar to our observations with the  $\Delta$ sspA strain, there was no difference in the outgrowth of spores from the *C. difficile*  $\Delta$ sspB strain in comparison to wildtype spores (Fig 7B).

Spores from *C. difficile*  $\Delta$ sspB were also tested for chemical resistance in the same manner as previously discussed. In comparison to *C. difficile* R20291 wildtype spores, *C. difficile*  $\Delta$ sspB spores exposed to 3% H<sub>2</sub>O<sub>2</sub>, 75% EtOH, 0.25% glutaraldehyde, 1 M HCl, 0.05% hypochlorite, 2.5% formaldehyde, and 250 mM nitrous acid did not have reduced viability (S3A–S3G Fig).

### Transcript levels from *C. difficile* mutant strains revealed SASP influence of $\sigma^G$ -controlled genes

To understand at what stage SspA and SspB exert their effects on *C. difficile* sporulation, we analyzed the abundance of transcript during sporulation for some select sporulation-related genes. RNA was extracted from the *C. difficile* R20291, *C. difficile*  $\Delta$ sspA, *C. difficile*  $\Delta$ sspB, *C. difficile*  $\Delta$ sspB<sub>sspAG52V</sub>, *C. difficile*  $\Delta$ sspA  $\Delta$ sspB strains, and the *C. difficile*  $\Delta$ spo0A negative control strain at 18 hours, 24 hours, and 48 hours post plating. Quantitative reverse transcriptase PCR was performed and the fold change determined between wildtype and the indicated strains. For evaluation of *sspA*, *sspB*, *CDR20291\_3080*, *spoVT*, and *pdaA* transcripts, cDNA was generated from a 48 hour time point. A 24 hour timepoint was used to determine *sleC* transcripts and the 18 hour timepoint for *spoIVA*. Technical triplicates of three biological replicates were performed for each strain and one replicate consistently presented as an outlier while the other two replicates had similar fold changes (S6 Fig). The transcript levels for *sspA*, *sspB*, *CDR20291\_3080*, *spoIVA*, and *sleC* did not change in the mutant strains, when compared to the wildtype strain. However, the *C. difficile*  $\Delta$ sspB<sub>sspAG52V</sub> strain had a trend for increased abundance of *spoVT* and *pdaA*, both  $\sigma^G$ -controlled genes, but this did not reach statistical significance (S6 Fig).

## Discussion

*C. difficile* infections occur in individuals with a disrupted microbiota, commonly due to the use of broad-spectrum antibiotics [1,4,5]. Vegetative *C. difficile* colonizes the lower GI tract and produces toxins that disrupt the epithelial lining integrity and cause colitis and diarrhea [4]. While the toxin producing vegetative cells are eliminated with antibiotic treatment, the spores can persist in the gut and become vegetative cells again, or they can shed into the environment [4,5]. This shedding allows for the transmission of disease by passing spores into an environment where they can then transfer to other individuals [4,8]. Spores can withstand environmental insults, such as UV light, and many chemicals, and persist long term in most environments [10,17,24].

The small acid-soluble proteins (SASPs) are well established to protect the spores from environmental insults (e.g., UV light or chemicals) [10,17,18,24]. Here we investigated the functions of *C. difficile* SASPs in response to UV light and chemicals. We found that *C. difficile* SspA and SspB have a role in UV resistance but none of the SASPs significantly contribute to chemical resistances. Surprisingly, we discovered that the deletion of *C. difficile*  $\Delta$ sspA  $\Delta$ sspB resulted in phase-gray, unreleased spores and we hypothesize that SspA and SspB are working together to influence sporulation, a SASP function not previously reported.

*C. difficile* SASPs are involved in protecting spores from UV exposure. This was not surprising since the SASPs in all other organisms have had a large role in UV resistance [28,34,37,60,61]. In *C. difficile*, SspA is the major contributor to UV resistance. UV protection

is attributed to the binding of the SASPs to the DNA, which changes its conformation and encourages spore photoproduct mutations over cyclobutane pyrimidine dimers [18,24,38,40,43,45,52,62]. The loss of the spore photoproduct repair system, SPL, interestingly only resulted in a 10x loss of viability. This highlights the importance of SspA in protecting the genome from lethal UV irradiation. However, in a *C. difficile*  $\Delta$ *sspA*  $\Delta$ *Spl* double mutant, the viability remained the same as for a *C. difficile*  $\Delta$ *sspA* single deletion, suggesting SspA is the major contributor to UV resistance. This also suggests that SspA primarily influences the change in DNA structure that encourages spore photoproduct formation upon UV exposure.

In *B. subtilis*, other SASPs than SspA and SspB are considered minor SASPs with many redundant roles [18,24,35]. Unexpectedly, whenever the double deletion *C. difficile*  $\Delta$ *sspA*  $\Delta$ *CDR20291\_3080* or the triple deletion *C. difficile*  $\Delta$ *sspA*  $\Delta$ *CDR20291\_1130*  $\Delta$ *CDR20291\_3080* were tested, they resulted in 10% viability. We hypothesized that these deletions would result in either the same defect of 0.1% viability seen with *C. difficile*  $\Delta$ *sspA* alone or that UV sensitivity would be increased upon more deletions. This increase in survival would suggest that an unknown factor may be compensating for the loss of these SASPs (there were no obvious candidates from the whole genome re-sequencing). We also found that the other 3 *C. difficile* SASPs, *sspB*, *CDR20291\_1130*, and *CDR20291\_3080* are unable to complement a *C. difficile*  $\Delta$ *sspA* deletion, but hypothesized that this could be due to differences in expression levels. Indeed, when the *sspA* promoter region was used to drive expression of the other SASPs, complementation occurred to approximately 5% of wildtype level. We hypothesize that these other SASPs can bind DNA but need higher expression to do so.

The transcript levels determined by RT-qPCR showed similar levels of *sspA*, *sspB*, *CDR20291\_3080*, *spoIVA* ( $\sigma^E$ -controlled), and *sleC* ( $\sigma^K$ -controlled) between the mutant and wildtype strains. However the  $\sigma^G$ -controlled genes, *spoVT* and *pdaA*, trended toward higher levels in the  $\Delta$ *sspB*<sub>*sspAG52V*</sub> strain, though this difference was not statistically significant. But, this trend, and that expression of *sspB* is required to be driven by a  $\sigma^G$ -dependent promoter (Fig 5), supports our hypothesis that SspA and SspB influence sporulation within the forespore compartment. Due to asynchronous sporulation of *C. difficile* cultures, we could not quantify the absolute abundance of the SASP transcripts—the variability between experiments was considerable.

Other analyses revealed that the SASPs SspA, *CDR20291\_1130*, and *CDR20291\_3080* only minorly contribute to outgrowth. A triple mutation was needed before a ~2 hour defect was observed. We predict that deletion of all three SASPs depletes the amino acid pool and results in a delay in protein production and outgrowth of the vegetative cell.

In other organisms, SASPs play large roles in protection from chemicals. Interestingly, *C. difficile* SASPs were not important for survival of the spores upon chemical exposure. Nitrous acid was the only chemical where the SASPs may play a minor role in protection. Of note, there were some reductions in spore survival of some mutants compared to wildtype across the other chemicals tested. However, in these cases complementation plasmids did not restore viability, leading to the conclusion that some other factor, besides the SASP deletion, was causing the reduced viability. For evaluating the effect of hypochlorite and glutaraldehyde, reproducibility was an issue. Every trial varied in response to these chemicals, making it difficult to draw a confident conclusion from these data. We conclude that *C. difficile* SASPs do not contribute to chemical resistance. Indeed, the *C. difficile* spore coat is established to contain proteins involved in some resistances [63,64].

In *B. subtilis*, the  $\Delta$ *sspA*  $\Delta$ *sspB* strain is commonly used to characterize the SASPs. It was very surprising that the *C. difficile*  $\Delta$ *sspB* with the *sspA*<sub>*G52V*</sub> allele ( $\Delta$ *sspB*<sub>*sspAG52V*</sub>) and the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* double mutant were unable to form phase-bright spores; instead, they formed phase-gray spores lacking DPA. The DPA phenotype could be caused by various

reasons such as DPA not being produced by the mother cell, the SpoVA proteins not being produced and therefore not packaging DPA into the spores, or the cortex/other surrounding spore layers not being produced and enclosing the DPA into the spore. Because DPA contributes to heat resistance and heat resistance is a classically tested feature of endospores and used in sporulation assays, this could have impacted the results of the sporulation assay by killing fully formed spores that do not package DPA and thus exacerbating the sporulation frequency phenotype [10,56,65–67]. However, due to the mutant strains withstanding ethanol treatment in our chemical assays, we performed another sporulation assay using ethanol resistance. Indeed, using ethanol resistance as a metric for spore formation, we observed similar levels of a sporulation defect for the *C. difficile*  $\Delta$ sspB<sub>sspAG52V</sub> and the *C. difficile*  $\Delta$ sspA  $\Delta$ sspB double mutant strains (compared to the heat based assay). Interestingly, the double mutant is fully complemented with only the *sspA* gene, however, the *sspB* gene only restores 10x the amount of sporulation from the mutant alone. This could suggest that *sspB* requires *sspA* in order to complete its role in sporulation.

Due to the phenotype, it was very difficult to purify spores from *C. difficile*  $\Delta$ sspB<sub>sspAG52V</sub> and *C. difficile*  $\Delta$ sspA  $\Delta$ sspB. The spores were treated with lysozyme to digest the mother cell peptidoglycan and release the phase-gray spore. These spores are then less dense, further complicating the purification process. Because of these difficulties, the spore amounts used in DPA content assays and immunoblots are rough estimates based on microscope counts. The Coomassie stained gel shows that more protein was present in the *C. difficile*  $\Delta$ sspA  $\Delta$ sspB sample than in the wildtype and the complemented samples even though the immunoblots show similar levels of protein present. This discrepancy could be due to vegetative cells/debris still present after purification through a density gradient or it is possible we underestimated the number of spores in the microscope counts.

Furthermore, evaluation of UV viability on the *C. difficile*  $\Delta$ sspB<sub>sspAG52V</sub> strain complemented with *sspB* shows that these spores have approximately 60% of wildtype viability. Thus, the *sspA*<sub>G52V</sub> allele does not impair the ability of SspA to protect against UV damage which indicates that DNA binding still occurs. Also, expression of the *sspA*<sub>G52V</sub> allele from a plasmid reduced sporulation frequency in strains that sporulated normally. Furthermore, whenever *sspA*<sub>G52V</sub> was expressed in strains with that encoded a wildtype *sspA* allele, the sporulation frequency was reduced leading to the conclusion that *sspA*<sub>G52V</sub> has a dominant-negative effect of the allele. However, this reduction could be due to the overexpression of the allele from a plasmid in comparison with the expression of a chromosomal copy. Due to the observed phenotypes, we hypothesize that SspA and SspB together regulate spore formation. Interestingly, the *C. difficile*  $\Delta$ sspA  $\Delta$ sspB phenotype is similar to the phenotype of a *C. difficile*  $\Delta$ spoVT mutant [16,68]. In *B. subtilis*, SpoVT is involved in a feed forward loop to regulate sporulation. While its expression is through  $\sigma^G$ , it works to enhance some, and repress other,  $\sigma^G$ -controlled genes [69]. SpoVT regulates the SASPs, SspA and SspB, with a mutant having a 30% reduction in these proteins [70]. This mutant strain is still able to form phase-bright spores but they have a reduced sporulation frequency [70]. In *C. difficile*, the *spoVT* mutant has a different phenotype than that observed in *B. subtilis*. This protein is under  $\sigma^G$  regulation and possibly also  $\sigma^F$  [15,16,68]. The *C. difficile*  $\Delta$ spoVT strain forms immature spores that are phase-dark [16]. Similarly to *B. subtilis*, SASP expression was reduced in *C. difficile*; *C. difficile* *sspA* had a 70 fold reduction and *sspB* a 12 fold reduction [16]. The authors suggested that this SASP reduction was probably not sufficient to explain the phenotype [16]. However, our data suggests that the *C. difficile*  $\Delta$ spoVT phenotype may be due to reduced *sspA* and *sspB* levels. This further highlights the differences in sporulation and SASP function in *C. difficile* compared to the model *B. subtilis* and also furthers our hypothesis of SspA and SspB regulating spore formation. Furthermore, in the *B. subtilis*  $\Delta$ sspA  $\Delta$ sspB double mutant, expression levels of some genes were



changed [71]. However, the expression levels suggest that the SASPs work to negatively regulate multiple forespore genes and even one mother cell gene [71]. They do note that the changes in transcription may not be due to regulation but to actual binding of the SASPs to the genome, which represses transcription.

*B. subtilis* sporulation program contains multiple checkpoints. Most of these checkpoints occur in the early stages of sporulation (before/during commitment). More recently, a checkpoint was found that functions later, during the assembly of the spore layers. CmpA is produced in sporulating cells and functions to prevent spores whose pathways have gone awry from completing the sporulation process. During normal sporulation, CmpA is detected at early sporulation but not in later stages [72,73]. *C. difficile* does not encode many of the early checkpoints that occur before sporulation commitment because Spo0A activation does not occur through phosphorelay, unlike *B. subtilis*. *C. difficile* also does not encode *cmpA*, but it is likely that it could contain a protein that would function in a similar capacity. Our findings indicate that *C. difficile* SspA and SspB are necessary for spore maturation and could be a checkpoint for the sporulation. What spores could be generated from the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* strains are without DPA. However, we do not know if cortex is synthesized or if coat layers are formed. Unlike with the CmpA pathway, the *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* strains remain stalled post-engulfment and seemingly do not proceed to lysis. Further investigations into the spore assembly process for *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* would reveal where SspA and SspB are vital for sporulation and potentially the pathway that they function in. The novel finding of immature spore formation in *C. difficile*  $\Delta$ *sspA*  $\Delta$ *sspB* double mutant suggests that *C. difficile* SASPs perform a previously unreported function in the sporulation process. This insight will open new doors for understanding the regulation of sporulation of *C. difficile*.

## Materials and methods

### Bacterial growth conditions

*C. difficile* strains were grown in a Coy anaerobic chamber at >4% H<sub>2</sub>, 5% CO<sub>2</sub>, and balanced N<sub>2</sub> at 37°C in either brain heart infusion supplemented with 5 g/L yeast extract (BHIS) and 0.1% L-cysteine or tryptone yeast medium with 0.1% thioglycolate [74]. When necessary, media was supplemented with thiamphenicol (10 µg/mL normally or 5 µg/mL for sporulation), taurocholate (TA) (0.1%), anhydrous tetracycline (100 ng/mL), kanamycin (50 µg/mL), or xylose (1%). *E. coli* strains were grown on LB at 37°C and supplemented with chloramphenicol (20 µg/mL) for plasmid maintenance. *B. subtilis* BS49 was grown on LB agar or in BHIS broth at 37°C and supplemented with 2.5 µg/mL chloramphenicol for plasmid maintenance and 5 µg/mL tetracycline during conjugations.

### Plasmid construction

The *C. difficile* *sspA* targeted CRISPR plasmid was constructed by amplifying 500 bp upstream and downstream from the *sspA* gene using primer pairs 5'*sspA*\_MTL, 3'*sspA*\_up and 5'*sspA*\_down, 3'*sspA*\_downMTL, respectively. The fragments were cloned into the *NotI* and *XhoI* site of pKM126 by Gibson assembly [75]. The gRNA was retargeted to *sspA* by inserting gBlock CRISPR\_ *sspA*\_165 into the *KpnI* and *MluI* sites by Gibson assembly, generating the pHN05 plasmid. The *spl*, *sspB*, *CDR20291\_1130* and *CDR20291\_3080* targeting plasmids were similarly constructed. For *spl* plasmid construction, 5'*spl*\_UP and 3'*spl*\_UP amplified the upstream homology arms and the downstream homology by 5'*spl*\_DN and 3'*spl*\_DN. These were cloned into pKM197 at the *NotI* and *XhoI* sites. The gRNA was retargeted by cloning into the *KpnI* and *MluI* sites gBlock CRISPR\_ *spl*\_647 resulting in plasmid pHN61. Primers 5'*sspB* UP and 3'*sspB* UP amplified the upstream region, while primers 5'*sspB* DN and 3'*sspB* DN

amplified the downstream portion. These fragments were also cloned into pKM126 at the same sites as previously used for homology. The gRNA was switched with CRISPR\_ sspB\_144 at *KpnI* and *MluI* sites, generating pGC05. For *CDR20291\_1130*, primer pairs 5'CDR20291\_1130\_UP, 3' CDR20291\_1130\_UP and 5'CDR20291\_1130\_DN, 3'CDR2021\_1130\_DN were used, respectively, to amplify upstream and downstream portions of *CDR20291\_1130* homology. For *CDR20291\_3080*, primer pairs 5'CDR20291\_3080\_UP, 3' CDR20291\_3080\_UP and 5'CDR20291\_3080\_DN, 3'CDR2021\_3080\_DN were used, respectively, to amplify upstream and downstream portions of *CDR20291\_3080* homology. These homology arms were cloned by Gibson assembly into pKM126 [50] at sites *NotI* and *XhoI* [75]. gRNAs CRISPR\_ CDR20291\_1130\_114 and CRISPR\_ CDR20291\_3080\_184 were cloned into the plasmids at the *KpnI* and *MluI* sites. Next, because of issues with the *tet* promoter (in pKM126) causing leaky expression of *cas9*, and causing potential problems during conjugation, *tetR* was replaced with *xylR* from pCE641 [76]. The *xylR* region was amplified with primers 5' sspB.xylR and 3'cas9\_Pxyl2 for sspB plasmid, 5'CD1130\_HR\_xylR and 3'cas9\_Pxyl2 for the CDR20291\_1130 plasmid and the primers 5'CD3080\_HR\_xylR and 3'cas9\_Pxyl2 for the CDR20291\_3080 plasmid to create pHN101, targeting *sspB*, pHN32, targeting *CDR20291\_1130*, and pHN34, targeting *CDR20291\_3080*.

For *C. difficile*  $\Delta$ sspA complementation plasmids, the genes and promoter regions were inserted by Gibson assembly into the *NotI* and *XhoI* sites of pJS116 [51] by Gibson assembly [75]. The *sspA* gene plus 500 bp upstream was amplified using primer pair 5' sspA\_MTL and 3' sspA.pJS116 to create plasmid pHN11. The vector, pHN14, consisting of the *sspB* gene and 500 bp upstream was generated using primers 5' sspB UP and 3' sspBpJS116. The *sspA* portion of the *sspA* and *sspB* double mutant complementation vector, pHN30, was amplified with 5' sspApJS116 and 3' sspAsspB. The *sspB* portion of the double mutant complement was amplified with 5' sspAsspB and 3' sspBpJS116. For pHN84, the *sspA* complement with a 6x histidine tag on the C-terminus, the primers 5' sspA\_MTL and 3' sspA.His\_pJS116 were used. The primers 5' sspB UP with 3' PsspB\_ sspA and 5' PsspB\_ sspA with 3' sspA.pJS116 were used to generate a plasmid with 500 bp upstream of the *sspB* gene driving expression of the *sspA* gene for pHN91. The 500 bp upstream of *sspA* was amplified with 5' sspA\_MTL and 3' sspB\_ sspA and used to drive expression of the *sspB* gene, amplified with 5' sspB\_ sspA and 3' sspBpJS116 for the pHN83 complementation vector. To generate the pHN56 vector of the *CDR20291\_1130* gene and 500 bp upstream, the primer pair 5' 1130comp and 3' 1130comp were used. For amplification of the *CDR20291\_3080* gene and 500 bp upstream, pHN57, the primers 5' 3080comp and 3' 3080comp were used. Generation of 500 bp upstream of *sspA* was amplified with primers 5' sspA\_MTL and 3' sspA\_CD1130 and used to drive expression of the *CDR20291\_1130* gene amplified with primers 5' CD1130\_ sspA and 3' 1130comp for plasmid pHN96. The plasmid pHN97 was generated with 500 bp upstream of *sspA*, with primers 5' sspA\_MTL and 3' sspA\_CD3080 and the *CDR20291\_3080* gene amplified with 5' CD3080\_ sspA and 3' 3080comp. For the negative control, pHN109, of *mCherry* driven by *sspA* promoter region, the *sspA* promoter region was amplified with primers 5' sspA\_MTL and 3' PsspA.mCherry, while the *mCherry* gene was amplified from pRAN473 [77] with primers 5' PsspA.mCherry and 3' mCherry.PsspA. Another negative control of just the *sspA* promoter region, pHN102, was generated by using 5' sspA\_MTL and 3' PsspA.pJS116 to amplify the *sspA* promoter region. To generate the  $\sigma^E$  sspB plasmid, the promoter region of *bclA2* was amplified with 5' sigE. bclA2\_pJS116 and 3' sigE.bclA2\_ sspB and the *sspB* gene with 5' sigE.bclA2\_ sspB and 3' sspB\_pJS116, creating pHN80. For the  $\sigma^K$  sspB complement vector, the promoter region of *sleC* was amplified with 5' pJS116\_sigK and 3' sigK\_ sspB and the *sspB* gene with 5' sigK\_ sspB and 3' sspB\_pJS116, generating pHN49. The promoter region of *gpr* was amplified with 5' pJS116\_sigF and 3' sigF\_ sspB and the *sspB* gene with 5' sigF\_ sspB and 3' sspB\_pJS116 to generate the  $\sigma^F$  sspB plasmid, pHN47. To generate pHN118, the plasmid containing the *sspA*<sub>G52V</sub>

allele and promoter, primers 5' sspA\_MTL and 3' sspA.pJS116 were used to amplify from HNN04. All plasmid sequences were confirmed by DNA sequencing. Cloning was done in *E. coli* DH5 $\alpha$ . A complete list of oligonucleotides used and the strains and plasmids generated can be found in [S1](#) and [S2](#) Tables, respectively.

## Conjugations

The resulting plasmids were conjugated separately into *C. difficile* R20291 using *B. subtilis* BS49 as a conjugal donor, as described previously [50]. Briefly, 0.25 mL of *C. difficile* R20291 overnight culture was back diluted into 4.75 mL fresh BHIS and incubated for 4 hours. Meanwhile, 5 mL of BHIS supplemented with chloramphenicol and tetracycline was inoculated with 1 colony of *B. subtilis* BS49 containing the plasmid and was incubated at 37°C for 3 hours. After incubation, the *B. subtilis* culture was passed into the anaerobic chamber and 100  $\mu$ L culture was plated on TY agar medium, along with 100  $\mu$ L of *C. difficile* R20291. This was incubated for approximately 24 hours. Growth was then suspended in 1.5 mL of BHIS and plated onto BHIS agar supplemented with thiamphenicol and kanamycin for selection. Resulting transconjugant colonies were streaked, twice, onto BHIS supplemented with thiamphenicol and kanamycin (TK) or BHIS supplemented with tetracycline (to screen for the conjugal transfer of the *Tn916* transposon). Thiamphenicol resistant and tetracycline sensitive colonies were tested by PCR for plasmid components.

## CRISPR induction

Overnight cultures of 5 mL TY medium supplemented with thiamphenicol were inoculated with one colony of a *C. difficile* R20291 strain containing a CRISPR mutagenesis plasmid with *tet*-driven *cas9* (pGC05 and pHN05). After approximately 16 hours of growth, 0.25 mL of overnight culture was back diluted with 4.75 mL TY broth and supplemented with thiamphenicol and anhydrous tetracycline and incubated for 6 hours [50]. A loopful (approximately 10  $\mu$ L) of culture was plated onto BHIS and the individual colonies were tested by PCR for the mutation. Once PCR confirmed, the plasmid was cured by passaging onto BHIS plates. For induction of *xyl*-driven *cas9* plasmids (pHN61, pHN101, pHN32 and pHN34), colonies were passaged 3 times on TY agar supplemented with 1% xylose and thiamphenicol. Mutants were detected by PCR and the plasmid was cured by passaging in BHIS broth supplemented with 0.5% xylose. Pairwise mutants were generated by conjugating the appropriate plasmid into the necessary mutant strain and inducing as described above.

## Spore purification

Cultures were plated onto 70:30 media or CDMM minimal media where indicated and incubated 5 days. Spores were purified as previously described [51,55,56,78]. Briefly, plates were scraped and contents suspended in dH<sub>2</sub>O overnight at 4°C. The pellets were resuspended then centrifuged at maximum speed. The upper, fluffy-white, layer was removed and resuspended again in dH<sub>2</sub>O. This process was repeated approximately 5 times. The spores were then separated by density gradient in 50% sucrose solution at 3,500  $\times$ g for 20 minutes. The spore pellet was washed approximately 5 times in dH<sub>2</sub>O. The spores were stored at 4°C until use.

To purify the  $\Delta$ sspB<sub>sspAG52V</sub> and the  $\Delta$ sspA  $\Delta$ sspB double mutant spores, the pellets were scraped into dH<sub>2</sub>O and left overnight at 4°C. The pellets were then resuspended with 1  $\mu$ g of lysozyme and incubated for 4 hours at 37°C. The suspension was centrifuged at max speed for 1 minute and the upper phase removed, and the pellet resuspended with dH<sub>2</sub>O. This process was repeated approximately 5 times before 5 mL of spores were layered onto a HistoDenz

gradient of 10 mL of 50% and 10 mL of 25%. This was centrifuged 30 minutes at 18,900 xg at 4°C. The pellet was then washed approximately 5 times in dH<sub>2</sub>O and stored at 4°C until use.

### Germination assay and DPA content

Germination was monitored using a Spectramax M3 plate reader (Molecular Devices, Sunnyvale, CA). 5 μL of OD<sub>600</sub> = 100 spores were added to 95 μL germination buffer consisting of a final concentration 1x HEPES, 30 mM glycine, 10 mM TA and the OD<sub>600</sub> was monitored for 1 hour at 37°C. To assay total DPA content, 1 x 10<sup>8</sup> spores in 20 μL, were boiled at 95°C for 20 minutes. 5 μL (an equivalent of 2.5 x 10<sup>7</sup> spores) of the solution was added to 95 μL of 1X HEPES buffer with 250 μM TbCl<sub>3</sub> and analyzed by excitation at 275 nm and emission at 545 nm with a 420 nm cutoff [54,55,59,66,79].

### Western blotting

For the  $\Delta$ sspB<sub>SNP</sub> strain, approximately 1 x 10<sup>9</sup> spores were incubated for 1 hour in 50 μL of BHIS with or without 10 mM TA at 37°C. The solutions were boiled for 20 minutes in 2x NuPAGE buffer at 95°C. 10 μL (equivalent to approximately 1 x 10<sup>8</sup> spores) of each solution was separated on a 15% SDS PAGE gel. For the double mutant strain,  $\Delta$ sspA  $\Delta$ sspB, approximately 4–8 x 10<sup>6</sup> total spores were separated. The protein was transferred to polyvinylidene difluoride (PVDF) membranes at 0.75 amps for 1.5 hours for the  $\Delta$ sspB<sub>sspAG52V</sub> strain and at 1 amp for 30 minutes for the  $\Delta$ sspA  $\Delta$ sspB strain. The membranes were blocked overnight in 5% milk in TBST. The following day, the membranes were washed 3 times, 15 minutes each, at room temperature. SleC anti-sera was added to 5% milk in TBST at a 1:20,000 dilution for 1 hour. The membranes were washed again, as above. The anti-rabbit secondary antibody was diluted to 1:20,000 in 5% milk in TBST and incubated for 1 hour. The membranes were again washed, as above. The membranes were incubated with Pierce ECL Western Blotting Substrate (ThermoScientific) for 1 minute and then x-ray film was exposed and developed.

### Phase contrast imaging

Strains were plated onto 70:30 (70% SMC medium and 30% BHIS medium) sporulation media and incubated for 6 days. After the incubation period, the growth was harvested and suspended in fixative (4% formaldehyde, 2% glutaraldehyde in 1x PBS). The samples were imaged on a Leica DM6B microscope at the Texas A&M University Microscopy Imaging Center.

### Heat resistance

Heat resistance assays were completed as previously described [67]. Briefly, three 70:30 plates were inoculated with fresh colonies of the indicated strains. These were grown for 48 hours before half of the plate was harvested into 600 μL of pre-reduced PBS solution and the pellets resuspended. 300 μL of suspension was removed from the chamber and heated for 30 minutes at 65°C and mixed every 10 minutes by inversion. After heating, these samples were passed into the anaerobic chamber for enumeration. Both the heat-treated and the remaining 300 μL of untreated suspension was serially diluted and plated in technical triplicates of 4.5 μL spots on rich, BHIS, medium supplemented with 0.1% TA. After 22 hours of growth, the CFUs were enumerated. The ratios of treated to untreated were calculated and then the efficiency of sporulation determined in comparison to the wildtype strain. The experiment was performed in biological triplicate.

### Ethanol resistance

Ethanol resistance assays were performed as previously described with modification [80]. A 70:30 plate was inoculated and incubated for 48 hours. After incubation,  $\frac{1}{4}$  of the plate was scraped and resuspended in 1 mL of PBS. To enumerate vegetative cells, 500  $\mu$ L of the culture was added to 500  $\mu$ L of BHIS and serially diluted and plated on BHIS. To enumerate spores, 500  $\mu$ L of the culture was added to 300  $\mu$ L of 95% ethanol and 200  $\mu$ L of dH<sub>2</sub>O and incubated 20 minutes. After incubation, the solution was serially diluted into PBS with 0.1% TA and plated onto BHIS TA. The sporulation frequency was generated by dividing the number of spores by the total cell count.

### UV exposure

The 302 nm UV lamp (Fisher Scientific) was allowed to warm-up for 20 minutes before UV exposure. Spores were diluted in PBS to  $1 \times 10^9$  spores/mL. A sample was collected at T<sub>0</sub> for initial, untreated, spore calculations. 1 mL of a spore solution was added to a miniature glass (UV penetrable) petri dish. These dishes were placed under the UV lamp and exposed with constant agitation for the indicated time. After exposure, the untreated and treated samples were serially diluted then introduced into the anaerobic chamber where they were plated onto BHIS medium supplemented with taurocholic acid and thiamphenicol, for plasmid maintenance. After 48 hours of incubation, the CFUs were enumerated. Treated spore counts were normalized to untreated and then this ratio was normalized to the ratio for wildtype spores.

### Outgrowth

Outgrowth, post-germination, was performed in half-strength BHIS supplemented with 30 mM glycine and 10 mM taurocholic acid. Thiamphenicol was supplemented where necessary to maintain plasmids. Spores were added to an OD<sub>600</sub> of 0.05 and the OD<sub>600</sub> was recorded over time.

### Chemical resistance

$1 \times 10^9$  spores were suspended in PBS with the indicated chemical concentration for various exposure times. The exposed spores were immediately serially diluted into PBS tubes and plated onto rich media supplemented with taurocholate. For generation of nitrous acid, a one to one volume of 1 M sodium acetate, pH 4.5 and 1 M sodium nitrite were combined and incubated for at least 30 minutes before spore exposure. For formaldehyde exposure, the exposed solution was serially diluted into PBS with 400 mM glycine and incubated for at least 20 minutes to quench the formaldehyde before plating.

### RNA extraction

Strains were incubated on 70:30 agar for 18, 24, or 48 hours. The cells were harvested and RNA extractions completed using the FastRNA Pro Blue Kit (MP Biologicals). Cells were lysed in a bead-beater with 3 rounds of 45 seconds on and 2 minutes off on ice. Contaminating DNA was treated with TURBO DNA-free kits (Invitrogen) with 3 rounds of DNase incubation. After DNase treatment, cDNA was generated using SuperScript III First-Strand Synthesis System (Thermo Scientific) with 100 ng of total RNA. Negative controls without the reverse transcriptase were also generated for the samples. 3 biological replicates were extracted and treated for each strain.

## qRT-PCR

The genes *rpoA*, *sspA*, *sspB*, *3080*, *sleC*, *spoVT*, *pdaA*, and *spoIVA* were analyzed with the following primer pairs, respectively, 5' *rpoA*, 3' *rpoA*, and 5' *sspA*\_qPCR, 3' *sspA*\_qPCR, and 5' *sspB*\_qPCR, 3' *sspB*\_qPCR, and 5' *CD3080*\_qPCR, 3' *CD3080*\_qPCR, and 5' *sleC*\_qPCR, 3' *sleC*\_qPCR, and 5' *spoVT*\_qPCR, 3' *spoVT*\_qPCR, and 5' *pdaA*\_qPCR, 3' *pdaA*\_qPCR, and 5' *spoIVA*\_qPCR, 3' *spoIVA*\_qPCR. PowerUP SYBR Green master mix (Applied Biosystems) was combined with 500 nM of each primer and 2  $\mu$ L cDNA to a volume of 10  $\mu$ L per reaction. Three technical replicates were performed for each strain using the QuantStudio 6 Flex real-time PCR machine (Applied Biosystems). Fold change was determined by the comparative cycle threshold method with *rpoA* serving as the internal control to which experimental genes were normalized.

## Supporting information

### S1 Fig. Heat based sporulation efficiency is not affected by deletion of *sspA*,

*CDR20291\_1130* or *CDR20291\_3080* alone. Strains were grown on sporulation medium for two days. Sporulating cultures were heat treated at 65°C. Sporulation rate was determined by comparison of the CFU of heat treated culture to CFU of untreated culture and then the ratios were compared to wildtype. pEV indicates an empty vector. All data represents the average of three independent experiments. Statistical analysis by one way ANOVA with Dunnett's multiple comparison test.

(TIF)

**S2 Fig. Ethanol-based sporulation assay.** The strains were treated with 28.5% final concentration of ethanol. The sporulation frequency was determined by dividing the number of spores by the total number of cells (spores + vegetative cells). *C. difficile*  $\Delta$ *spo0A* was set to the limit of detection to determine sporulation frequency. All data represent the average of three independent experiments and the standard error from the mean. Statistical analysis by one way ANOVA comparison to wildtype with Dunnett's multiple comparison test. \*  $p < 0.001$ .

(TIF)

**S3 Fig. Chemical resistance is not impacted by loss of individual SASPs.**  $1 \times 10^9$  spores were exposed to chemicals for 1, 5, 10, or 30 minutes. After exposure, solutions were serially diluted and plated onto rich medium with germinants. The CFUs were enumerated and compared to unexposed samples and then this ratio was compared with that of the wildtype spores. A) 3% H<sub>2</sub>O<sub>2</sub> B) 75% EtOH C) 0.25% Glutaraldehyde D) 1 M HCL E) 0.05% hypochlorite F) 2.5% Formaldehyde G) 250 mM Nitrous Acid. pEV indicates an empty vector. All data represents the average of three independent experiments. Statistical analysis by two way ANOVA with Dunnett's multiple comparison. A) *C. difficile*  $\Delta$ *sspA* pEV and *C. difficile*  $\Delta$ *sspA* *psspA*  $P < 0.0001$  at 30 minutes. C)  $P < 0.001$  for *C. difficile*  $\Delta$ *sspB* *psspB* 5 minutes and *C. difficile*  $\Delta$ *sspA* *psspA* at 10 minutes.  $P < 0.05$  for *C. difficile*  $\Delta$ *CDR20291\_3080* pEV at 10 minutes. D)  $P < 0.05$  for *C. difficile*  $\Delta$ *sspA* *psspA* at 5 minutes and *C. difficile*  $\Delta$ *sspB* *psspB* at 30 minutes.  $P < 0.01$  for *C. difficile*  $\Delta$ *sspA* *psspA* at 10 minutes.  $P < 0.001$  for *C. difficile*  $\Delta$ *sspA* pEV and *C. difficile*  $\Delta$ *sspA* *psspA* at 30 minutes. E)  $P < 0.01$  for *C. difficile*  $\Delta$ *sspB* *psspB* at 1 minute. F)  $P < 0.05$  for *C. difficile*  $\Delta$ *CDR20291\_3080* at 10 minutes. G)  $P < 0.05$  for *C. difficile*  $\Delta$ *CDR20291\_1130* at 5 minutes, *C. difficile*  $\Delta$ *sspA* pEV and *C. difficile*  $\Delta$ *CDR20291\_1130* pEV at 10 minutes.  $P < 0.01$  for *C. difficile*  $\Delta$ *CDR20291\_3080* at 5 minutes.  $P < 0.001$  for *C. difficile*  $\Delta$ *sspA* pEV at 5 minutes.

(TIF)

**S4 Fig. Sporulation efficiency of mutant strains as determined by heat treatment.** Strains were grown on sporulation medium for two days. Sporulating cultures were heat treated at 65°C. Sporulation rate was determined by taking the ratio of the CFU of heat treated culture to CFU of untreated culture and then the ratios were compared to wildtype. pEV indicates an empty vector. All data represents the average of three independent experiments. Statistical analysis by one way ANOVA with Dunnett's multiple comparison test.  
(TIF)

**S5 Fig. Coomassie-stained gel of  $\Delta$ sspA  $\Delta$ sspB samples.** The same sample volumes used for the  $\Delta$ sspA  $\Delta$ sspB SleC cleavage assay were separated by a 15% SDS PAGE and stained with Coomassie. pEV indicates an empty vector.  
(TIF)

**S6 Fig. Fold change of transcripts by qRT-PCR between mutant strains.** RNA was extracted from wildtype and mutant strains. DNA was depleted from the RNA samples and then cDNA was generated. Transcript levels were determined through quantitative reverse transcriptase PCR and the fold change between mutant transcript levels and wildtype levels was determined. A) RNA extracted after 48 hours of incubation, *sspA* transcript levels. B) RNA extracted after 48 hours of incubation, *sspB* transcript levels. C) RNA extracted after 48 hours of incubation, *3080* transcript levels. D) RNA extracted after 18 hours of incubation, *spoIVA* transcript levels. E) RNA extracted after 24 hours of incubation, *sleC* transcript levels. F) RNA extracted after 48 hours of incubation, *spoVT* transcript levels. G) RNA extracted after 48 hours of incubation, *pdaA* transcript levels. BLQ is below limit of quantification; the cycle threshold is below the limit of which we can accurately quantify. All data represents the average of three independent experiments and the standard error from the mean. Statistical analysis by one way ANOVA with Dunnett's multiple comparison test.  
(TIF)

**S1 Table. Primers used in this study.**  
(DOCX)

**S2 Table. Strains and plasmids used in this study.**  
(DOCX)

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## Author Contributions

**Conceptualization:** Hailee N. Nerber, Joseph A. Sorg.

**Data curation:** Hailee N. Nerber.

**Formal analysis:** Hailee N. Nerber, Joseph A. Sorg.

**Funding acquisition:** Joseph A. Sorg.

**Investigation:** Hailee N. Nerber.

**Project administration:** Joseph A. Sorg.

**Supervision:** Joseph A. Sorg.

**Writing – original draft:** Hailee N. Nerber, Joseph A. Sorg.

**Writing – review & editing:** Hailee N. Nerber, Joseph A. Sorg.

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